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Duplications and Defects in the *CYP2A6* Gene: Identification, Genotyping, and In Vivo Effects on Smoking

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ABSTRACT

In humans, 80% of nicotine is metabolized to the inactive metabolite cotinine by the enzyme CYP2A6, which can also activate tobacco smoke procarcinogens (e.g., 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone). Previously, we demonstrated that individuals who are nicotine-dependent and have defective *CYP2A6* alleles (**2, *3*) smoked fewer cigarettes; however, we recognize that the genotyping method used for the *CYP2A6*3* allele gave a high false-positive rate. In the current study we used improved genotyping methods to examine the effects of the defective *CYP2A6*2* and *CYP2A6*4* alleles on smoking behavior. We found that those with the defective alleles ($N = 14$) smoked fewer cigarettes per day than those homozygous ($N = 277$) for wild-type alleles (19 versus 28 cigarettes per day, $P < .001$). In addition, we identified a duplicated form of the *CYP2A6* gene, corresponding to the gene deletion *CYP2A6*4* allele, developed a genotyping assay, assessed the gene copy number, and examined its prevalence in Caucasian smokers ($N = 296$). We observed an ascending rank order for plasma cotinine and breath carbon monoxide levels (an index of smoke inhalation) in individuals with null (*CYP2A6*2* and *CYP2A6*4*) alleles ($N = 14$), those homozygous for wild-type (*CYP2A6*1/*1*) alleles ($N = 277$), and those with our newly identified *CYP2A6* gene duplication ($N = 5$). The phenotype, as determined by plasma nicotine/cotinine ratios, had a descending rank order for these three genotype groups that did not reach significance. Although further characterization is required for the duplication gene variant, these results extend our previous findings and suggest a substantial influence of *CYP2A6* genotype and phenotype on smoking behavior.

Genetic variation of *CYP2A6* alters coumarin and nicotine (NIC) metabolism (Yamano et al., 1990; Iscan et al., 1994; Messina et al., 1997). Initially, a wild-type (*CYP2A6*1*) and two defective alleles (*CYP2A6*2* and *CYP2A6*3*) were identified. *CYP2A6*2* is a null allele with no activity toward probe substrates, although the methodology for detection, function, and allele frequency of the *CYP2A6*3* allele are controversial (Yamano et al., 1990; Fernandez-Salguero et al.,1995; Oscarson et al., 1998; Benowitz et al., 2000). Recently, a *CYP2A6* gene deletion (*CYP2A6*4*) was characterized (Yokoi and Kamataki, 1998; Nunoya et al., 1999; Oscarson et al., 1999b); the mechanism proposed for the creation of the deleted allele is similar to that found for the deleted (*CYP2D6*5*) and duplicated (*CYP2D6*2X2*) alleles of *CYP2D6*, involving unequal crossover between *CYP2D6* and adjacent *CYP2D* genes (Gaedigk et al., 1991). The existence of a *CYP2A6* gene deletion variant infers the existence of a *CYP2A6* gene duplication (Fig. 1A).

In humans, 80% of NIC is inactivated by metabolism to cotinine (COT; Benowitz et al., 1994). Determining the variation in NIC inactivation is important because of NIC's role in producing tobacco dependence and regulating smoking behavior. We, and others, have demonstrated that CYP2A6 is responsible for the majority of the metabolic inactivation of NIC to COT (Nakajima et al., 1996b, 2000; Messina et al., 1997; Benowitz et al., 2000) and for the metabolism of COT to *trans*-3-hydroxyCOT, 5′-hydroxyCOT and possibly norCOT (Nakajima et al., 1996a; Murphy et al., 1999).

Dependent smokers adjust their smoking behavior to maintain constant blood and brain NIC levels (McMorrow and Foxx, 1983; Russel, 1987). Consistent with this, we previously found that heterozygotes for defective (*CYP2A6*2* or *CYP2A6*3*) alleles smoked fewer cigarettes (CIGs) per week than smokers homozygous for wild-type *CYP2A6*1* alleles (129 versus 159 CIGs per week) and were less likely to become NIC dependent (Pianezza et al., 1998). Repeating the

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ABBREVIATIONS: NIC, nicotine; CIG, cigarette; COT, cotinine; CO, carbon monoxide; PCR, polymerase chain reaction; dNTP, deoxyribonucleotide triphosphate; bp, base pair(s); ppm, parts per million.

*CYP2A6*2* genotyping on these samples (Pianezza et al., 1998) with new techniques, an allele-specific assay (Oscarson et al., 1998) and a restriction digestion assay (Chen et al., 1999), demonstrated a conversion of nine individuals previously genotyped as *CYP2A6*2/*2* to *CYP2A6*1/*2* but no change in the individuals previously genotyped as *CYP2A6*1/*2*. The revised *CYP2A6*2* allele frequencies were 2.7% in the never tobacco-dependent group (N = 184) and 2.1% in the tobacco-dependent group (N = 164), consistent with recent studies of Caucasians [2.3% (Chen et al., 1999); 1.1, 1.4, and 3.0% (Oscarson et al., 1998)].

It is clear that the original genotyping assay for *CYP2A6*3* was inaccurate; it has been proposed that a gene conversion in the 3′-flanking region (in the position of the original reverse primer R4; Fernandez-Salguero et al., 1995), occurring in 30 to 40% of the *CYP2A6*1* alleles, results in the *CYP2A6*3* genotype misclassification (Oscarson et al., 1999a). Newer assays suggest that the frequency of the *CYP2A6*3* is extremely low (0–0.7%, Chen et al., 1999; Oscarson et al., 1999b). Our kinetics data indicated that liver samples previously genotyped as having the *CYP2A6*3* allele demonstrated slower NIC metabolism (Messina et al., 1997; R. F. Tyndale and E. M. Sellers, unpublished data). In addition, genotyping of samples in which *CYP2A6*3* had been previously identified (Pianezza et al., 1998) for other variant alleles indicated that some of these samples contained the *CYP2A6*4* allele; the identification of a *CYP2A6*4* allele in an individual originally classified as having a *CYP2A6*3* allele has also been observed by Oscarson et al. (1999a). In addition there appear to be a number of other nucleotide changes, and resultant amino acid changes, in the *CYP2A6*-coding region from these subjects which are currently being investigated (R. F. Tyndale and E. M. Sellers, unpublished data). These data suggest: 1) that some of the individuals who we previously genotyped as having the *CYP2A6*3* allele may have alternative null allelic variants that may, or may not, account for our previous observations and 2) that we need to reassess the role of genetically variable *CYP2A6* in the risk for tobacco dependence with larger numbers of subjects (because of the lower estimates of the allelic variants).

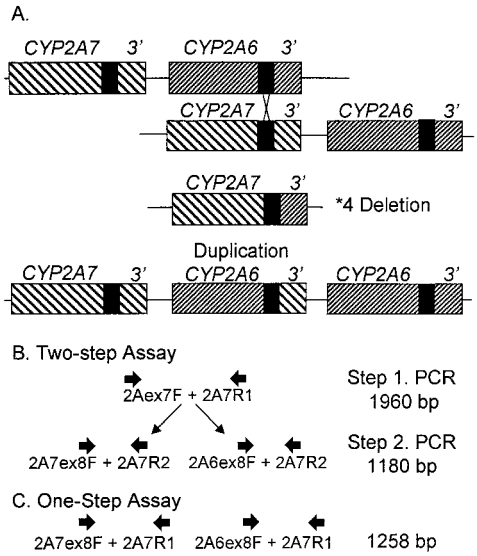

Fig. 1. A, schematic of the putative unequal crossover event leading to the creation of the *CYP2A6* deletion and duplication variants. The boxed region indicates putative crossover positions spanning from intron 8 to the 3′-flanking region. B, schematic of the two-step genotyping assay that was modified to detect the duplication variant. The first step uses a common *CYP2A6/7* exon 7 forward primer with a *CYP2A7*-selective reverse primer in the 3′-flanking region. The second step uses the PCR product from the first step as template DNA in two separate reactions using a selective *CYP2A7* or *CYP2A6* exon 8 forward primer with a nested *CYP2A7*-selective reverse primer in the 3′-flanking region to detect the wild-type *CYP2A7* gene and duplicated *CYP2A6/7* variant, respectively. C, schematic of a one-step genotyping assay for detection of the duplication variant. A *CYP2A7* exon 8 forward primer, or *CYP2A6* exon 8 forward primer, was used with a *CYP2A7* reverse primer in the 3′-flanking region to detect the wild-type *CYP2A7* gene and the duplicated *CYP2A6/7* variant, respectively.

In this study, we focused on retesting the second observation from our previous study (Pianezza et al., 1998), which suggested that NIC-dependent (DSM-IV, American Psychiatric Association, 1994) individuals with *CYP2A6*2* or *CYP2A6*3* null alleles smoked fewer CIGs per day. Specifically, we demonstrated decreased CIGs per day and lower plasma COT and breath carbon monoxide (CO) levels in individuals with *CYP2A6*2* or *CYP2A6*4* null alleles. We also identified a putative *CYP2A6* gene duplication variant, established a genotyping method for this variant, determined the allele frequencies, and then examined the impact of this novel variant on in vivo indices of smoking in Caucasians (N = 296).

Materials and Methods

Primers and Sequencing. Oligonucleotide primers for polymerase chain reaction (PCR) assays and DNA sequencing (Table 1) were synthesized by the Hospital for Sick Children Biotechnology Service Center (Toronto, Canada). Cosmid DNA from clones 19296, 19019, 17943, and 27292 (gratefully received from Dr. Linda Ashworth, Human Genome Center, Liverpool, CA) containing *CYP2A6*, *CYP2A7*, *CYP2A7P*, and *CYP2A13*, respectively (Hoffman et al., 1995) were used to test the *CYP2A* gene specificity of the primers and to obtain intronic DNA sequence. *CYP2A* gene-specific and nonspecific forward primers (exon and intron 1, 3, 6, 7, 8, and 9) and 3′-flanking reverse primers were used to amplify genomic DNA containing wild-type, *CYP2A7/6* (*CYP2A6*4* gene deletion), and *CYP2A6/7* (*CYP2A6* gene duplication) DNA. Sequencing was performed by the Core Molecular Biology Facility, York University (Toronto, Canada). Sequence alignments were performed using DNASIS for windows (Hitachi Software, Genetic Systems, San Francisco, CA).

Subjects and Sampling. All study protocols were approved by the Ethics Review Committee of the Sunnybrook and Women's College Health Science Center. A structured questionnaire was used to obtain information concerning demographics, as well as history and pattern of psychoactive drug use [drug dependence assessment with DSM-IV (American Psychiatric Association, 1994)]. All subjects (N = 400) met the following criteria: 1) healthy male or female, 2) 16 to 70 years of age, 3) current smoker (50% light smokers, currently smoking ≤15 CIGs/day, and 50% heavy smokers, currently smoking >15 CIGs/day for each gender), and 4) willingness to sign the consent form. For this study we restricted the analysis to Caucasians (N = 296 of the 400 with three or more Caucasian grandparents) consisting of 155 female smokers (66 light and 89 heavy) and 141 male smokers (61 light and 80 heavy). Between 4 and 8 PM, subjects were assessed for breath CO with a Micro II Smokelyzer (Bedford Scientific Ltd., Upchurch, England). A single venous blood sample was acquired, and plasma NIC and COT were assessed by high-performance liquid chromatography (Pacifici et al., 1993). Genomic DNA was extracted from venous blood samples using the QIAamp Blood Kit (Qiagen Inc., Santa Clarita, CA). *CYP2A6*2* and *4* assays were performed as previously described (Oscarson et al., 1998, 1999b).

CYP2A6 Gene Duplication Assay. A two-step genotyping assay was developed for the duplicated allele (Fig. 1B) based on inverting the gene specificity of the assay used for detecting the *CYP2A6*4* allele (Oscarson et al., 1999b). Specifically, the first step used a forward primer with sequence common to both *CYP2A6* and *CYP2A7* in exon 7 (2Aex7F) with a 3′-flanking reverse primer that is *CYP2A7* specific (2A7R1, analogous to 2A6R1, which is used for the *CYP2A6*4* allele, Fig. 1B). The PCR reaction mixtures (25 μl) contained 0.25 μM each primer, 200 μM deoxyribonucleotide triphosphates (dNTPs), 1.2 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Gibco BRL, Life Technologies, Burlington, Ontario, Canada), and 50 ng of DNA. The reaction conditions were as follows: initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 20 s, and extension at 72°C for 3 min, with a final extension of 7 min at 72°C. The second PCR step used nested gene-specific *CYP2A7* (2A7ex8F) or *CYP2A6* (2A6ex8F) forward primers with a nested *CYP2A7*-specific reverse primer (2A7R2) to identify the *CYP2A7* wild type and *CYP2A6* duplicated variants, respectively. The PCR reaction mixtures (25 μl) contained 0.25 μM each primer, 200 μM dNTPs, 1.8 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Gibco BRL, Life Technologies), and 1 μl of first step PCR-generated DNA. The reaction conditions were as follows: initial denaturation at 95°C for 1 min, followed by 15 cycles of denaturing at 95°C for 15 s, annealing at 44°C for 20 s, and extension at 72°C for 4.5 min, with a final extension of 10 min at 72°C.

A modified one-step assay for the detection of the duplication variant was also developed (Fig. 1C) that used a *CYP2A7*-specific (2A7ex8F) or *CYP2A6*-specific (2A6ex8F) forward primer with the *CYP2A7* (2A7R1) reverse primer for detection of the wild-type *CYP2A7* and duplicated *CYP2A6* variants, respectively. The PCR reaction mixtures (25 μl) contained 0.25 μM each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Gibco BRL, Life Technologies), and 50 ng of DNA. The reaction conditions were as follows: initial denaturation at 94°C for 1 min, followed by 31 cycles of denaturing at 94°C for 15 s, annealing at 44°C for 20 s, and extension at 72°C for 4.5 min, with a final extension of 7 min at 72°C. PCR products (8 μl, 1258 bp) were separated by gel electrophoresis on a 1.2% agarose gel stained with ethidium bromide. This assay can also be performed using the *CYP2A7* R2 primer producing a PCR product of 1180 bp.

Quantification of Genomic CYP2A DNA. To assess whether the *CYP2A6/7* variant identified was a hybrid variant or was a duplicated variant (e.g., existing in addition to the *CYP2A6* wild-type gene), we quantified *CYP2A6* and *CYP2A7* DNA from samples genotyped as being *CYP2A6*1/*1* or *CYP2A6*1/*1* plus the variant (*CYP2A6/7*) allele. To assess the amount of *CYP2A6*-coding region DNA that was present in the samples, we used PCR to amplify *CYP2A6* genomic DNA from exon 1 to 4 using the 2A6ex1F and 2A6ex4R primers (the PCR product is 1.7 kb). Both the wild-type *CYP2A6* gene and the *CYP2A6/7* variant would be amplified by these primer pairs. The PCR reaction mixtures (25 μl) contained 0.25 μM each primer, 200 μM dNTPs, 1.2 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Gibco BRL, Life Technologies), and 50 ng of DNA. The reaction conditions were as follows: initial denaturation at 94°C for 1 min, followed by 33 cycles of denaturing at 94°C for 15 s, annealing at 60°C for 20 s, and extension at 72°C for 3.5 min, with a final extension of 7 min at 72°C.

To assess the amount of 3′-flanking *CYP2A6* DNA that was present in the samples, we amplified genomic DNA using a common forward primer (2Aex7F) with *CYP2A6*-specific (2A6R2) reverse primer. This primer pair amplifies DNA from the wild-type *CYP2A6* gene (and also *CYP2A6*4*, although not tested here) but not from the duplicated *CYP2A6/7* gene, which has *CYP2A7* 3′-flanking sequence (the PCR product is 1883 bp). To assess the amount of 3′-flanking *CYP2A7* DNA that was present in the samples, we amplified genomic DNA with a common forward primer (2Aex7F) with *CYP2A7*-specific (2A7R2) reverse primer. This primer pair amplifies DNA from the wild-type *CYP2A7* gene and also the *CYP2A6/7* duplication variant. The PCR reaction mixtures (25 μl) contained 0.25 μM each primer, 200 μM dNTPs, 1.2 mM $MgCl_2$, 0.6 U of *Taq* DNA polymerase (Gibco BRL, Life Technologies), and 50 ng of DNA. The reaction conditions were as follows: initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturing at 94°C for 15 s, annealing at 55°C for 20 s, and extension at 72°C for 3 min, with a final extension of 4 min at 72°C.

Conditions of linearity were established for each primer pair using serial dilutions of the cosmid clone containing *CYP2A6* (for 2A6ex1F with 2A6ex4R and 2Aex7F with 2A6R2 PCR reactions) and *CYP2A7* (for 2Aex7F with 2A7R2 PCR reactions). Genomic *CYP2A6, CYP2A7*, and *CYP2A13* DNA from the cosmid clones were used to confirm isozyme specificity of the PCR reactions and primer pairs. The reaction mixtures for each of the three sets of CYP2A primer pairs (assayed separately) were the same as used for the one-step genotyping assay. In a separate experiment we controlled for the amount and quality of the genomic DNA (50 ng) from the samples by amplifying the housekeeping gene β-actin (conditions from Tyndale et al., 1994); the assay linearity was established using a serial dilution of liver DNA (20 cycles of PCR used).

TABLE 1
Primers used for PCR amplification, sequencing, and genotyping

Primer[a] Name	Sequence	Location
2A6ex1F[b]	5′-GCT GAA CAC AGA GCA GCT GTA CA-3′	Exon 1
2AE3F[c]	5′-GCG TGG TAT TCA GCA ACG GG-3′	Exon 3
2A6WTF[b]	5′-CTC ATC GAC GCC CT-3′	Exon 3
2Aex7F[d]	5′-GGC CAA CAT GCC CTA CAT G-3′	Exon 7
2A6ex8F[d]	5′-CAC TTC CTG AAT GAG-3′	Exon 8
2A7ex8F[d]	5′-CAT TTC CTG GAT GAC-3′	Exon 8
2A6ex9F	5′-CAC CTA AGG ACA TTG ACG TGT CCC-3′	Exon 9
2A6In9F	5′-AAA AGG AGA TGA CGG CAC AGC-3′	Intron 8
2AE3R[c]	5′-TCG TCC TGG GTG TTT TCC TTC-3′	Intron 3
2A6ex4R[b]	5′-GGA GGT TGA CGT GAA CTG GAA GA-3′	Exon 4
2A6R1[d]	5′-GCA CTT ATG TTT TGT GAG ACA TCA GAG ACA A-3′	3′ flanking
2A7R1	5′-GCA CTT ATG TTT TGT GAG ACA TCA GAT AGA G-3′	3′ flanking
2A6R2[d]	5′-AAA ATG GGC ATG AAC GCC C-3′	3′ flanking
2A7R2	5′-AAA ATG GGC ATG AAC GCT T-3′	3′ flanking
2A6S1F	5′-GAA GAG TAG TAA TAA TAG CAG-3′	3′ flanking
2A6S2F	5′-AGG GAC ACA ACG AGA CAT GA-3′	3′ flanking
2A6S3F	5′-GCA CAA TCC TTG AAA GAA GC-3′	3′ flanking

[a] F, forward upstream primer; R, reverse downstream primer.
[b] Primers from Oscarson et al. (1998).
[c] Primers from Fernandez-Salguero et al. (1995).
[d] Primers from Oscarson et al. (1999b).

After quantitative PCR conditions were established each sample was assessed with each primer pair two to three times on different days. In addition, using our duplication variant genotyping assay, we identified an additional 11 individuals in our database with the duplication variant (N = 16 total genotyped as homozygous *CYP2A6*1/*1* plus the duplication variant); these individuals were included in the assessment of the amount of genomic *CYP2A* DNA, but their smoking demographics were not available.

Statistics. The null hypothesis was tested (i.e., increased number of *CYP2A6* gene copies results in increased indices of smoking) by one-tailed t tests based on the pooled error term from a one-way ANOVA. Significance was set at $P \leq .05$.

Results

Identification of a Duplication Variant. To test whether unequal crossover events between the *CYP2A6* and *CYP2A7* genes had occurred (Fig. 1A), resulting in deleted and duplicated *CYP2A6* alleles, we amplified DNA from individuals with low and high NIC oxidase activity using *CYP2A7* forward and *CYP2A6* 3′-flanking reverse primers for deletion variants and *CYP2A6* forward and *CYP2A7* 3′-flanking reverse primers for duplication variants. Amplification products as well as the *CYP2A6* and *CYP2A7* cosmid clones were sequenced from exon 8 to 350 bp downstream of the stop codon (Fig. 2). The duplication crossover junction extends 219 bp upstream of the stop codon to 49 bp downstream of the stop codon (268 bp), in contrast to the crossover junction for the *CYP2A6*4A* deleted allele that occurs more than 106 bp downstream of the stop codon but consistent with the crossover position of the recently identified *CYP2A6*4D* allele (Oscarson et al., 1999a,b). The duplication crossover junction is defined by 15 positions of upstream sequence, which are identical with *CYP2A6*, and 35 downstream positions, which are identical with *CYP2A7*. It includes 4-bp positions (810, 819, 836, 892), which are uninformative because of reported *CYP2A6* and *CYP2A7* sequence polymorphisms (GenBank accession numbers: U22028, M33317, M33318; Nunoya et al., 1999; Oscarson et al., 1999a,b). Of note, after DNA sequencing, it was observed that two of the five DNA samples with the duplication variant contained a T at nucleotide 819 (Fig. 2) in contrast to the wild-type G, which would result in an amino acid change from glycine (GGC) 479 to valine (GTC). This is the same

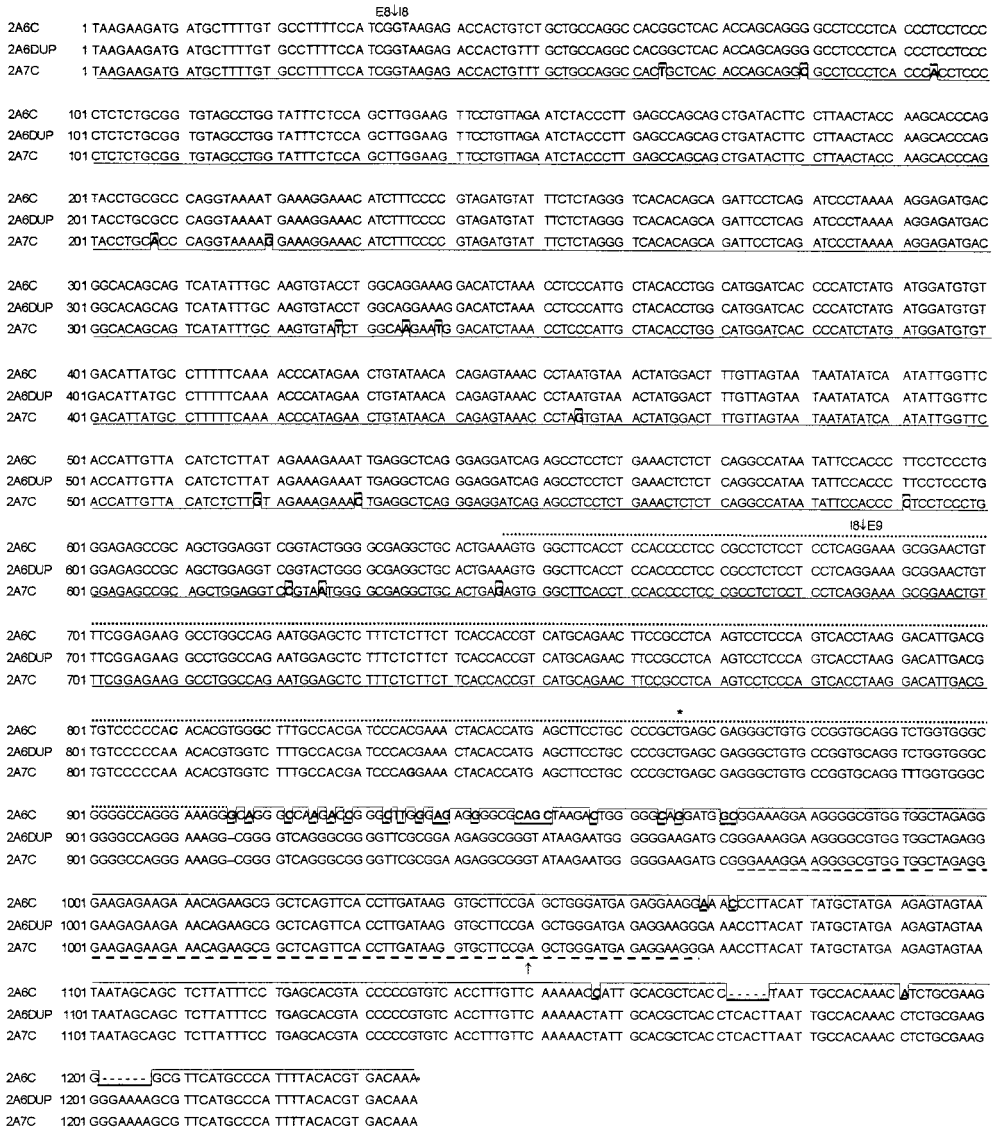

Fig. 2. Alignment of DNA sequence for the *CYP2A6* and *CYP2A7* wild-type cosmid clones and the duplicated *CYP2A6/7* gene. Positions of the intron and exon boundaries (i.e., In8 ↓ Ex9) and stop codon (asterisk) are noted. The duplication crossover region (stippled bar above sequence) contains 4 bp (810, 819, 836, 892) that are not informative for defining the region due to sequence polymorphisms. This region is consistent with the crossover region defined for the deletion variant *CYP2A6*4D* (Oscarson et al., 1999a). The other crossover junction, creating the *CYP2A6*4A* deletion variant (stippled underline), was identified by Oscarson et al. (1999b) with the specific position (as identified by Nunoya et al., 1999) indicated by an arrowhead. In the five duplication variants that were sequenced, three had the *CYP2A6* wild-type G at nucleotide 819, and two had a T that causes an amino acid change from glycine to valine, which we also find in the *CYP2A7* gene.

nucleotide change identified by Oscarson et al., (1999a), and when found in the *CYP2A6* gene was referred to as the *CYP2A6*5* variant; their paper suggests that this nucleotide alteration changes glycine 479 to a leucine amino acid, resulting in a null allele.

Genotyping Assay for the Duplication Variant. With an approach based on the *CYP2A6*4* deletion assay (Fig. 1B; Oscarson et al., 1999b), we designed and tested a two-step genotyping assay for the detection of the *CYP2A6/7* duplicated and *CYP2A7* wild-type genes. Assay specificity was tested using DNA from individuals of known genotypes (*CYP2A6*1, *2*, and **4*), as well as sequenced duplication variants (Fig. 3A). We also developed a rapid one-step assay (Fig. 1C) for the wild-type *CYP2A7* and duplicated *CYP2A6* variants using the gene-specific exon 8 *CYP2A6* or *CYP2A7* forward primer paired with the gene-specific *CYP2A7 R1* 3′ reverse primer (Fig. 3B). This assay can also be performed using the *CYP2A7 R2* reverse primer. Using either the two-step or one-step assays we detected the duplicated variants but had no false-positive results from the samples without the duplication. However, we were able to detect a wild-type *CYP2A7* gene product in a sample genotyped as *CYP2A6*4/*4* (Fig. 3), which is not predicted from the scheme illustrated in Fig. 1. We assayed two other homozygous *CYP2A6*4/*4* samples from our database and detected a *CYP2A7* exon 8–3′ PCR product but no *CYP2A6*-coding region product (e.g., Fig. 4B).

Is the Duplication Variant Present with, or instead of, the Wild-Type *CYP2A6* Gene? To assess whether the novel hybrid variant *CYP2A6/7* that we had identified existed (as predicted, Fig. 1A) with the wild-type *CYP2A6* gene, as opposed to replacing it, we determined the amount of DNA from the coding region of *CYP2A6*, as well as from the 3′-flanking region of *CYP2A6* and *CYP2A7*, in individuals with the duplication variant and compared the amount of DNA to those with a homozygous wild-type genotype. Fig. 4A illustrates the standard curve for the PCR primer pair spanning exon 1 to 4 using a serial dilution of DNA from the cosmid clone containing the *CYP2A6* gene. A typical ethidium stained agarose gel of the PCR products from this amplification is shown in Fig. 4B. Using this assay we measured the amount of DNA PCR product in five samples with the wild-type (*CYP2A6*1/*1*) genotype and five samples containing the duplication variant (Dup) as illustrated in Fig. 4B. Amplification using these primers with a *CYP2A6*4/*4* homozygous individual or *CYP2A7* cosmid clone as template DNA indicates gene specificity of the assay. For each sample assayed we also assessed the amplification of genomic DNA using β-actin primers. The standard curve for β-actin was created using dilution curves of hepatic genomic DNA and is illustrated in Fig. 4C.

When five samples in each group were reassayed six times (as illustrated in Fig. 4B), and adjusted for individual levels of β-actin, we found that those samples with the duplication had higher levels of *CYP2A6*-coding DNA (exon 1–4) relative to those with a wild-type genotype (247 ± 40 versus 122 ± 6 optical density units, respectively, $P < .004$). This provided the first evidence for a gene duplication event resulting in more copies of the coding region of *CYP2A6* rather than the

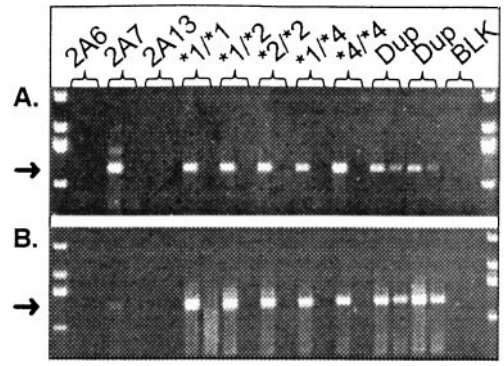

Fig. 3. A, genotyping assay results from the two-step duplication genotyping method. The first-step uses a forward primer (2A-ex7F) common to *CYP2A6* and *CYP2A7* and a *CYP2A7*-specific reverse primer (2A7R1). The second step utilizes *CYP2A7*-specific (2A7ex8F) or *CYP2A6*-specific (2A6ex8F) forward primers with a nested *CYP2A7*-specific primer (2A7R2) for detection of the wild-type *CYP2A7* gene (first lane of each pair) or the duplicated *CYP2A6* gene (second lane of each pair), respectively. The arrow indicates the 1180-bp second step PCR product (outer lanes show the 3054-, 2036-, 1636-, and 1018-bp molecular markers) B, the one-step assay combines the *CYP2A7*-specific (2A7-ex8F) or the *CYP2A6*-specific (2A6ex8F) forward primer with the *CYP2A7*-specific reverse primer (2A7R1). The first lane of each pair contains the *CYP2A7* positive control and the second lane indicates detection of the duplication variant (arrow indicates the 1258-bp product).

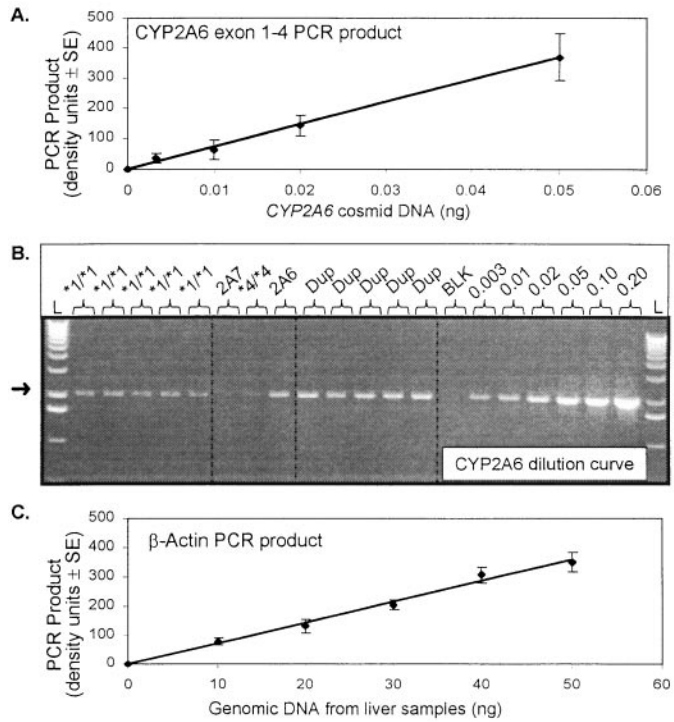

Fig. 4. A, standard curve demonstrating a linear relationship between amount of *CYP2A6* as template and detection of PCR product using exon 1–4 PCR primer pairs (see text for details). B, an ethidium-stained gel illustrating the PCR product formation using *CYP2A6* exon 1 and 4 primer pairs. DNA samples of different genotypes, as well as a representative dilution curve of *CYP2A6* cosmid genomic DNA (0.003–0.20 ng DNA), are shown. Samples with the wild-type genotype (**1/*1*) have lower amounts of PCR product (50-ng DNA template) than samples with the duplication variant (Dup). The primers do not amplify DNA from the *CYP2A7* gene, as indicated by the lanes containing DNA from the *CYP2A7* cosmid clone (2A7) and a sample that is homozygous for the deletion variant (**4/*4*) in contrast to the lanes containing the *CYP2A6* cosmid DNA (2A6). C, A standard curve indicating the relationship between the amount of DNA (hepatic genomic DNA from two different samples, repeated three times) used and the amount of β-actin PCR product produced (for conditions see text). β-actin was used as a control for the DNA in the assessment of *CYP2A* copy number (Fig. 5).

novel variant being the result of a gene-conversion event with no increase in *CYP2A6* gene copy. The ratio of PCR product was 4:2 (247:121) rather than the expected 3:2 that would be predicted if one assumes that the PCR product is derived from two copies in the *CYP2A6*1/*1* group and two copies plus the duplicated allele in the other group. This suggested that additional duplicated copies may be present; hence, we repeated these studies in larger numbers of samples.

Having established the linearity of the assays, we screened our database for additional DNA samples containing *CYP2A6* duplication variants. We used 28 samples that were homozygous *CYP2A6*1/*1* and 16 samples from individuals who we genotyped as having the *CYP2A6/7* hybrid duplication variant (five from the current data set and 11 that were identified in our database). β-actin PCR product was also determined using DNA from each sample. As expected from the postulated mechanism (Fig. 1A), we found that significantly more DNA was amplified, using primers for *CYP2A6* exon 1 to 4, in the samples with the duplicated variant compared to those without it (Fig. 5A).

To further extend these observations, we amplified DNA with a selective *CYP2A6* reverse primer in the 3′-flanking region that would not amplify the duplication variant. We found similar amounts of PCR DNA product for the 3′-flanking region of *CYP2A6* in samples from both groups, consistent with similar copy numbers of the wild-type *CYP2A6* gene being present in samples from both groups (Fig. 5B). This indicated that the source of the increased amount of *CYP2A6*-coding region DNA observed in samples genotyped as having the duplication variant (Fig. 5A) was most likely due to the duplication variant itself rather than to multiple copies of the wild-type *CYP2A6* gene. When we amplified the 3′-flanking region of *CYP2A7,* we expected, and found, increased amounts of this PCR DNA product in the samples genotyped with the duplication variant, consistent with the duplicated *CYP2A6* allele having a 3′-flanking region of *CYP2A7* sequence (Fig. 5C). Together these data provide initial evidence that the duplicated *CYP2A6/7* gene variant exists with a wild-type *CYP2A6* gene on chromosome 19, rather than replacing it as an allelic variant per se. As with the smaller sample set originally tested, we observed a ratio higher than 2:3 for both the *CYP2A6*-coding region (Fig. 5A, mean ± S.E.M., 4.5 ± 0.3) and the *CYP2A7* 3′-flanking region (Fig. 5C, 3.5 ± 0.4), suggesting that there may be more than one copy of the duplicated variant in all or some of the individuals genotyped with the duplication variant.

***CYP2A6* Allelic Frequencies in Caucasian Smokers.** We examined the frequency of the variant alleles in Caucasian smokers from the original data set (N = 296; Table 2); only four individuals in the study were nondependent smokers (all had *CYP2A6*1/*1* genotypes). An allele frequency of 1.35% was observed for *CYP2A6*2* [(one homozygote and six heterozygotes (8/592)], whereas the *CYP2A6*4* allele was found at a frequency of 1.18% [seven heterozygotes (7/592)]. The genotype frequencies for either *CYP2A6*2* or *CYP2A6*4* were not significantly different from the genotype frequencies predicted by Hardy-Weinberg equilibrium. These individuals (with *CYP2A6*2* or **4* alleles) were combined to form a decreased activity group 1 (N = 14, one or fewer active *CYP2A6* allele). The majority of the smokers were *CYP2A6*1/*1* individuals and constituted group 2 (N = 277, two active alleles). The *CYP2A6*-duplicated gene was detected in five persons, indicating a gene duplication prevalence of 1.7% (5/296) in this population (group 3, N = 5; three or more active copies). As mentioned above, two of the five samples with the duplicated gene contained a nucleotide change G819T, consistent with the previously identified mutation in the wild-type gene (*CYP2A6*5*; Oscarson et al., 1999a).

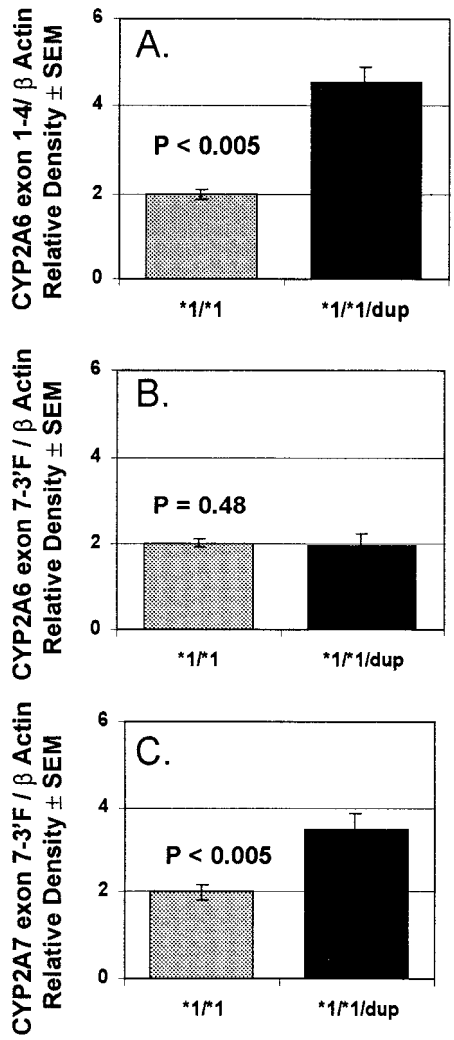

Fig. 5. Individuals genotyped as having the *CYP2A6/7* gene variant have greater amounts of *CYP2A6*-coding region DNA, consistent with a *CYP2A6* gene duplication event. PCR amplification was performed on DNA from individuals genotyped as *CYP2A6*1/*1* (N = 28) and those genotyped as *CYP2A6*1/*1* plus the duplicated allele (N = 16). The data are expressed by the amount of *CYP2A6* (exon 1–4 or exon 7–3′-flanking) and *CYP2A7* (exon 7–3′-flanking) relative to β-actin grouped by genotype. To simplify interpretation of the data, the ratio of PCR product (e.g., *CYP2A6* exon 1–4/β-actin) detected in the samples was adjusted so that the samples genotyped as *CYP2A6*1/*1* had a ratio of 2 to illustrate the two chromosomal copies; the relative ratios between groups for each experiment was not affected by this graphical adjustment. A, the amount of PCR product from amplification of *CYP2A6* exon 1–4 (using 2A6-ex1F and 2A6-ex4R primers) relative to β-actin indicates significantly greater amounts of *CYP2A6*-coding region DNA in the group with the duplication (dup). B, similar amounts of *CYP2A6* 3′-flanking DNA PCR product (using 2A-ex7F and 2A6R1 primers) relative to β-actin DNA occurs in both groups, consistent with the 3′-flanking region of the duplication variant being *CYP2A7* sequence rather than *CYP2A6*. C, Significantly more *CYP2A7* 3′-flanking DNA PCR product (using 2A-ex7F and 2A7R1 primers) is detected in individuals with the *CYP2A6/7*-duplicated variant, consistent with a gene duplication as described in Fig. 1.

We also assessed the impact of *CYP2A6* genetic variation on in vivo smoking with both the current smoking levels and the number of CIGs smoked during the heaviest period of life-time smoking. The reported number of current CIGs per day was significantly lower for those in group 1 (13.5 ± 2.3) compared to those in group 2 (19.5 ± 0.7, $P < .03$). This was also true for the period of heaviest regular smoking (19 versus 28, $P < .001$, group 1 versus group 2, respectively). However, people smoke CIGs with different intensities, and self-reported CIG numbers smoked may not be an accurate or robust smoking index; therefore, CO levels were used as an additional index of smoking behavior and inhalation. An ascending rank order was observed for the impact of the *CYP2A6* genetic variation on CO levels [parts per million (ppm), Table 2; Fig. 6A]. Group 1 (one or fewer active alleles) had significantly lower CO levels than did individuals homozygous for wild-type alleles (group 2) and individuals with duplicated *CYP2A6* (group 3). To further examine smoking and kinetic variables among the three groups, we compared COT plasma levels (Table 2; Fig. 6B). Significantly higher COT levels were observed in group 3 compared with both the group of homozygous wild-type individuals (group 2) or group 1, those with at least one null allele. Although not reaching significance, the ratio of plasma NIC to COT (a measure of phenotype) followed a descending rank order with the highest ratio found in group 1 (0.20 ± 0.11); group 2 had intermediate ratios (0.12 ± 0.03) and group 3, with the duplicated variants, had the lowest ratios (0.09 ± 0.01).

However, although the individuals with duplicated alleles (group 3) had higher breath CO and COT levels indicating greater smoking behavior than the other two groups, they reported smoking fewer CIGs per day, both at the current time (mean, 13.3 ± 3.3; range, 6 to 25) and at the time of heaviest smoking (23 CIGs/day; range, 15 to 35), than would be expected. However, they appeared to smoke the CIGs more intensely, as suggested by an almost double CO to CIG ratio (2.1 ± 0.7) compared with either group 2 (1.2 ± 0.1) or group 1 (1.2 ± 0.2, $P \leq .05$) and by higher plasma NIC to CIG ratios (group 3, 3.0 ± 1.0) compared with group 2 (1.6 ± 0.4) or group 1 (1.4 ± 0.3, $P < .02$) (current CIGs per day used to match current COT and CO results). Thus, group 3 appears to compensate for more *CYP2A6* gene copies by increasing the intensity of smoking, whereas those individuals with null alleles and slower NIC metabolism (group 1) compensated by decreasing the number of CIGs per day, but smoking them with the same intensity as those individuals with normal *CYP2A6* levels (group 2). There were no statistically significant differences in smoking demographics between those individuals with the duplication variant with, or without, the $T^{819}G$ mutation. For example, CO levels were 23 ppm in the individuals with the mutation ($N = 2$) and 22 ppm in individuals without the mutation ($N = 3$, $P = .9$).

Discussion

CYP2A6-mediated coumarin 7-hydroxylase and NIC oxidase activities are highly variable (Yamano et al., 1990; Iscan et al., 1994; Messina et al., 1997), suggesting many *CYP2A6* gene variants of increased and decreased activity may exist. To determine the molecular mechanisms involved in the very low and high *CYP2A6* activity individuals, we searched for deleted and duplicated copies of the *CYP2A6* gene, derived from unequal crossover of the *CYP2A7* and *CYP2A6* genes (Fig. 1A), analogous to the mechanism proposed for *CYP2D6* (Gaedigk et al., 1991). We identified both deletion and duplication variants using a PCR approach and developed a genotyping assay for the duplicated variant to assess the impact of these variants on NIC metabolism and smoking.

The location of the crossover point between *CYP2A7* and *CYP2A6* in the deletion variant (*CYP2A6*4A*) is 106 to 201 bp downstream from the stop codon (Fig. 2; Nunoya et al., 1999; Oscarson et al., 1999a,b). In contrast, the duplication variant (*CYP2A6/7*) that we have identified is derived from an upstream unequal crossover that spans the stop codon and is consistent with the reported crossover point of the *CYP2A6*4D* variant (Oscarson et al., 1999a). In addition, this region contains the *CYP2A6*1B* and *CYP2A6*5* variants, which are also thought to have arisen by unequal crossover between the *CYP2A6* and *CYP2A7* genes (Oscarson et al., 1999a). From exon 8 to the 3′-flanking region is highly conserved among the *CYP2A* genes (Fig. 2), making them good candidates for unequal crossover events; it is very possible that there are additional uncharacterized duplication and deletion variants with crossover positions in this same region. Therefore, we adapted the genotyping method of Oscarson et al. (1999b) to identify *CYP2A6* gene duplications occurring from unequal crossover anywhere within this region. We propose that screening of large populations for the duplicated allele could be done with a one-step assay (Figs. 1C and 3B), followed by confirmation with the two-step assay (Figs. 1B and 3A). The current *CYP2A6*2, *3, *4, *5* and duplicated alleles do not account for all of the metabolic outliers identified in our studies or those of other investigators (Benowitz et al., 2000), indicating that other *CYP2A6* variant alleles exist.

Although the frequencies of the variant alleles in Caucasian smokers were low, we were able to detect an effect of the different genotypes on smoking indices. Our current data retest one portion of our previous findings (Pianezza et al., 1998). In the present study we demonstrate that CIG smok-

TABLE 2
Impact of *CYP2A6* genotype on indicies of smoking

	Group 1	Group 2	Group 3
Genotype	*CYP2A6*2/2*, $N = 1$ *CYP2A6*1/2*, $N = 6$ *CYP2A6*1/*4*, $N = 7$	*CYP2A6*1/*1*, $N = 277$	*CYP2A6*1/*1* plus duplication, $N = 5$
Gender	F = 8/M = 6	F = 143/M = 144	F = 4/M = 1
CO (ppm)	14 ± 2	20 ± 0.6[a]	23 ± 4[a]
Cotinine plasma levels (ng/ml)	217 ± 76[b]	265 ± 9[b]	378 ± 76

F, female; M, male.
[a] Significantly different from group 1 ($P < .05$).
[b] Significantly different from group 3 ($P < .05$).

ers with *CYP2A6* null alleles (**2 or *4*) smoke fewer CIGs per day than do homozygous wild-type smokers both currently (13.5 ± 2.3 versus 19.5 ± 0.7, $P < .03$) and at the time of heaviest smoking (19 versus 29, $P < .001$). In addition, we have shown that they have lower breath CO levels (Fig. 6A), a measure that does not rely on self-report. They also have lower COT levels (Fig. 6B), which indicates decreased smoking and metabolism of NIC to COT. These data indicate a role for *CYP2A6* gene variants in affecting smoking behavior, with slower metabolizers smoking less than faster metabolizers. We have confirmed these data independently using inhibition of *CYP2A6* in smokers in vivo, observing a significant decrease in smoking (e.g., decreased CO levels, increased latency between CIGs) in the presence of a *CYP2A6* inhibitor (Sellers et al., 2000a).

The individuals with duplicated *CYP2A6* (group 3) smoked more, as evidenced by higher CO levels (Fig. 6A) and plasma COT levels (Fig. 6B); however, they reported fewer CIGs per day than expected, leading us to hypothesize that they may smoke more intensely rather than more frequently. This is supported by higher NIC/CIG and CO/CIG ratios than found in the other two groups. Despite the lack of controlled phenotyping conditions (i.e., dose and timing not controlled by investigator, inhalation route used avoiding the first pass effect that often contributes substantially to the metabolic ratio, single time-point plasma collection), the ratios of NIC/COT demonstrated a rank order (not significant), with the lowest ratio in the duplicated group 3, followed by the intermediate wild-type group 2 and the group with null allele carriers (group 1). This suggests that, with some refinement of the methodology, plasma NIC/COT ratios in smokers may be useful for finding those individuals with variant *CYP2A6* alleles in the absence of investigator-administered NIC or coumarin. Our data suggest that the individuals in this study have between one and three copies of the duplicated variant as well as the wild-type *CYP2A6* (Fig. 5). It is clear that further analysis of the variant duplicated allele is required, including formal in vivo NIC and coumarin kinetic studies and smoking demographic analysis of larger numbers of individuals, as well as Southern blotting and expression studies to clarify copy number and impact of the nucleotide changes.

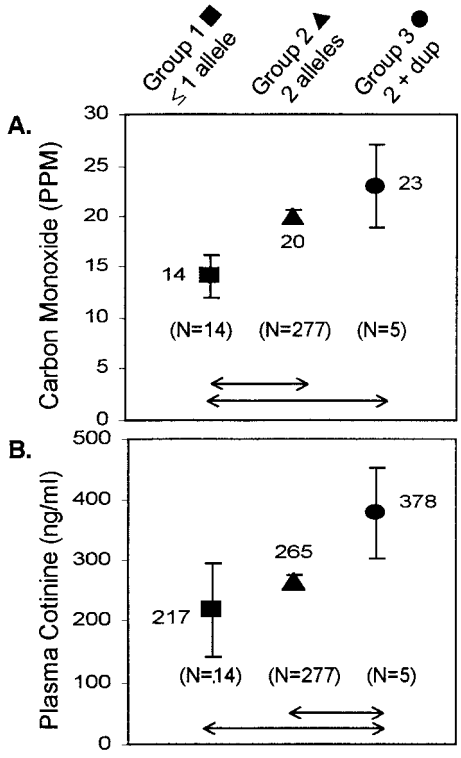

Fig. 6. Impact of *CYP2A6* genetic variation on breath CO levels (A) and plasma COT levels (B). Group 1 (■, one or fewer active gene copies) contains individuals with null alleles, group 2 (▲, two active gene copies) contains *CYP2A6*1/*1* individuals, and group 3 (●, three or more active gene copies) contains individuals with duplicated *CYP2A6* gene variants. Data are given as mean ± S.E.M. Arrows indicate pairs that are significantly different ($P \leq .05$).

Tobacco smoke contains a number of tobacco-specific procarcinogen nitrosamines, e.g., *N*-nitrosodiethylamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, and *N*′-nitrosonornicotine, that CYP2A6 can activate via α-hydroxylation (Crespi et al., 1990; Patten et al., 1997). Therefore, individuals who have *CYP2A6* null alleles may also be less efficient at bioactivating tobacco smoke procarcinogens to carcinogens, whereas those with duplications may be more efficient. This is of particular interest because ethnic variation in frequencies of *CYP2A6* variant alleles exist (Oscarson et al., 1998; Yokoi and Kamataki, 1998, 1999b) and may be related to the ethnic differences in lung cancer incidence and histology (Groeger et al., 1997). The role of *CYP2A6* in levels of smoking and procarcinogen activation is supported by the recent study of Miyamoto et al. (1999), who found that having the *CYP2A6*4* allele resulted in a significant reduction in risk for lung cancer. The decreased risk observed could be due to the gene's impact on amount smoked (decreasing exposure to procarcinogens) and/or on the decreased activation of procarcinogens. To examine the in vivo role of *CYP2A6* in the activation of procarcinogens, we have blocked *CYP2A6* activity in smokers using methoxsalen, a *CYP2A6* inhibitor. Our preliminary data suggest a significant rerouting of the *N*-nitrosodiethylamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone nitrosamines from the mutagenic α-hydroxylation pathways to the nonmutagenic 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol glucuronidation pathway (Sellers et al., 2000b).

In summary, we have demonstrated reduced smoking behavior (CO levels, CIGs per day, and COT levels) for those with fewer copies of the active *CYP2A6* gene compared with individuals homozygous for the wild-type allele. We have also identified a putative *CYP2A6* gene duplication and established a genotyping assay for its detection. Individuals with the duplication variant had higher breath CO and COT levels, suggesting higher levels of smoking, although they reported fewer CIGs per day, suggesting that they smoke each CIG with greater intensity (higher CO/CIG and NIC/CIG ratios). These data demonstrate that *CYP2A6* gene variants exist that have an impact on smoking behavior, suggesting a significant role for *CYP2A6* in smoke exposure and potentially in the etiology of tobacco-related cancers. Our data suggest also that mimicking the decreased activity variants by inhibiting the activity of *CYP2A6* may produce the same benefits that are imparted by the null alleles, providing novel therapeutic approaches to prevention and treatment of tobacco smoking.

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