Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5237–5241, June 1991
Medical Sciences

Molecular mechanism of slow acetylation of drugs and carcinogens in humans

(genetic polymorphism/arylamine *N*-acetyltransferase/chemical carcinogenesis/allele-specific amplification)

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Communicated by Gottfried Schatz, March 11, 1991

ABSTRACT **The acetylation polymorphism is one of the most common genetic variations in the transformation of drugs and chemicals. More than 50% of individuals in Caucasian populations are homozygous for a recessive trait and are of the "slow acetylator" phenotype. They are less efficient than "rapid acetylators" in the metabolism of numerous drugs and environmental and industrial chemicals. The acetylation polymorphism is associated with an increased risk of drug toxicity and with an increased frequency of certain cancers. We report the identification of the primary mutations in two alleles of the gene for the *N*-acetyltransferase (NAT; acetyl-CoA:arylamine *N*-acetyltransferase, EC 2.3.1.5) isozyme NAT2 associated with slow acetylation. These alleles, M1 and M2, account for more than 90% of slow acetylator alleles in the European population we have studied. M1 and M2 were identified by restriction fragment length polymorphisms with *Kpn* I and *Msp* I and subsequently cloned and sequenced. M1 and M2 each are characterized by a combination of two different point mutations, one causing an amino acid substitution (Ile-113 → Thr in M1, Arg-197 → Gln in M2), the other being silent (C 481 → T in M1, C 282 → T in M2). Functional expression of M1 and M2 and of chimeric gene constructs between mutant and wild-type *NAT2* in COS-1 cells suggests that M1 causes a decrease of NAT2 protein in the liver by defective translation, whereas M2 produces an unstable enzyme. On the basis of the mutations described here and a rare mutant allele (M3) reported recently, we have developed a simple DNA amplification assay that allows the predictive genotyping of more than 95% of slow and rapid acetylator alleles and the identification of individuals at risk.**

The acetylation polymorphism is one of the most common inherited variations in the biotransformation of drugs and chemicals. Its association with drug toxicity and an increased risk to develop certain cancers has made it one of the oldest and best-studied examples of a pharmacogenetic condition (1–3). Forty to 70% of Caucasians in Europe and North America are of the "slow acetylator" phenotype and are less efficient than "rapid acetylators" in the metabolism of numerous drugs and chemicals containing primary aromatic amine or hydrazine groups. These include agents such as isoniazid, sulfamethazine (SMZ) and other sulfonamides, procainamide, hydralazine, dapsone, and caffeine, as well as several chemicals with carcinogenic potential such as benzidine, 2-aminofluorene, and β-naphthylamine, present in dyes, antioxidants, pesticides, and explosives (2–4). Highly mutagenic and carcinogenic arylamines also are generated during cooking of food (5). Slow acetylators are at higher risk to develop bladder cancer (1–4), whereas rapid acetylators are at higher risk for colorectal cancer (6).

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In previous studies, we have shown that slow acetylators have a quantitative decrease in their liver of a cytosolic arylamine *N*-acetyltransferase (NAT; acetyl-CoA:arylamine *N*-acetyltransferase, EC 2.3.1.5) (7). This enzyme was purified (8) and two human genes, *NAT1* and *NAT2*, encoding two NAT proteins, were subsequently cloned and characterized (9, 10). *NAT2* was identified as the gene encoding the "polymorphic" NAT2 isozyme (10, 11).

To study the molecular mechanism of slow acetylation, we have performed restriction fragment analysis with a *NAT2* probe on genomic DNA samples of individuals whose acetylator phenotype had been established. This led to the identification of two mutant alleles (M1 and M2), which account for over 90% of the alleles associated with slow acetylation. We describe the mutations in the M1 and M2 alleles and how they cause deficient NAT2 in livers of slow acetylators. On the basis of this information and the sequence of a rare *NAT2* allele recently reported from Japan (12, 13), we have developed a simple allele-specific DNA amplification assay that allows the predictive genotyping of slow and rapid acetylators.

METHODS

Acetylator Phenotype. The acetylator phenotype was determined by measurement of the urinary molar ratio of the caffeine metabolites 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1-methylxanthine as described (14), except that urine samples were acidified to pH 3.5 immediately after collection and stored frozen to ensure chemical stability of AFMU, and urine was extracted with 30 vol of 100% chloroform. Liver samples were from the human liver bank at the Biocenter in Basel (7). The activity of NAT2 (*in vitro* phenotype) was determined with the substrate SMZ (7, 8). Experimental protocols for *in vivo* testing and for use of biopsy material were approved by the appropriate ethical review boards at the institutions involved.

Nucleic Acid Isolation and Analysis. High molecular weight genomic DNA was isolated from human liver or blood leukocytes as in ref. 15, and after digestion with restriction endonucleases was analyzed on Southern blots (ref. 16, p. 382). Total liver RNA (17) and poly(A)$^+$ RNA (18) were purified and analyzed on Northern blots (ref. 16, p. 202). Probe DNA for Southern and Northern blots was radiolabeled with [α-^{32}P]dATP by random oligonucleotide priming (19) to specific activities of 1–5 × 10^8 cpm/μg of DNA. Filters were hybridized in 1 M NaCl containing 1% SDS, 10% dextran sulfate, and denatured salmon sperm DNA at 200 μg/ml at 69°C for 18 hr and washed at 65°C twice for 30 min in 2× SSC, twice for 30 min in 2× SSC/1% SDS, and once for 30 min in 0.1× SSC/0.2% SDS (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0).

Abbreviations: NAT, arylamine *N*-acetyltransferase; RFLP, restriction fragment length polymorphism; SMZ, sulfamethazine; wt, wild-type.

Cloning and Sequencing of Mutant *NAT2* Alleles. Mutant allele M1 was isolated from a genomic library constructed in λEMBL3, using DNA from a heterozygous wild-type (wt)/M1 individual. A cDNA encoding the rabbit NAT2 enzyme (20) was used for screening. Details of the cloning of M1 are given in ref. 10. For sequence analysis of M1 the 1.9-kilobase (kb) *Eco*RI fragments containing the single coding exon of *NAT2* were isolated from 12 positive λEMBL3 clones and subcloned in pBluescript (Stratagene). M2 was cloned from DNA of a homozygous M2/M2 individual by screening of a λgt10 library constructed from *Eco*RI-digested genomic DNA after size selection (1.6–2.1 kb) on a 1% agarose gel. The 1.9-kb EcoRI fragment containing the wt *NAT2* coding exon was used as hybridization probe and the inserts of positive phages were also subcloned in pBluescript. Sequences of M1 and M2 were determined by using the dideoxy chain termination method (21) and Sequenase (United States Biochemical).

Cloning of Chimeric *NAT2* Gene Constructs. Chimeric constructs of wt and M1 and M2 alleles were generated by *Xba* I digestion of 1.9-kb *Eco*RI fragments harboring *NAT2* coding exons, fragment isolation, and religation as indicated in Fig. 3.

Functional Expression of *NAT2* Alleles and Chimeric Constructs in Monkey Kidney COS-1 Cells. *Eco*RI fragments (1.9 kb) containing *NAT2* coding exons were subcloned in the vector p91023 (B) (22) and transiently expressed in COS-1 cells as described (23).

NAT Enzyme Assay and Immunoquantification of NAT2 on Western Blots. NAT2 enzyme activity was measured in cytosol from liver and from transfected COS-1 cell cultures by using SMZ as substrate. The NAT assay and the determination of kinetic parameters (K_m, V_{max}) were performed as described (7, 8). Immunoreaction on Western blots used a polyclonal rabbit antiserum raised against purified human NAT2 (8). The serum was prepurified on nitrocellulose strips containing purified rabbit NAT2 (23, 24). Blot intensities of NAT2-specific protein bands at 31 kDa were quantified by using a Camag (Muttenz, Switzerland) TLC scanner in the transmission mode.

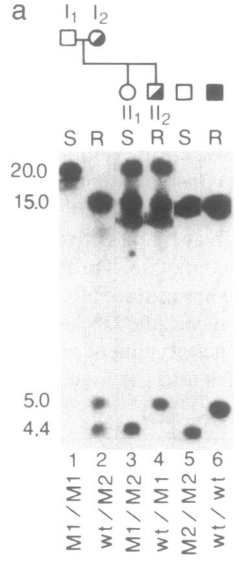
Allele-Specific Amplification of *NAT2* Alleles. Specific primers for the wt and the mutant alleles M1, M2 (this study), and M3 (12) were used in separate polymerase chain reactions (PCRs) (25). Primer "M1 wt" (CTGATTTGGTCCAG) is complementary to the *NAT2* gene at position 481–494, primer "M1 mut" (CTGATTTGGTCCAA) recognizes the mutation C 481 → T of M1; primers "M2 wt" (574–590, TTTACGCTTGAACCTCG) and "M2 mut" (574–590, TTTACGCTTGAACCTCA) test for the presence of the mutation G 590 → A of M2, primer pairs "M3 wt" (857–870, AATAGTAAGGGATC) and "M3 mut" (857–870, AATAGTAAGGGATT) for the mutation G 857 → A (12) of M3. The common primer used for the reactions with primers M1 wt, M1 mut, M3 wt, and M3 mut is "primer 1" (−74 to −58, AATTAGTCACACGAGGA), for the reactions with primers M2 wt and M2 mut it is "primer 2" (1119–1138, TCTAGCATGAATCACTCTGC).

All PCRs were carried out in a total volume of 50 μl in the presence of 10 mM Tris·HCl at pH 8.3, 50 mM KCl, 0.01% gelatin, each dNTP at 0.2 mM, each primer at 0.5 μM, 1.25 units of *Taq* DNA polymerase (Bethesda Research Laboratories), 300–600 ng of genomic DNA, and 1.5 mM $MgCl_2$ (primers M1 wt and M1 mut), 1.25 mM $MgCl_2$ (M2 wt and M2 mut), or 1.75 mM $MgCl_2$ (M3 wt and M3 mut). Thirty cycles [60 s at 94°C, 90 s at 48°C (for M1)/55°C (for M2)/35°C (for M3), 180 s at 72°C] were carried out and were followed by a final extension period of 7 min at 72°C. Ten microliters of each sample was analyzed on a 1.5% agarose gel.

RESULTS

Identification of Mutant Alleles M1 and M2 by Restriction Fragment Length Polymorphism (RFLP) Analysis, Cloning, and Sequence Determination. To identify mutant alleles we initially performed RFLP analysis on genomic DNA samples from 25 healthy individuals whose acetylator phenotype had been established by measuring the acetylated metabolite of caffeine in urine (14), and on DNA from 33 human liver samples with known NAT2 enzyme activity measured with the substrate SMZ (7). Hybridization and wash conditions were chosen so that *NAT2*-specific signals were 10- to 50-fold stronger than signals derived from the two related human genes *NAT1* and *NATP*, which share 87% and 79% nucleotide identity with *NAT2* (10). RFLPs generated by *Msp* I and *Kpn* I segregated with acetylator phenotype; for simplicity and because *Msp* I detected only one mutant allele (M1), only *Kpn* I RFLPs are shown in Fig. 1*a*. The patterns in lanes 1–4 show segregation of *NAT2*-specific fragments in a two-generation family. The wt allele thus was characterized by two bands, 15 and 5 kb, whereas mutant allele M1 was reflected by a single band, 20 kb (lane 1), and mutant allele M2 by two fragments, 15 and 4.4 kb. Examples of DNA of individuals homozygous for M2 and for wt, respectively, are shown in lanes 5 and 6 of Fig. 1*a*.

In both M1 and M2 a point mutation causing a single amino acid change in the deduced protein sequence was combined with an additional silent base substitution (Fig. 1*b*). The silent mutation in M1 alters the recognition sequence for the



b	wt	M1	M2
pos. 282	C	C	T
pos. 341	T (Ile)	C (Thr)	T (Ile)
pos. 481	C	T	C
pos. 590	G (Arg)	G (Arg)	A (Gln)

FIG. 1. Identification of the two mutant *NAT2* alleles M1 and M2 by RFLP analysis and sequence determination of cloned mutants. (*a*) *Kpn* I patterns of a two-generation family (lanes 1–4) and of DNAs of two further individuals. Genomic DNAs (5 μg) were digested with the restriction enzyme *Kpn* I, electrophoresed on 0.5% agarose gels, and hybridized with radiolabeled *NAT2* after transfer to a nylon membrane (ref. 16, p. 382). Hybridization and washes were performed as described in the text. Phenotypes (R, rapid acetylator; S, slow acetylator) are given above each lane; numbers represent size of bands in kb. (*b*) Nucleotide and amino acid changes in M1 and M2, as compared with the wt gene; nucleotide numbering refers to the coding exon (pos. 1 = A in initiator ATG), as in ref. 10.

a

liver no.	4	16	26	30	5
genotype	wt/wt	wt/M1	wt/M2	M1/M1	M2/M2
NAT2 activity (pmol AcSMZ/min·mg)	461	483	293	97	32
NAT2 protein (relative blot intensities of 31kD NAT2 band)	1.0	0.86	0.38	0.06	0

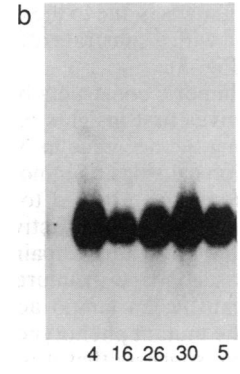


FIG. 2. Comparison of cytosolic NAT2 enzyme activity and immunoreactive NAT2 protein (*a*) and *NAT2*-specific mRNA on a Northern blot [2.5 μg of poly(A)$^+$ RNA per lane] (*b*) in five human liver samples whose genotypes were determined. In *a*, blot intensities of NAT2 protein are expressed as percentage of the most intense band in liver 4, which has the genotype wt/wt.

restriction enzyme *Kpn* I, explaining the observed RFLP (Fig. 1*a*), but the mechanism by which these mutations cause slow acetylation remained unknown.

Relationship Between *NAT2* mRNA, NAT2 Protein, and V_{max} of SMZ Acetylation in Livers of Slow and Rapid Acetylators of Defined Genotype. When liver samples with defined genotype were compared in regard to NAT2 enzyme activity, immunoreactive NAT2 protein, and *NAT2* mRNA (Fig. 2), homozygous and heterozygous rapid acetylator livers with the genotypes wt/wt, wt/M1, and wt/M2 displayed high NAT2 enzyme activities for the acceptor amine SMZ and showed NAT2-specific 31-kDa protein bands on Western blots (Fig. 2). In slow acetylator livers with the genotypes M1/M1, M2/M2, and M1/M2, NAT2 enzyme activity expectedly was markedly reduced and NAT2 protein was barely detectable or absent. But in spite of the correlation between genotype, NAT2 enzyme activity, and immunoreactive NAT2 protein, all samples contained similar quantities of *NAT2*-specific mRNA (Fig. 2*b*). Impaired translation of mutant mRNA and more rapid degradation of the mutant NAT2 protein were therefore considered as potential mechanisms leading to these results. They were tested by functional expression of wt and mutant alleles in monkey kidney COS-1 cells (Fig. 3), using the expression vector p91023(B) (22). The results obtained closely mimicked the *in vivo* situation—apparently equal amounts of mRNA (Fig. 3*c*) were associated with reduced cytosolic enzyme activity (Fig. 3*a*) and decreased immunoreactive protein (Fig. 3*b*) when M1 and M2 were expressed. The affinities of expressed NAT2 for SMZ and for the cofactor acetyl-CoA were found to be identical for the normal (wt) and the mutant proteins (data not shown). These data support our previous findings in human liver samples (7) and suggest that slow acetylation in humans is caused by a decrease in the quantity of a kinetically normal enzyme rather than the presence of a functionally altered protein.

a

allele / construct	wt	M1	M2	C1	C2	C3	C4	C5	C6
NAT2 activity (nmol AcSMZ/min·mg)	22.5	3.4	5.0	34.9	27.2	29.2	4.1	3.9	34.4
% of wt	100	15	22	155	121	130	18	17	153

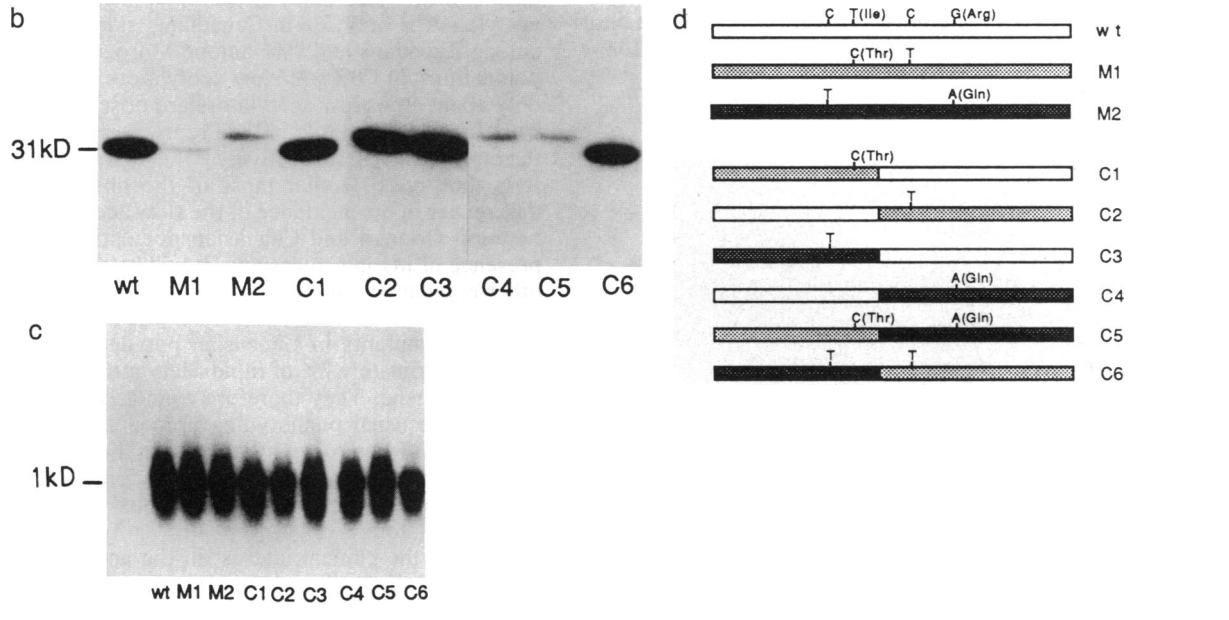


FIG. 3. Expression of wt and mutant *NAT2* alleles and of chimeric gene constructs by transient transfection in monkey kidney COS-1 cells. (*a*) Comparison of NAT2 enzyme activities. (*b*) Immunoreactive NAT2 protein on Western blots (100 μg per lane). (*c*) *NAT2*-specific RNA on Northern blots (1 μg of total RNA per lane). (*d*) Schematic depiction of wt, M1, and M2 alleles and chimeric constructs C1–C6.

Functional Significance of the Mutations in M1 and M2. To dissect the contribution of single mutations, the chimeric gene constructs schematically depicted in Fig. 3*d* were analyzed by transient expression as described above. Constructs C1 and C2, each bearing only one of the two mutations of allele M1, on expression both behaved like the normal enzyme, demonstrating that both nucleotide substitutions of M1 are required to decrease enzyme protein. Analysis of the M2-derived constructs C3 and C4 showed that the silent mutation alone (C3) results in wt behavior and that it is the amino acid change Arg-197 → Gln (C4) which causes the reduction of enzyme protein. In agreement with these results, the construct C5, which carries the two mutations causing amino acid changes of M1 and M2, led to decreased protein and function, whereas the product of C6, which contains the silent mutations of M1 and M2, was indistinguishable from that of the wt gene. We then compared the stabilities of expressed proteins and found that the half-lives at 37°C of wt and M1-derived activities were identical (22 hr), whereas that of M2 was reduced to 6 hr. Thus, the Arg-197 → Gln mutation of M2 apparently produces a less stable protein, whereas the combination of the two nucleotide substitutions in M1 presumably causes defective translation. Obviously, the mechanism by which M1 and M2 cause decreased NAT2 will have to be further studied.

Predictive Genotyping Test by Allele-Specific Amplification. The knowledge of the mutations of M1 and M2, as well as that of a third mutant allele, M3, recently reported by Ohsako and Deguchi (12), was used to develop a set of mutation-specific primers for allele-specific amplification of small amounts of DNA by the PCR (Fig. 4). The study population was composed of 18 phenotyped individuals *in vivo* [caffeine test (14)] and of 26 liver samples phenotyped *in vitro* [SMZ acetylation (7)]. Twenty-four were classified as slow and 20 as rapid acetylators. Our test predicted correctly 19 of the 20 rapid and 22 of the 24 slow acetylator phenotypes. Of the 65 slow alleles identified, M1 accounted for 30 alleles (46%), M2 for 32 alleles (49%), and M3 for only 3 alleles (5%).

DISCUSSION

In the present report we provide evidence that "slow acetylation" in humans is due to mutations in the single coding exon of the *NAT2* gene, one of two closely related genes on chromosome 8 recently characterized in this laboratory (10).

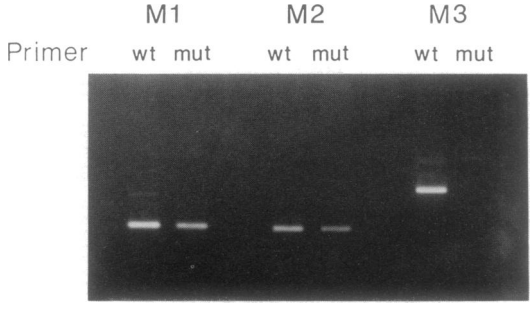


FIG. 4. Allele-specific amplification by PCR for the three mutations associated with the slow acetylator phenotype. The mutation-specific primers (mut) for M1 (lane 2, counting from left) and M2 (lane 4) both yield the correct amplification products (568 and 565 base pairs), whereas no band is visible on the ethidium bromide-stained agarose gel after amplification with the M3-specific primer (lane 6). The DNA of the subject exemplified in lanes 1–6 (KDL 17) therefore has one M1 and one M2 allele. The primer complementary to the wt *NAT2* gene at the site of mutation M1 predictably leads to amplification from allele M2 (lane 1), the corresponding wt primer of M2 binds to allele M1 (lane 3), and the wt primer of M3 amplifies both alleles of this subject (lane 5, 944-base-pair fragment).

Two mutant alleles, M1 and M2, were identified by RFLPs and cloned and sequenced. They account for the majority or >90% of slow acetylator alleles, and on expression in COS-1 cells they cause the marked decrease of NAT2 protein previously observed in livers of slow acetylators (7).

The experiments with expression of wt and mutant *NAT2* genes described in Fig. 3 resolve the old controversy on whether slow acetylation is caused by a quantitative decrease of a functionally normal enzyme (26), or the presence of a functionally altered enzyme (27). According to our results, slow acetylation in humans is due to the quantitative decrease of an enzyme protein with a substrate affinity identical to that of the wt enzyme (Fig. 3).

The analysis of chimeric constructs between wt, M1, and M2 in COS-1 cells gives first insights on how the individual mutations are causing the decrease in NAT2. In the case of M1, both the mutation causing an amino acid change and the silent base substitution are required to result in decreased protein and consequently decreased activity. We assume that translation of mutant mRNA is impaired, possibly by an alteration in mRNA secondary structure. In contrast to M1, the one mutation causing an amino acid change in M2 is sufficient to cause the mutant phenotype, and the analysis of the expressed protein suggests that it is less stable than the wt and M1 derived enzymes.

The molecular mechanism of slow acetylation in humans is fundamentally different from that of the widely used animal model, the New Zealand White rabbit (28), where we have observed that a deletion causes the total absence of the NAT2 protein (23).

A recent study from Japan (12, 13) described M2 and a third mutant allele, M3, by RFLP analysis. A point mutation (G 857 → A) in M3 was reported by these investigators (12). We have applied the knowledge of the primary mutations of M1 and M2 described here and of M3 to develop a simple DNA genotyping assay. It is based on allele-specific DNA amplification by PCR. So far, we have analyzed 92 alleles, of which 65 were identified as mutant alleles, M1, M2, or M3. Over 93% of phenotypes were correctly predicted. Additional mutant alleles presumably account for the nonpredicted slow acetylator phenotypes.

The proportion of rapid and slow acetylators varies remarkably in populations of different ethnic background or geographic origin (1, 2). For example, the percentage of slow acetylators is only 5% in Canadian Eskimos, but it is 87% among Egyptians and 90% among Moroccans. In European populations 40–70% are slow acetylators, whereas in Japan only about 10% slow acetylators are observed. In the study from Japan (12, 13) M2 and M3 represented 78% and 22% of the mutant alleles, respectively, while M1 was not observed. It is thus possible that most of the observed interethnic differences in the incidence of the slow acetylator phenotype between Oriental and Caucasian populations is due to the presence of M1 in Caucasians. It will be of interest to study other populations in the future with respect to frequency of M1, M2, and M3. Moreover, the high incidence of the slow acetylator mutants in Caucasian populations signifies that only approximately 7% of rapid acetylators are homozygous for the wt gene. They therefore cannot easily be differentiated by the usual phenotyping procedure (2, 14) from the predicted 40% heterozygous carriers of the slow *NAT2* allele. The availability of a simple genotyping procedure allows the easy distinction between homozygous and heterozygous rapid acetylators.

Much of the clinical interest in the acetylation polymorphism comes from the association of acetylator phenotypes with a number of spontaneous and drug-induced diseases (1–3). Slow acetylators have a higher risk of developing bladder cancer, particularly evident in individuals occupationally exposed to aromatic amines (reviewed in ref. 2). On

the other hand, rapid acetylators have a higher risk of developing colorectal cancer (6). The described DNA assay will permit a careful reexamination of this large body of clinical observations. It allows the prediction of the acetylator phenotype in over 95% of individuals tested and requires only a small sample of DNA, which may be derived from leukocytes, single hair roots, buccal epithelia, or any other tissue. This will allow the genotyping of patients and volunteers in clinical studies or of workers at high risk on exposure to arylamine chemicals.

This work was supported by Swiss National Science Foundation Grant 3.817.87.

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