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# Cytosolic Arylamine *N*-Acetyltransferase (NAT) Deficiency in the Dog and Other Canids Due to an Absence of *NAT* Genes

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**ABSTRACT.** The purpose of this study was to determine the molecular basis in the dog for an unusual and absolute deficiency in the activity of cytosolic *N*-acetyltransferase (NAT), an enzyme important for the metabolism of arylamine and hydrazine compounds. NAT activity towards two NAT substrates, *p*-aminobenzoic acid and sulfamethazine, was undetectable in dog liver cytosol, despite substrate concentrations ranging from 10 μM to 4 mM and a wide range of incubation times. Similarly, no protein immunoreactive to NAT antibody was evident on western blot analysis of canine liver cytosol. Southern blot analysis of genomic DNA from a total of twenty-five purebred and mixed bred dogs, and eight wild canids, probed with a full-length human *NAT2* cDNA, suggested an absence of *NAT* sequences in all canids. Polymerase chain reaction amplification of genomic DNA using degenerate primers designed to mammalian *NAT1* and *NAT2* consensus sequences generated products of the expected size in human, mouse, rabbit, and cat DNA, but no *NAT* products in any dog or wild canids. These results support the conclusion that cytosolic NAT deficiency in the domestic dog is due to a complete absence of *NAT* genes, and that this defect is shared by other canids. BIOCHEM PHARMACOL **54**;1:73–80, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** arylamine acetyltransferase; dog; gene; deficiency; sulfonamide; PABA

NAT‖ (EC 2.3.1.5; acetylCoA:arylamine *N*-acetyltransferase) is a cytosolic phase II drug-metabolizing enzyme that catalyzes the acetylation of arylamine and hydrazine compounds. NAT is involved in the metabolism of a number of clinically important drugs, including the antihypertensive agent hydralazine, the antiarrhythmic procainamide, the tuberculostatic drug isoniazid, and sulfonamide antibiotics [1]. NAT also plays an important role in the bioactivation and/or detoxification of various arylamine carcinogens [2]. Although the endogenous role of NAT is not fully defined, there is recent evidence to suggest that this enzyme may also be involved in the metabolism of folate derivatives [3, 4].

NAT activity has been demonstrated in a large number of diverse species, including primates, rabbits, rodents, birds, fish, amphibians, molluscs, and even bacteria [5–7]. Two major NAT enzymes, NAT1 and NAT2, have been well characterized in rabbits, rodents, and humans [8–10], and a third novel isozyme, NAT3, has also been identified in mice [11]. These 31- to 33-kDa isozymes are highly homologous (for example, NAT1 and NAT2 exhibit approximately 80% amino acid identity in humans), but vary in tissue distribution and substrate specificity [12]. *NAT1* and *NAT2*, the genes that encode the human NAT isozymes, have small (870 bp) intronless coding regions, exhibit 87% nucleotide identity [13], and are thought to have arisen by gene duplication. *NAT* genes have also been cloned in the chicken, rabbit, mouse, hamster, and rat [14–18]. The mammalian *NAT* genes that have been characterized share approximately 75–97% nucleotide identity, and all have intronless coding regions.¶

Despite the high degree of conservation of NAT activity among many species, a number of researchers, using both *in vivo* and *in vitro* techniques and a number of NAT substrates, have been unable to detect any NAT activity in dogs [19–23]. We report herein that the dog is completely deficient in cytosolic NAT activity due to an apparent

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‖ *Abbreviations:* BHT, butylated hydroxytoluene; DTT, dithiothreitol; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; NAT, *N*-acetyltransferase; PABA, *p*-aminobenzoic acid; PCR, polymerase chain reaction; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene fluoride; SMZ, sulfamethazine; and SSC, saline sodium citrate.

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¶ Grant DM, Molecular genetics of the *N*-acetyltransferases. In: *Tenth International Symposium on Microsomes and Drug Oxidations, Toronto, Canada, 18–21 July 1994*, pp. 12–14.

absence of any *NAT* genes, and that this defect is shared by other members of the canid family.

## MATERIALS AND METHODS

### *Animals*

Whole blood samples in EDTA were collected for genomic DNA extraction from purebred and mixed breed dogs and domestic cats seen at the Veterinary Medical Teaching Hospital at Cornell University, and from wild canids housed at several zoological societies in the Northeastern United States. Liver samples and whole blood were obtained at the time of euthanasia from healthy mixed breed dogs, New Zealand White rabbits, and SWR mice. Human whole blood for DNA extraction was collected in EDTA from a single individual (one of the authors).

### *NAT Activity Assays*

Liver samples from dogs, rabbits, and mice were collected immediately following euthanasia with pentobarbital (dogs, rabbits) or $CO_2$ (mice). Livers were perfused with cold PBS and were snap frozen in liquid nitrogen. Liver cytosol was prepared by differential centrifugation after homogenizing thawed livers on ice in TEDK buffer (10 mM triethanolamine–HCl, 1 mM EDTA, 1 mM DTT, 50 mM KCl, pH 7.0) with 10 μM leupeptin, 100 μM PMSF, and 18 μM BHT; cytosols were stored in single-use aliquots at −80° and protein concentrations were determined by the method of Bradford [24]. For assays of NAT activity, rabbit or canine liver cytosol was incubated with 10 μM to 4 mM concentrations of either PABA or SMZ, which are preferred substrates for NAT1 and NAT2, respectively, in both humans and rabbits. Rabbit cytosol was diluted such that the turnover of substrate to product was less than 10%. Incubations also included 100 μM acetyl CoA and an acetyl CoA regenerating system consisting of 5 mM acetyl-DL-carnitine and 0.22 U/mL of carnitine acetyltransferase [25]. Incubation times at 37° varied from 5 min to 24 hr. For canine activity assays, some reactions were also run with 1 mM acetyl CoA. Reactions were stopped with 15% perchloric acid and were centrifuged at 14,000 *g* for 5 min at 4°. The supernatants were analyzed the same day for acetylated metabolites by reverse phase HPLC using a Beckman Ultrasphere ODS C-18 column (250 × 4.6 mm; 5 μm particle size) and a Shimadzu SCL-10A solvent delivery system (Shimadzu Scientific, Columbia, MD) with UV detection at 270 nm for *N*-acetyl PABA and 265 nm for *N*-acetyl SMZ. The mobile phase for both assays consisted of acetonitrile:water:glacial acetic acid:triethylamine at a ratio of 95:905:10:0.5 (by vol.), run isocratically at 2 mL/min. The assay limits of detection were 110 nM (5.5 pmol as injected in 50 μL) for *N*-acetyl PABA, and 90 nM (4.5 pmol as injected) for *N*-acetyl SMZ.

### *Western Blot Analysis of Liver Cytosols*

Canine and rabbit liver cytosols were prepared as for NAT activity assays. Cytosols (10–800 μg of canine cytosol protein per lane) were separated by SDS–PAGE in 15% acrylamide and were transferred to PVDF membranes (Immobilon-P, Millipore Corp., Bedford, MA) for immunoblotting. Membranes were blocked overnight at 4° in 5% blocking solution (5% nonfat dry milk in PBS with 0.05% Tween), prior to incubation for 1 hr with polyclonal rabbit anti-human NAT antibody diluted 1/500 in the same blocking solution. A two-step conjugate system was used consisting of biotinylated donkey anti-rabbit IgG at 1/20,000 in 5% blocking solution, and streptavidin-HRP conjugate at 1/5000 in PBS, followed by ECL detection (all reagents from Amersham Life Sciences, Arlington Heights, IL). Membranes were washed between incubations with PBS/0.1% Tween. Human recombinant NAT2 protein (1 μg from an *Escherichia coli* lysate), human liver cytosol (100 μg protein), and rabbit liver cytosol (10–100 μg) were used as positive controls. Anti-human NAT antibody, recombinant NAT, and human liver cytosol were gifts from Dr. Denis Grant, The Hospital for Sick Children, Toronto, Canada.

### *Genomic Southern Blot Analysis*

Genomic DNA was extracted from whole blood using a modified salting out technique [26]. Ten micrograms of each DNA sample was digested with *Eco*RI, electrophoresed in an 0.8% agarose gel, and transferred to nylon membranes (Hybond-N, Amersham Life Sciences). For some blots, genomic DNA was also digested with *Bam*HI, *Hin*dIII, and *Kpn*I. Membranes were prehybridized overnight at 50° in hybridization solution containing 120 mM $Na_2HPO_4$, 525 mM NaCl, 1.5 mM EDTA, 7% SDS, 5x Denhardt's solution [27], and 10% PEG 8000. Membranes were then hybridized at 50° overnight in fresh hybridization solution to which was added $2 \times 10^6$ cpm/mL of [$^{32}$P]dATP random prime labeled full-length human *NAT2* cDNA prepared from *Eco*RI and *Sal*I digestion of pNAT2 [28], which is the plasmid pKEN2 containing the full-length human *NAT2* coding sequence. Blots were washed under low stringency conditions (final wash, 0.5x SSC, 0.1% SDS for 2–5 min at 50°) and were exposed to X-OMAT-AR film (Eastman Kodak, Rochester, NY) with a single intensifying screen at −80° for up to 10–14 days. Following stripping of blots, a full-length chicken actin cDNA probe, prepared by *Hin*dIII digestion of a 1.2 kb insert from pBR322 (Promega Corp., Madison, WI), was used as a positive control for DNA integrity on canine and canid blots.

### *PCR Analysis of Genomic DNA*

Two primers were designed to anneal to consensus regions in mammalian *NAT* genes: sense primer, 5′-CCCT(T/A)TGAGAA(C/T)(C/T)T(T/C/G)AACAT(G/C)CA(C/T)

TGTG-3′ (corresponds to bp 106–133 in the coding region of mammalian *NAT*); antisense primer, 5′-GCCCAC(C/T) AAACAGTGAAC(C/A/T)CCTTCTG-3′ (corresponds to bp 683–708), and were used to attempt to amplify *NAT* sequences from genomic DNA from domestic dogs and wild canids using PCR. Genomic DNA samples from mouse, rabbit, human, and cat were used as positive controls. Final concentrations of PCR reagents were as follows: 1x reaction buffer (50 mM KCl, 10 mM Tris–HCl, pH 9.0, and 0.1% Triton X-100), 2.5 mM $MgCl_2$, 2.5% DMSO, 600 nM of each primer, 250 μM of each dNTP, and 2.5 U of *Taq* DNA polymerase per 50 μL reaction. Reactions were hot started at 94° before the addition of 500 ng of DNA template in water. Cycling conditions consisted of an initial denaturation at 94° for 4 min, followed by 35 cycles of the following sequence: 94° for 1 min, 60° for 1 min, and 72° for 1 min; followed by a final extension step at 72° for 7 min. Canine and wild canid genomic DNA samples were also amplified at lower annealing temperatures when no product was obtained at 60°. Amplified products were checked for homology to *NAT* by hybridization with [$^{32}$P]dATP random prime labeled full-length human *NAT2* cDNA, followed by low stringency washes as performed for genomic blots, and exposure to autoradiographic film. In addition, the integrity of all canid DNA samples was evaluated by the use of control primers designed to anneal to consensus regions of the coding sequence of the mammalian *5HT$_1$* (serotonin receptor) gene [29], which, like *NAT*, is highly conserved and intronless [30]. $5HT_1$ primers were provided by Dr. Jeffrey Ward, Department of Pharmacology, Cornell University.

## RESULTS

### *NAT Activity in Canine Liver Cytosol*

No *N*-acetylated metabolites could be detected in canine liver cytosol after incubation with either PABA or SMZ (Fig. 1), despite substrate concentrations ranging from 10 μM to 4 mM, incubation times varying from 5 min to 24 hr, and final acetyl CoA concentrations of 100 μM or 1 mM (data not shown). In contrast, rabbit liver cytosol generated 1.61 ± 0.25 nmol of *N*-acetyl PABA/mg protein/min and 1.54 ± 0.05 nmol of *N*-acetyl SMZ/mg protein/min, when incubated with 100 μM substrate and 100 μM acetyl CoA.

### *Immunoblot Analysis of Canine Liver Cytosol*

No immunoreactive NAT protein was found in canine liver cytosol, using an antibody that detected human recombinant NAT, NAT in human and rabbit liver cytosol (Fig. 2), and NAT in mouse liver cytosol (data not shown). It should be noted that although this polyclonal antibody reacts with both NAT1 and NAT2 [31], it is difficult to detect NAT1 alone in native liver cytosol in humans and rabbits without further purification based on NAT activity [10, 32]. Because NAT activity could not be detected in the dog, further purification was not attempted; however, when the amount of canine cytosol loaded was increased to up to 800 μg of protein, and autoradiography exposure times were prolonged for up to 1 hr, we still could not detect immunoreactive NAT in the dog (data not shown).

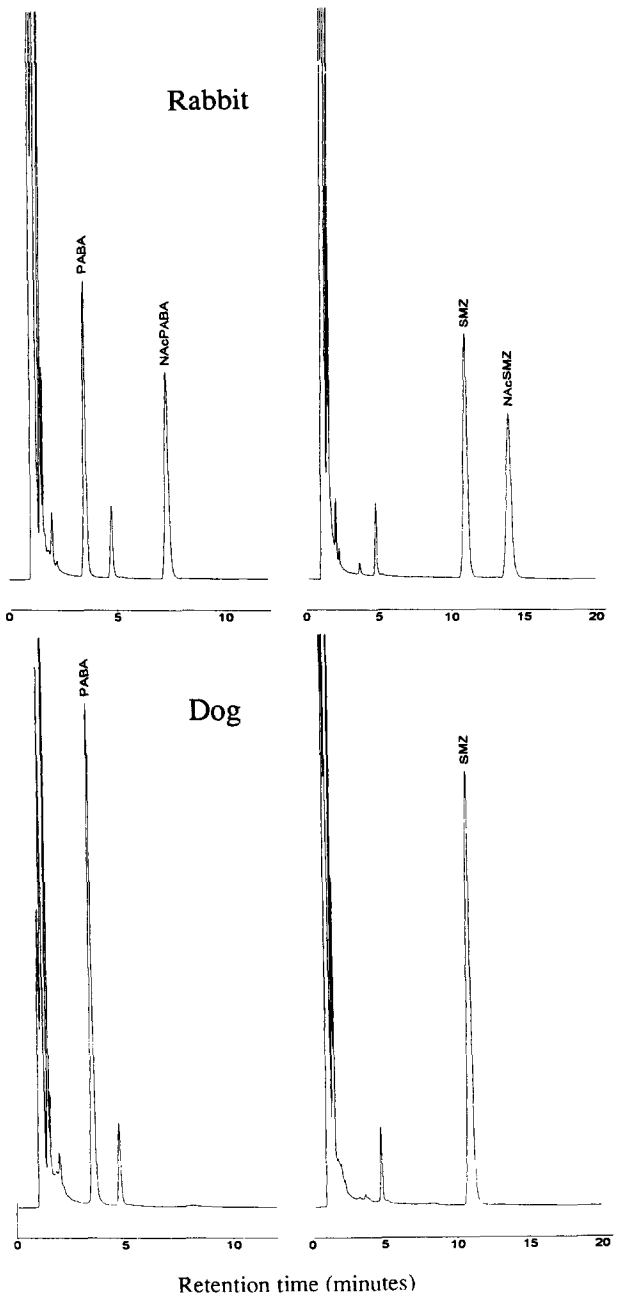


**FIG. 1. Absence of NAT activity in canine liver cytosols. HPLC elution patterns following incubation of rabbit or canine liver cytosol with 100 μM PABA or 100 μM SMZ showed an absence of *N*-acetylated products in the dog (bottom panels). Reactions included 100 μM acetyl CoA and an acetyl CoA regenerating system (see Materials and Methods), and were incubated at 37° for 15 min. Under these conditions, rabbit liver cytosol generated 1.61 ± 0.25 nmol of *N*-acetyl PABA/mg protein/min and 1.54 ± 0.05 nmol of *N*-acetyl SMZ/mg protein/min (top panels). Sensitivity of this assay for *N*-acetyl PABA and *N*-acetyl SMZ was 110 nM (5.5 pmol as injected in 50 μL), and 90 nM (4.5 pmol as injected), respectively.**

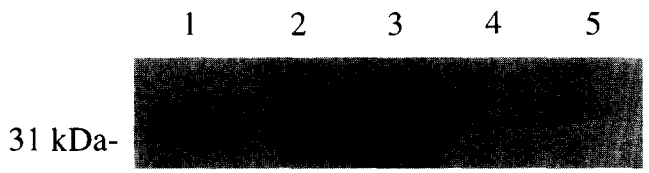


FIG. 2. Absence of immunoreactive NAT in canine liver cytosols. A polyclonal rabbit anti-human NAT antibody was unable to detect any immunoreactive NAT protein in canine liver cytosols (lane 4, 100 μg, and lane 5, 300 μg of protein). Positive controls included 1 μg of *E. coli* lysate containing human recombinant NAT2 (lane 1), 100 μg of human liver cytosol (lane 2), and 10 μg of rabbit liver cytosol (lane 3). A two-step conjugate system was used, consisting of biotinylated donkey anti-rabbit IgG and streptavidin-HRP conjugate, followed by ECL detection and exposure to autoradiography film. Only 10 μg of rabbit cytosol was loaded for these experiments because 100 μg of rabbit cytosol gave an overly strong signal under these conditions.

### *Southern Blot Analysis of Canine and Wild Canid Genomic DNA*

No *NAT* homologues were detected by Southern blot analysis of genomic DNA from five mixed breed dogs (Fig. 3A) and twenty purebred dogs (data not shown), although under these conditions the human *NAT2* probe detected all three human *NAT* sequences (*NAT1*, *NAT2*, and the pseudogene *NATP*), which correspond to the three fragments detected in an *Eco*RI genomic digest [12] (Fig. 3A). This probe also detected *NAT* homologues in rhesus monkey, rat, mouse, rabbit, and cow (data not shown), and in the domestic cat (Fig. 3A). To assure that *NAT* sequences in the dog had not been missed on small fragments in the *Eco*RI digest, *Bam*HI, *Hin*dIII, and *Kpn*I digests of canine genomic DNA were also subjected to Southern blot analysis, but again no canine *NAT* homologues were detected (data not shown). *NAT* sequences were also not detected in genomic DNA from eight wild canids (arctic wolf, red wolf, African wild dog, golden jackal, coyote, arctic fox, silver fox, and kit fox; Fig. 4). As a positive control, an actin probe detected multiple discrete bands in all domestic dog (Fig. 3B) and wild canid (data not shown) genomic DNA, indicating that poor DNA transfer or DNA degradation was not responsible for the negative results in canids using the *NAT* probe.

### *PCR Analysis of Genomic DNA*

Using degenerate primers designed to anneal to mammalian *NAT1* and *NAT2* consensus regions, PCR products of expected size (603 bp) were amplified from mouse, rabbit, human, and cat genomic DNA (Fig. 5). However, no specific products were detected in any mixed breed or purebred dog, despite decrements in annealing temperatures to 50° to account for possible marked differences in sequence homology in this species. Although there were some weak products of varying sizes amplified from canine samples annealed at 55° and 50°, no *NAT* sequences were

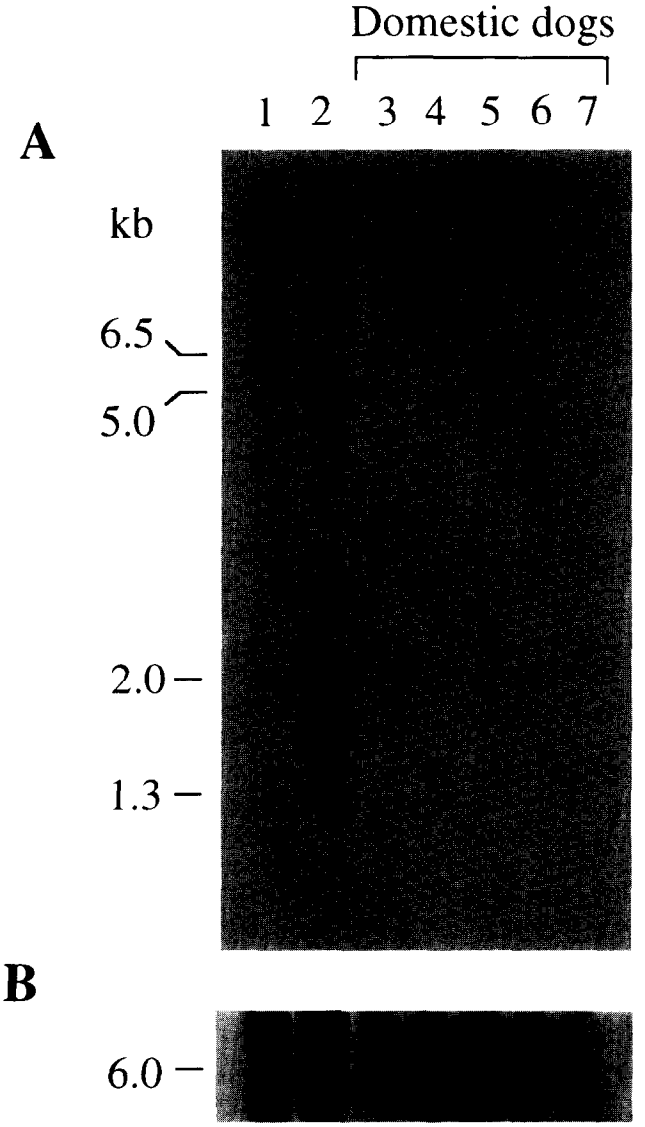


FIG. 3. Southern blot of canine genomic DNA. *Eco*RI-digested canine genomic DNA (10 μg per lane) was probed with a full-length human NAT2 cDNA under low stringency conditions (final wash, 0.5x SSC, 0.1% SDS for 5 min at 50°). No NAT homologues could be detected in any of five mixed breed dogs (lanes 3–7, panel A). Under these conditions, however, this probe detected all three human NAT sequences (lane 2; *NAT1*, *NAT2*, and the human pseudogene, *NATP*, which are present on three separate fragments in an *Eco*RI genomic digest [13], and also detected an NAT homologue in the domestic cat (lane 1). After stripping the same blot, a full-length chicken actin cDNA probe was used as a positive control to confirm the integrity of canine DNA. Multiple bands were present in all lanes as shown in a segment of the blot in panel B.

detected by Southern blot analysis of any canine PCR reactions; however, both human and cat PCR products were confirmed by Southern blot analysis using the full-length human *NAT* probe to be *NAT* sequences (data not shown). Genomic DNA from red wolf, arctic wolf, arctic fox, kit fox, golden jackal, coyote, and African wild dog was also negative for *NAT* sequences using similar PCR conditions, followed by Southern blotting (Fig. 6, A and B). As

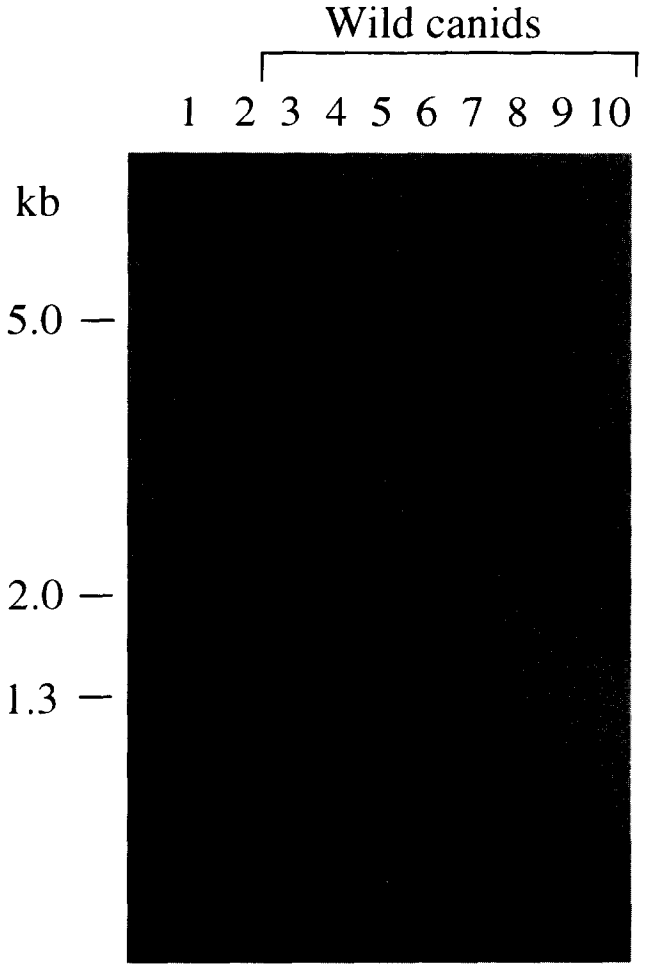


**FIG. 4. Southern blot of genomic DNA from wild canids. *Eco*RI-digested genomic DNA (10 μg per lane) from human (lane 1), domestic dog (lane 2), and eight wild canids (lanes 3–10: red wolf, arctic wolf, coyote, golden jackal, African wild dog, arctic fox, kit fox, and silver fox, respectively) was probed with full-length human *NAT2* cDNA. No *NAT* homologues could be detected in any canid despite low stringency wash conditions (see Materials and Methods) and prolonged autoradiographic exposures (1-week exposure shown).**

mentioned earlier, these degenerate consensus primers, however, readily detected *NAT* sequences in a number of other mammals, including the cat (Fig. 5). To rule out the possibility that the canid DNA samples were degraded, the integrity of all canid DNA was confirmed by the successful amplification from each species of a single 600 bp product using control primers designed to an intronless, highly conserved gene [29, 30] encoding the mammalian $5HT_1$ receptor (data not shown).

## DISCUSSION

The results of this study support the conclusion that domestic dogs are completely deficient in cytosolic NAT activity due to an apparent lack of *NAT* genes. No *N*-acetylation activity could be detected in canine liver cytosols using either PABA (NAT1 substrate in humans)

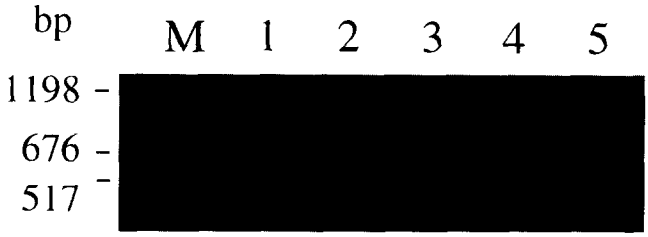


**FIG. 5. PCR amplification of *NAT* sequences. Genomic DNA was amplified by PCR using degenerate primers designed to anneal to mammalian *NAT* consensus sequences (see Materials and Methods), electrophoresed in a 2% agarose gel, and stained with ethidium bromide. PCR products of the expected size (603 bp) were amplified from mouse (lane 1), rabbit (lane 2), human (lane 3), and cat (lane 4) genomic DNA, but no specific *NAT* products were amplified in the dog (lane 5). Lane M: pGEM® marker digested with *Hin*fI, *Rsa*I, and *Sin*I (Promega Corp.).**

or SMZ (NAT2 substrate in humans), despite a wide range of incubation times and increasing substrate and acetyl CoA concentrations. The HPLC assays used in this study were sensitive to nanomolar concentrations of metabolite, and thus would have detected as little as 0.01% turnover of 1 mM substrate. Similar results have been reported by a number of investigators using various sulfonamides and arylamine carcinogens evaluated both *in vivo* and *in vitro*, wherein no NAT activity could be detected in any dog [19–23, 33]. Although it has been proposed that the lack of detectable *N*-acetylation in the dog is due to rapid deacetylation [34], canine deacetylases are microsomal, not cyto-

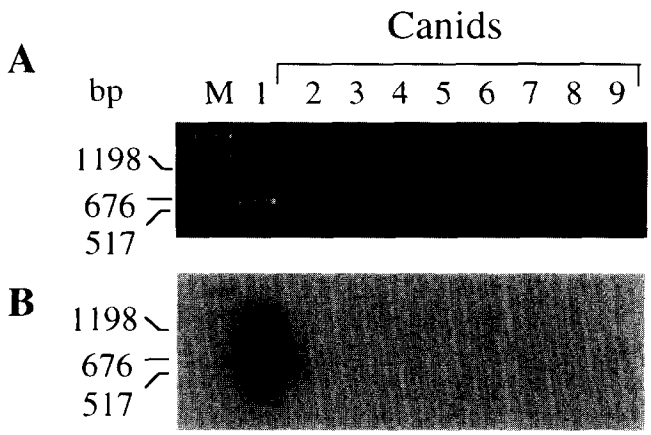


**FIG. 6. PCR and Southern blot analysis of PCR products in wild canids using *NAT* primers. Genomic DNA was amplified by PCR using degenerate primers designed to anneal to mammalian *NAT* consensus sequences. PCR products were electrophoresed in 2% agarose and stained with ethidium bromide (panel A), followed by Southern blot analysis using a full-length human *NAT2* cDNA probe (panel B). No products of the expected size (603 bp) were amplified from the domestic dog (lane 2; panel A), or from seven wild canids (lanes 3–9: red wolf, arctic wolf, arctic fox, kit fox, golden jackal, coyote, and African wild dog, respectively). Very weakly amplified products of different sizes, seen in some canids, did not hybridize with the *NAT* probe (panel B). A product of the expected size was amplified from human genomic DNA (lane 1; panel A), and readily hybridized with the *NAT* probe (lane 1, panel B). Lane M: pGEM® marker per Fig. 5.**

solic, enzymes [35]. In addition, in previous studies in our laboratory, we were unable to detect any deacetylation activity in canine liver cytosol fractions.* Thus, the inability to detect *N*-acetylated products in canine liver cytosol cannot be attributed to rapid deacetylation, and these findings suggest an absent or inactive cytosolic NAT enzyme. Interestingly, another canid, the fox, also appears to lack NAT activity [5].

On western blot analysis, no immunoreactive NAT protein was detectable in canine liver, despite increases in protein loading up to 80-fold relative to human and rabbit samples. These negative results in the dog could have been attributed to species differences in NAT immunogenicity, although all mammalian cytosolic NATs characterized do share high amino acid sequence identity, and this polyclonal antibody did detect NAT in three other mammalian species (human, rabbit, and mouse), and has been shown by others to react with hamster NATs as well [36]. The possibility that dog liver expresses very low levels of one or both NATs or that the dog expresses a very unstable enzyme could not be discounted based on these data alone. However, these possibilities were not pursued further because of the inability to purify cytosols based on NAT activity profiles in the dog.

As a next step to explaining these findings, we went directly to genomic Southern blot analysis to see if *NAT* sequences were present in the dog, and we were unable to detect *NAT* homologues in any of twenty-five domestic dogs and eight wild canids, despite low stringency wash conditions and prolonged autoradiographic exposures. Although dramatic differences in *NAT* sequences in the canid family compared with human *NAT* sequences could explain these results, the human *NAT2* cDNA probe detected all human *NAT* sequences (*NAT1*, *NAT2*, and *NATP*, the human pseudogene) under these conditions, and also detected *NAT* sequences in a variety of other species (e.g. rhesus monkey, rat, mouse, rabbit, and cow), and in another carnivore, the domestic cat.

PCR amplification of genomic DNA supported these findings. Using degenerate primers designed to amplify *NAT1* and *NAT2* consensus regions reported for all mammalian *NAT*s characterized to date, we were able to amplify *NAT* sequences from all species examined except for any domestic dogs or wild canids. Although these negative results could be explained by marked sequence differences between dogs and other mammals (including cats), decrements in annealing temperatures resulted in only multiple weak PCR products in all canids that did not hybridize with *NAT*. These results are consistent with the activity, immunoblot, and genomic Southern blot data and further suggest that canids lack any homologous *NAT* sequences.

Deficiencies of both NAT1 and NAT2 have been documented in humans [2], and there are a number of pharmacologic implications for diminished NAT activity. For example, defects in NAT2 activity have been well characterized in humans, and have been associated with an increased risk of idiosyncratic toxicity from arylamine drugs such as sulfonamides [37, 38]. Interestingly, dogs can also develop idiosyncratic sulfonamide toxicities that are similar to the syndromes seen in humans, and include hepatotoxicity, blood dyscrasias, and skin eruptions [39]. Because of difficulties in accurately determining the incidence of these reactions in either species, however, it is unclear whether absolute cytosolic NAT deficiency in all dogs contributes to an overall higher risk of sulfonamide toxicity in this species compared with humans. It is clear, however, that absent cytosolic *N*-acetylation in dogs does influence the pharmacologic effects of other arylamine compounds. For example, the administration of arylamine carcinogens such as 2-aminofluorene does not lead to hepatic tumor development in dogs unless the compound is administered in the *N*-acetylated form [40], suggesting that cytosolic *N*-acetylation is required for the hepatic carcinogenicity of some arylamine carcinogens. The dog can thus be used to assess the relative importance of cytosolic *N*-acetylation in determining the carcinogenic potential of various arylamine chemicals, but the dog is otherwise not a good model for predicting the safety of arylamine and hydrazine drugs targeted for use in humans.

It should be noted that although dogs are completely deficient in cytosolic NAT activity towards arylamine and hydrazine compounds, there are present in mammals other acetylating pathways that are distinct from NAT and that do appear to be conserved in dogs. For example, dogs and other species are able to *N*-acetylate some non-aromatic amines such as cyanamide [41], but the enzyme involved does not acetylate arylamine NAT substrates and is presumably a protein distinct from NAT. Dogs and other species also have microsomal acetylase activities attributable to carboxylesterases, but these enzymes have biochemical and sequence characteristics that are different from those of mammalian NATs [42]. Finally, dogs are able to *N*-acetylate some sulfonamides at the *N*-1 (sulfonamido) group, but this reaction also appears to be catalyzed by a different, as yet uncharacterized, enzyme [5]. These other acetylating pathways have not as yet been associated with polymorphic detoxification defects, and the endogenous roles of these enzymes have not been characterized.

The absence of NAT in canids is unusual, considering the great variety of species in which NAT activity has been demonstrated, including rhesus monkey, wild and domestic cats, civets, horses, pigs, ruminants, penguins, pigeons, fish, turtles, snails, and bacteria [6–7, 43–46]. Even in those species in which relative deficiencies in NAT activity have been described, there is still some residual *N*-acetylation detectable which is attributed to the activity of a second NAT isozyme (NAT1 or NAT2) [1]. This degree of conservation of enzyme activity, as well as the high degree of NAT homology among mammals at the amino acid and nucleotide levels, implies that selective pressures are acting to conserve NAT function in most species. The only other

* Trepanier LA, Cribb AE and Spielberg SP, unpublished observations, 1993.

species in which no cytosolic NAT activity has been detected is the suncus [47], a shrew in the insectivore order, which is much more closely related to primates and rodents than it is to canids [48]. Interestingly, the defect in the suncus also appears to be the absence of any *NAT* genes [47].

NAT may play an important endogenous role in most species, but apparently not in canids. Lack of cytosolic NAT in dogs does not mean that NAT is not essential in other mammals, since the endogenous role(s) for NAT and the comparative aspects of these pathways in different species have not been fully characterized. Recent evidence suggests that NAT1 can *N*-acetylate *p*-aminobenzoyl glutamate, a major metabolite of folate in humans, and that this pathway may be important for the urinary excretion of this catabolite [3]. If removal of this catabolite were an important endogenous function of NAT1 in humans (and possibly in other animals), this could explain the high degree of conservation of NAT activity across many species. If this pathway of folate catabolism were different in canids, however, this might explain the lack of NAT conservation in this group of carnivores.

The mechanism for the absence of both *NAT* genes in canids is not clear. It seems implausible that canids would lose two or more *NAT* sequences as independent events during the course of evolution. *NAT* genes in mice and in humans are closely situated (only about 9 kb apart in the mouse and both in the region 8p21.3–23.1 in humans) [16, 49], so it is possible that canids lost two or more closely situated genes as a single event. However, preliminary evidence in our laboratory indicates that the cat and other felids may have only a single *NAT* gene. This raises the possibility that ancestral carnivores may have had only one *NAT* and that only a single gene was lost during canid evolution. It is believed that *NAT1* and *NAT2* in humans and other species arose by gene duplication, although the timing of this event is not known. We are now working to characterize the presence or absence of *NAT* sequences in a number of carnivore families to elucidate the likely mechanism of *NAT* loss in canids, and its timing relative to the duplication of *NAT* in mammals.

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