N-acetylation pharmacogenetics: A gene deletion causes absence of arylamine N-acetyltransferase in liver of slow acetylator rabbits

(genetic polymorphism/drug therapy/xenobiotic biotransformation/toxicology/cDNA expression)

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ABSTRACT The New Zealand White rabbit provides a widely used animal model for the human acetylation polymorphism, which confers marked interindividual variation in the effect and toxicity of numerous drugs, chemicals, and potential carcinogens. The relationship of a recently isolated cDNA clone, designated rnat, to genetically polymorphic arylamine N-acetyltransferase (NAT; acetyl-CoA:arylamine N-acetyltransferase, EC 2.3.1.5) of rabbit liver was established by its expression in monkey kidney COS-1 cells: (i) cytosols from transfected cultures contained high levels of an Ac-CoA-dependent NAT activity, which was kinetically indistinguishable from that observed in cytosols from livers of genetically rapid-acetylator rabbits; (ii) transfected cells also contained an immunoreactive protein, recognized by NAT-specific antibodies, with identical electrophoretic mobility to NAT from rabbit liver. The rnat clone and anti-NAT antibodies were then used to study the relationship between NAT activity, liver enzyme protein, and the level of mRNA in livers from in vivo phenotyped rapid- and slow-acetylator rabbits. Livers from slow acetylators were devoid of both immunodetectable NAT protein and its corresponding mRNA. Analysis of genomic DNA with a panel of restriction enzymes revealed the loss of specific hybridizing bands in the DNA of slow-acetylator rabbits. These data strongly suggest that defective arylamine N-acetylation in the rabbit model is caused by a gene deletion resulting in an absence of specific mRNA and NAT enzyme protein.

Genetically determined variability in the biotransformation of drugs and xenobiotics has become the focus of intense investigations because of its consequences for clinical therapy, drug development, and toxicology (1-3). Arylamine N-acetyltransferase (NAT; acetyl-CoA:arylamine N-acetyltransferase, EC 2.3.1.5) is the target of one of the most common human genetic polymorphisms of drug metabolism. More than 50% of individuals in Caucasian populations are slow acetylators (4, 5) and have impaired metabolism of a wide variety of arylamine and hydrazine drugs and chemicals. These include the drugs sulfamethazine (SMZ) and several other sulfonamides, isoniazid, procainamide, hydralazine, phenelzine, caffeine, nitrazepam, and dapsone, as well as the potential carcinogens benzidine, 2-aminofluorene, and β-naphthylamine (5).

Phenotypically slow acetylators have been shown to be homozygous for an autosomal recessive gene (6). In addition to increased adverse reactions in slow acetylators treated with drugs whose elimination is primarily determined by acetylation, there are a number of associations of the acetylator phenotype with drug-induced and spontaneous disease. However, apart from the increased incidence of bladder cancer in the slow-acetylator phenotype (7)—carcinogenic amines causing bladder cancer are substrates for NAT—the associations of polymorphic acetylation with Gilbert disease (8), advanced breast cancer (9), diabetes (10), and leprosy (11) remain mechanistically unexplained.

In contrast to the extensive clinical studies, little is yet known about the molecular mechanism underlying the acetylation polymorphism. One approach to study the biochemical basis of the defect has been the use of suitable animal models (5). Among these, the New Zealand White rabbit is the most widely used because the in vivo and the in vitro patterns of arylamine N-acetylation (12) and the predisposition to toxicity in genetically slow-acetylator rabbits (4, 5) closely resemble those in humans.

Using an NAT-specific antiserum and an oligonucleotide designed on the basis of internal amino acid sequences from purified rabbit liver NAT (13), we recently isolated a cDNA clone (designated rnat) from a cDNA library constructed with liver mRNA from an in vivo phenotyped rapid-acetylator rabbit (14). In this report, we have examined the relationship of rnat to the polymorphic rabbit NAT enzyme and we provide evidence for a gene deletion in slow-acetylator rabbits causing an absence of NAT protein and the mRNA encoding it.

MATERIALS AND METHODS

Animals and Acetylator Phenotyping. A total of 24 New Zealand White rabbits (strain Kfm:NZW; Madorin, Fullinsdorf, Switzerland) were tested in vivo for their acetylator phenotype by an HPLC modification (15) of published methods (12) using sulfinpyrazone as probe drug. Two animals with sulfinpyrazone half-life values >90 min were classified as slow acetylators; the remaining 22 animals with values <70 min were classified as rapid acetylators. Five of these rabbits were used in the present study (sulfinpyrazone half-life values given in parentheses): rapid-acetylator rabbits r145 (46 min), r278 (41 min), and r281 (28 min), and slow-acetylator rabbits r82 (113 min) and r100 (141 min).

NAT Assay and Enzyme Kinetics. The NAT assay and the determination of kinetic parameters (K_m, V_max) were performed as recently described (15).

Enzyme Purification, Antibody Production, and Immunoreaction on Western Blots. NAT protein was partially purified from liver of rapid-acetylator rabbit r281 by a modification of the method described by Andres et al. (13) and subjected to preparative SDS/PAGE. The 33-kDa band corresponding to NAT was excised and used directly to immunize another rabbit. The resulting polyclonal antiserum was affinity purified against the original antigen immobilized on nitrocellulose, and Western blot analysis with detection by 125I-labeled protein A autoradiography was performed following standard procedures after electrophoretic separation of cytosolic proteins on 12% polyacrylamide gels (16).

Abbreviations: NAT, arylamine N-acetyltransferase; SMZ, sulfamethazine.
Nucleic Acid Isolation and Analysis. Genomic DNA was prepared from liver or blood leukocytes as described (17) and analyzed on genomic DNA blots by standard protocols and conditions (18). Total cellular RNA from liver tissue was isolated by the guanidinium isothiocyanate method (19), and mRNA was prepared according to ref. 20 and analyzed on Northern blots (18). Hybridization probes were radiolabeled with [α-32P]dATP using the random oligonucleotide-priming protocol (21) to specific activities of 1-5 x 10^6 cpm per μg of DNA.

Expression of ratn in Monkey Kidney COS-1 Cells. COS-1 cells were cultured as described (22). The expression vector p91023(B) (23) was kindly provided by R. J. Kaufman (Genetics Institute, Cambridge, MA). Semi confluent cultures were transfected by using either the DEAE-dextran or the calcium phosphate coprecipitation method (24). Twenty micrograms of supercoiled plasmid was applied per 90-mm culture dish. As controls, cultured cells transfected with the antisense construct, without DNA (mock transfection), and untreated cells were included in every experiment. Fifty to 70 hr after transfection, the cultures were harvested, the cells were lysed by sonication, and cytosols were prepared as described (15).

RESULTS
Functional Expression of ratn in COS-1 Cells. To establish the relationship between ratn and the polymorphic rabbit liver NAT enzyme, ratn was expressed in monkey kidney COS-1 cells. Transient transfections of the cDNA, inserted in sense and antisense orientation into the expression vector p91023(B) (23), were performed.

NAT activity in cultured cells was determined by adding SMZ at a concentration of 500 μM directly to the medium after transfection and by measuring N-acetyl-SMZ (Ac-SMZ) in the culture supernatant. The acetylated metabolite first appeared (>0.5 nmol/ml) at ~20 hr posttransfection and its concentration increased continuously until, after 1 week of culture, all of the added substrate had been converted to Ac-SMZ. Levels of Ac-SMZ formation in control cultures were relatively low (1-5% of the sense construct).

To perform kinetic analysis of the expressed enzyme protein, cell cytosols were prepared 50-70 hr after transfection. The affinity of the expressed NAT for SMZ (Km = 85 μM) was found to be essentially the same as that observed in liver cytosols of three rapid-acetylator rabbits (Fig. 1), with Km values of 82 μM (rabbits r145 and r278) and 89 μM (rabbit r281), including the rabbit (r145) from which the original cDNA was derived. Km values for the two slow-acetylator rabbits were 182 μM (rabbit r282) and 154 μM (rabbit r100). The maximal velocity (Vmax) of acetylated product formation in COS-1 cell cytosols prepared from cultures transfected with the sense construct was 1.8- to 3-fold higher than in cytosol of rapid-acetylator rabbit liver cytosol (Fig. 1).

The high level of expressed NAT activity was associated with the presence of an immunoreactive protein with electrophoretic behavior identical to that of the NAT protein observed in rabbit liver cytosol, with an apparent molecular mass of 33 kDa (Fig. 1 Inset). No immunoreactive NAT protein was detectable in cytosols of COS-1 cells transfected with the antisense construct or in un- or mock-transfected control cells. These cytosols also did not have detectable NAT activity for SMZ in vitro.

Relationship Between Vmax, Immunoreactive NAT Protein, and mRNA in Livers of in Vivo Phenotyped Rapid- and Slow-Acetylator Rabbits. Vmax values for SMZ acetylation and immunodetectable NAT protein in liver cytosols from three rapid- and two slow-acetylator rabbits are shown in Fig. 2A and B. Vmax values in the three rapid acetylators (6.4, 9.4, and 13.0 nmol·min⁻¹·mg⁻¹) in r278, r281, and r145, respectively, correlated with the amount of immunoreactive protein detected on Western blots. Cytosols from the two slow-acetylator rabbits did not show any detectable reaction with the anti-NAT antiserum; the respective Vmax values (0.29 and 0.30) were essentially the same as in untransfected COS-1 cell cytosols...

![Fig. 1. Functional expression of ratn in monkey kidney COS-1 cells. Linear transformation of kinetic data according to Hofstee (25) obtained in cytosols prepared from COS-1 cells transfected with the sense (COS/rNAT syn) or the antisense (anti) construct and compared to values derived from liver cytosols (100 μg of protein) of rapid-acetylator rabbits r145, r278, and r281, and slow-acetylator rabbits r82 and r100. In such plots, the slope of the line equals 1/Vmax, and the y intercept equals Vmax. (Inset) Immunoreaction on Western blots of cytosols (100 μg of protein per lane) of rabbit r145 and COS-1 cells transfected with the sense (COS/rNAT syn) and antisense (COS/rNAT anti) ratn constructs using the antiserum described in Materials and Methods.

![Fig. 2. Correlation between Vmax, NAT protein, and mRNA in livers of in vivo phenotyped rapid- (rabbits r278, r281, and r145) and slow- (rabbits r82 and r100) acetylator rabbits. (A) Vmax values determined with SMZ. (B) Immunoreactive NAT protein on Western blots reacted with NAT-specific antiserum (100 μg of protein per lane). (C) mRNA hybridized with ratn on Northern blots (5 μg of mRNA per lane).]
0.20 nmol·min\(^{-1}·mg\(^{-1}\) in r82 and r100, respectively) were 22- to 65-fold less than those of the rapid acetylators.

Notably, the same difference between rapid- and slow-acetylator rabbits was found when mRNA isolated from liver tissue was analyzed on Northern blots using the \(mat\) probe (Fig. 2C). A single mRNA species of 1.1 kilobases (kb) was observed in the three rapid acetylator rabbits, whereas no mRNA was detectable in the liver of the slow acetylators, even after overexposure of the autoradiograph.

**Restriction Analysis of Genomic DNA in Rapid- and Slow-Acetylator Rabbits.** The organization of \(mat\)-related sequences in genomic DNA of defined homozygous slow- and rapid-acetylator rabbits was analyzed on Southern blots after DNA digestion with a total of 12 different restriction enzymes. Of the six rabbits analyzed, four (two rapid, two slow) were from the animal facilities of our institute. Two additional DNA samples from rabbits in which the genotype was positively identified by pedigree studies (26) as homozygous rapid (RR) and homozygous slow (rr) were kindly provided by K. J. Martell and W. W. Weber (University of Michigan, Ann Arbor).*

Examples of banding patterns obtained with five enzymes upon hybridization with \(mat\) are shown in Fig. 3. The three rapid- and the three slow-acetylator DNAs each revealed identical phenotype-specific patterns, therefore, only one representative of each group is depicted. In all digests, at least one hybridizing fragment was missing in genomic DNA of slow-acetylator rabbits, suggesting deletion of \(mat\)-specific sequences. All restriction enzymes that have no recognition sites in \(mat\) (Apa I, EcoRI, Xba I) gave rise to two hybridizing bands in DNA of rapid acetylators, one of which was missing in the slow-acetylator rabbits. The same result was obtained with four additional enzymes without internal restriction sites in \(mat\) (BamHI, Hpa I, Pst I, Pvu I; data not shown). The sizes of the absent fragments ranged from 1.9 (Apa I) to 22 kb (BamHI). The smallest constant band in these digests was observed with Pst I (3.0 kb) and the largest was found with BamHI (13 kb).

**DISCUSSION**

In the present report we show, first, that the recently isolated cDNA clone \(mat\) encodes the polymorphic NAT; second, that livers of slow-acetylator rabbits have no \(mat\)-related mRNA and no immunodetectable NAT protein; finally, we can explain these findings by demonstrating the deletion of \(mat\)-specific fragments in the genomic DNA of slow-acetylator rabbits.

The deduced amino acid sequence of \(mat\) (14) had already indicated its close resemblance or identity with the polymorphic enzyme since 14 of 16 internal peptide sequences of purified rabbit NAT (13) were identified (27). In addition, \(mat\) showed 61% and 46% sequence homology at the nucleotide and deduced amino acid levels, respectively, to those of NAT from chicken liver (28). Functional expression proved conclusively that \(mat\) encodes the target enzyme of the acetylation polymorphism. The cytosolic protein produced in COS-1 cells was identical in enzyme activity, electrophoretic mobility, and immunoreactivity to NAT from rapid-acetylator rabbit liver (Fig. 1).

A central question in the study of the molecular mechanisms underlying genetic enzyme defects is whether the gene product is absent or whether it is present in a functionally altered form. Using the \(mat\) cDNA clone and NAT-specific antisera, we obtained convincing evidence that, in livers of slow-acetylator rabbits, \(mat\)-specific mRNA and NAT protein are absent (Fig. 2). The possibility that a structurally altered NAT protein is not recognized by the antibodies against the wild-type enzyme appears unlikely, since a cross-reacting polyclonal rabbit antiserum raised against purified human NAT (15) gave the same result, even though the rabbit and human deduced proteins differ by 20% in amino acid sequence (29). Moreover, the corresponding mRNA giving rise to a mutant protein would have been readily detected on Northern blots.

An explanation at the gene level for absent mRNA and NAT protein is provided by the analysis of genomic DNA from slow- and rapid-acetylator rabbits. Of 12 restriction enzymes tested, all revealed the absence of at least one \(mat\)-specific hybridizing fragment in slow acetylators. This finding suggests a deletion mechanism causing the NAT-acetylation defect in the rabbit. The simplicity of the hybridization patterns, especially for restriction enzymes without recognition sequences in \(mat\), could signify that most or all of the entire coding sequence is absent in slow-acetylator rabbits.

The data also indicate that the size of NAT-related gene(s) is relatively small. In the case of \(Apa\) I-digested DNA, the fragments add up to as little as 5.1 kb and, with five additional enzymes tested (EcoRI, Hpa I, Pst I, Pvu I, HindIII), to <10 kb.

Our findings of a gene deletion and absent mRNA and protein as the cause of the NAT polymorphism in the rabbit are in contradiction to previous suggestions. A combination of kinetic (30) and immunological (31) data earlier studies had led to the hypothesis that the activity differences between rapid- and slow-acetylator rabbits are a result of structural differences between apparently equal quantities of immunoreactive enzyme molecules in the two groups. A structural mutation in the respective gene was therefore proposed to lead to the production of a variant, enzymatically defective NAT protein in slow-acetylator rabbits (32). One possible explanation for the conflicting results may be that the antiserum used in the earlier studies immunoprecipitated unrelated proteins even in the absence of the polymorphic NAT protein.

The difference in substrate affinity of the residual NAT enzyme activity, the absence of immunoreactive NAT protein and mRNA, and the deletion of NAT-specific genomic

*As far as we can determine, the animals used in this study and those from the University of Michigan were derived from different founder stocks.*
sequences in slow-acetylator rabbits strongly suggest that the residual activity observed in the slow-acetylator phenotype is due to a different, probably related, NAT enzyme encoded by a different gene. The presence of a constant band hybridizing with mat on Southern blots in both phenotypes (Fig. 3) raises the possibility that this fragment contains a gene encoding a second NAT enzyme. The existence of a second NAT enzyme has been suggested previously, based on the observation that slow-acetylator rabbits have a residual NAT activity in liver and extrahepatic tissues (ref. 33; Fig. 2) with different substrate specificity and stability characteristics from that expressed to high levels in rapid-acetylator rabbit liver.

The fact that the polymorphic NAT gene is absent in slow-acetylator rabbits with no detectable deleterious effects excludes an important housekeeping function of the encoded NAT enzyme. The constancy of the second hybridizing band, if its sequence indeed encodes a related NAT protein, may indicate a particular physiological function for this enzyme. The polymorphic NAT gene might have been derived from an ancestral gene by gene duplication upon challenge with environmental xenobiotics. The absence of the duplicated gene in slow-acetylator rabbits could then be due either to incomplete selection for its presence or to its subsequent loss.

How the human acetylator polymorphism is related to the rabbit model remains an open question. Analysis with the mat probe of genomic DNA of slow- and rapid-acetylator human individuals did not reveal any differences in the hybridization patterns obtained in the two phenotypes (27). This result indicates a different molecular mechanism underlying the human acetylator defect. On the other hand, as in the rabbit, liver cytosols of slow-acetylator humans show a marked decrease in immunoreactive NAT protein (34). It therefore appears at present that different molecular mechanisms lead to similar consequences at the protein level. Thus, in practical terms, the rabbit may in fact provide a valid animal model for the human acetylation polymorphism.

In conclusion, the presented data suggest that a deletion of the gene for polymorphic NAT causes its deficiency in slow-acetylator rabbits. The availability of the rabbit cDNA clone mat described here has already led to the isolation of a human genomic clone, designated hmat-b (29), as a first step in understanding the human polymorphism and its multiple implications in drug therapy and xenobiotic toxicity.

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