Effect of Arylamine Acetyltransferase Nat3 Gene Knockout on N-Acetylation in the Mouse

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ABSTRACT:

Arylamine N-acetyltransferases (NAT) catalyze the biotransformation of many important arylamine drugs and procarcinogens. NAT can either detoxify or activate procarcinogens, complicating the manner in which these enzymes may participate in enhancing or preventing toxic responses to particular agents. Mice possess three NAT isoenzymes: Nat1, Nat2, and Nat3. Whereas Nat1 and Nat2 can efficiently acetylate many arylamines, few substrates appear to be appreciably metabolized by Nat3. We generated a Nat3 knockout mouse strain and used it along with our double Nat1/2(−/−) knockout strain to further investigate the functional role of Nat3. Nat3(−/−) mice showed normal viability and reproductive capacity. Nat3 expression was very low in wild-type animals and completely undetectable in Nat3(−/−) mice. In contrast, greatly elevated expression of Nat3 transcript was observed in Nat1/2(−/−) mice. We used a transcribed marker polymorphism approach to establish that the increased expression of Nat3 in Nat1/2(−/−) mice is a positional artifact of insertion of the phosphoglycerate kinase-neomycin resistance cassette in place of the Nat1/Nat2 gene region and upstream of the intact Nat3 gene, rather than a biological compensatory mechanism. Despite the increase in Nat3 transcript, the N-acetylation of p-aminosalicylate, sulfamethazine, 2-aminofluorene, and 4-aminobiphenyl was undetectable either in vivo or in vitro in Nat1/2(−/−) animals. In parallel, no difference was observed in the in vivo clearance or in vitro metabolism of any of these substrates between wild-type and Nat3(−/−) mice. Thus, Nat3 is unlikely to play a significant role in the N-acetylation of arylamines either in wild-type mice or in mice lacking Nat1 and Nat2 activities.

Genetic variation in drug metabolism has been extensively studied as an important factor in determining interindividual susceptibility to environmental toxicant exposure (Nebert, 2000). The arylamine N-acetyltransferases (NAT) mediate the acetylation of clinically relevant drugs and procarcinogenic chemicals with homocyclic and heterocyclic amine structures. Two isoforms, NAT1 and NAT2, are found in humans and are characterized by distinct tissue expression and pharmacological profiles (Grant et al., 2000). Whereas NAT2 has historically been associated with the rapid versus slow acetylator phenotype, the pharmacogenomic revolution has revealed that both NAT1 and NAT2 are highly polymorphic (Grant et al., 2000; Boukouvala and Fakis, 2005). As such, the genetic variation of NAT has been the basis for extensive epidemiological investigations into the role of NAT genotypes as modifying risk factors for the development of certain cancers because NAT, paradoxically, can both detoxify and bioactivate procarcinogenic arylamines (Hein, 2002, 2006).

Metabolic activation by NAT into DNA-binding electrophiles occurs primarily via O-acetylation of hydroxylamines, whereas detoxification into innocuous metabolites is mediated by N-acetylation of the parent amine (Hein, 2002), except for diarylamines, in which N-acetylated DNA adducts are formed (Rothman et al., 1996). N-Hydroxylation via one or more isoforms of the cytochromes 450 is believed to be the first step in the bioactivation process (Kim and Guengerich, 2005) and may compete with N-acetylation in the liver. The nature of the outcome, either activation or detoxification, may be substrate-specific and dependent on the balance of interacting drug-metabolizing enzymes (DMEs) that can activate and detoxify procarcinogens and, in particular, on the tissue-specific relative expression of NAT and other DMEs. Although epidemiological studies have alluded to a role for NAT acetylator status in altering risk for the development of cancers such as those in colon and bladder, results from these studies are inconsistent because of differences in sampling size, genotyping procedures, phenotyping/genotyping discordances, and exposure situations (for reviews, see Hein, 2002, 2006; Boukouvala and Sim, 2005).

To circumvent some of the problems associated with epidemiological studies in human populations, animal models of acetylation polymorphisms have been used to study the relationship between acetyl-
The slow acetylator phenotype and arylamine-induced indices of toxicity (Hein et al., 1997). In particular, the mouse has been used as a model organism to study the acetylation polymorphism because NAT2 rapid and slow acetylation also occurs in various inbred strains of mice (Glowinski and Weber, 1982). The slow acetylator phenotype is associated with the Nat2*8 allele found in A/J and A/HeJ mice, whereas rapid acetylator strains, C57BL/6, C3H/HeJ, BALB/c, and 129/Ola, carry the Nat2*8 allele (Martell et al., 1991; Kelly and Sim, 1994; Freeland et al., 1997; Boukouvala et al., 2002). The effect of controlled arylamine exposure on toxic endpoints such as the formation of DNA adducts and tumorigenesis has been investigated using these inbred strains of mice and acetylator congenic mouse strains (Levy and Weber, 1989, 1992). However, mouse Nat2 is not functionally equivalent to human NAT2 with respect to substrate specificity and tissue localization patterns (for review, see Boukouvala and Fakis, 2005), thereby confounding the interpretation of these studies in mice.

More recently, the use of gene knockout and transgenic mouse technology has been instrumental for confirming the role of some DMEs, in particular toxic responses, and for revealing paradoxical responses for other DMEs, thereby contradicting results from some in vitro experiments (see Nebert, 2006 for review). These animal models offer the advantage of investigating genetic variation under controlled exposures and environmental and secondary genetic influences. We and others have generated knockout mouse models lacking both Nat1 and Nat2 (Sugamori et al., 2003) or Nat2 alone (Cornish et al., 2003). However, mice possess a third Nat gene, Nat3 (Kelly and Sim, 1994; Freeland et al., 1997). Expression of Nat3 and its polymorphic variants (Boukouvala et al., 2002) in both prokaryotic and eukaryotic expression systems have failed to reveal any prototypical NAT substrates that are efficiently catalyzed by this enzyme. Only a few substrates, namely, 5-aminosalicylic acid (5-ASA) and 2-aminofluorene (AF), appear to be acetylated by Nat3 at low levels in such systems (Kelly and Sim, 1994; Estrada-Rodgers et al., 1998), and Nat3 mRNA has only been detected in spleen (Boukouvala et al., 2002). Recently, a third Nat showing 91% nucleotide identity to mouse Nat3 has been cloned from rat (Walraven et al., 2006). The rat Nat3, however, can N-acetylate a number of substrates, including AF, 4-aminobiphenyl (4-ABP), 5-ASA, 3-ethylnitrosamine, 3,5-dimethylaniline, and 4,4′-dimethylenedianiline, as well as O-acetylate proteins (Kelly and Sim, 1995; Kelly and Weber, 1999). The rat Nat3, however, can N-acetylate a number of substrates, including AF, 4-aminobiphenyl (4-ABP), 5-ASA, 3-ethylnitrosamine, 3,5-dimethylaniline, and 4,4′-dimethylenedianiline, as well as O-acetylate proteins.

**Materials and Methods**

**Materials.** The pPNT vector was provided by Dr. Janet Rossant (Hospital for Sick Children, Toronto, ON, Canada). R1 embryonic stem (ES) cells were from Dr. Andras Nagy (Mount Sinai Hospital, Toronto, ON, Canada). Most ES cell culture reagents (nonessential amino acids, l-glutamine, sodium pyruvate, Dulbecco’s modified Eagle’s medium, and G418) were purchased from Invitrogen (Burlington, ON, Canada) with the exception of β-mercaptoethanol and ganciclovir (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), fetal bovine serum (HyClone, Logan, UT), and leukemia inhibitory factor (ESGRO) (Chemicon International, Inc., Temecula, CA). Acetyl-l-carnitine, carnitine acetyltransferase, acetyl-CoA sodium salt, p-aminosalicylic acid (PAS), sulfanethazine (SMZ), N-acetyl-SMZ, ABP, AF, and N-acetyl-AF used for NAT activity assays were acquired from Sigma-Aldrich Canada Ltd. N-acetylated PAS was produced in definable quantities from PAS using wild-type recombinant human NAT1 as a catalyst (Dupret et al., 1994), and N-acetyl-ABP was provided by Dr. M. Novak (Miami University, Oxford, OH). Restriction enzymes for cloning and restriction digests were purchased from Invitrogen. Oligonucleotide primers were synthesized by Invitrogen. CD-1, C57BL/6 (B6), 129/SvJ (129), and CAST/Ei mice were obtained either from Charles River (St. Constant, QC, Canada) or The Jackson Laboratory (Bar Harbor, ME). Nat1(-/-) mice were produced as described (Sugamori et al., 2003). All the procedures involving animals were in accordance with the Canadian Council for Animal Care guidelines for use and care of animals.

**Construction of Targeting Vector.** A Nat3 targeting construct was generated using 129/SvJ genomic DNA and the vector pPNT (Tybulewicz et al., 1991), which contains a bacterial phosphoribosyltransferase II gene conferring neomycin resistance (neo) and a herpes simplex virus thymidine kinase (tk) gene, each under the transcriptional control of the mouse phosphoglycerate kinase (PGK) promoter. A 6.5-kilobase (kb) SacI/KpnI fragment corresponding to −6 kb of the 5′-untranslated region (UTR) and 0.48 kb of coding sequence of the Nat3 129/SvJ gene was blunt end–ligated into the XhoI site of pPNT (see Fig. 1A), located upstream to the bacterial phosphoribosyltransferase II gene. A 1.4-kb fragment corresponding to the 3′ 0.39 kb of coding sequence and 1 kb of the 3′-UTR was generated by polymerase chain reaction (PCR) (30 cycles of 94°C 10 s, 55°C 10 s, and 72°C 30 s) using the NAT3/KpnI 5′ and 3′ primers (see Table 1). This fragment was subcloned into the KpnI site of pPNT located upstream to the tk gene. Restriction digests and DNA sequencing confirmed insertion and correct orientation of the sequences.

The resulting construct was purified using a QIAGEN MaxiPrep kit (Qiagen Inc., Mississauga, ON, Canada).
Production of Chimeric Mice. R1 ES cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, 2 mM l-glutamine, 20% fetal bovine serum, and 1000 U/ml leukemia inhibitory factor. The sex-matched (ABP, AF) was administered by i.p. injection to 8- to 9-week-old age- and sex-matched Nat3(−/−) mice and wild-type B6 mice (n = 3 each). Blood samples were drawn from the saphenous vein using heparinized microvolumes (Sarstedt Inc., Montreal, QC, Canada) at four different time points (15, 30, 60, and 90 min for PAS; 2, 6, 22, and 24 h for ABP and SMZ; 1, 2, 4, and 6 h for AF) and centrifuged to separate plasma. Plasma samples were diluted 1:50 in HPLC mobile phase and analyzed for parent and acetylated products by HPLC as described above for the in vitro assays. For SMZ and N-acetyl-SMZ, a mobile phase consisting of 88% sodium perchlorate buffer/12% acetonitrile was used and an ultraviolet detector setting of 254 nM were used. Blank plasma samples were spiked with known amounts of parent and acetylated metabolites to quantify the amount of parent and acetylated products present in the samples. Area under the curve values were determined by the trapezoidal rule using the computer program Prism (GraphPad Software Inc., San Diego, CA). Statistical analyses were performed using a Student’s t test with GraphPad Prism.

RNA and Reverse Transcriptase-PCR. Total RNA was prepared using TRIzol (Invitrogen) from various tissue sources (liver, kidney, colon, bladder, cerebral cortex, cerebellum, and spleen). Approximately 1 μg of total RNA was treated with DNase for 30 min at 37°C, heat-inactivated in the presence of 2.5 mM EDTA for 10 min at 65°C, followed by reverse transcription using a RevertAid cDNA synthesis kit (MBI Fermentas, Burlington, ON, Canada) and a reverse-phase Beckman Ultrasphere ODS 5 μm column (15 cm × 4.6 mm i.d.; Beckman Instruments, Fullerton, CA). A flow rate of 2 ml/min with a mobile phase consisting of 66% sodium perchlorate buffer/34% acetonitrile and an ultraviolet detector setting of 280 nm were used for ABP- and AF-NAT activity assays. For PAS-NAT activity assays, a mobile phase consisting of 7% acetonitrile/1% acetic acid/0.1% triethylamine and an ultraviolet detector setting of 270 nm were used.

Determination of in Vitro NAT Activity. Tissue cytosols were prepared from wild-type B6 and Nat3(−/−) animals as described (Sugamori et al., 2003). NAT activity for PAS, ABP, and AF N-acetylation was determined using various tissue cytosols (n = 3 per gender, genotype, and tissue). Initial rates for the cytosolic fractions were performed in duplicate with 0.1 mM arylamine substrate, 0.1 mM acetyl-CoA, and 20 μl of regenerating system in a reaction volume of 100 μl. The regenerating system consisted of 5 mM acetyl-1-tet-carantine and 1 U of carantine acetyltransferase per milliliter of assay buffer (250 mM triethanolamine-HCl, 5 mM EDTA, and 5 mM dithiothreitol, pH 7.5). Reactions were preincubated for 3 min at 37°C and initiated with the addition of diluted cytosol at a protein concentration of 1 mg/ml. The reactions were incubated for 10 min at 37°C and terminated by the addition of 10 μl of 15% perchloric acid. After precipitation of the denatured protein, the supernatant fractions were assayed for the N-acetylated product (N-acetyl-PAS, N-acetyl-ABP, or N-acetyl-AF) by high-performance liquid chromatography (HPLC) using a Shimadzu LC-20A system (Mandel Scientific Company Inc., Guelph, ON, Canada) and a reverse-phase Beckman Ultrasphere ODS 5 μm column (15 cm × 4.6 mm i.d.; Beckman Instruments, Fullerton, CA). The flow rate of 2 ml/min with a mobile phase consisting of 66% sodium perchlorate buffer/34% acetonitrile and an ultraviolet detector setting of 280 nm were used for ABP- and AF-NAT activity assays. For PAS-NAT activity assays, a mobile phase consisting of 7% acetonitrile/1% acetic acid/0.1% triethylamine and an ultraviolet detector setting of 270 nm were used.

Drug Administration and Plasma Elimination Kinetics. PAS, ABP, AF, or SMZ (50 mg/kg) dissolved in saline (PAS, SMZ) or dimethyl sulfoxide (ABP, AF) was administered by i.p. injection to 8- to 9-week-old age- and sex-matched Nat3(−/−) mice and wild-type B6 mice (n = 3 each). Blood samples were drawn from the saphenous vein using heparinized microvolumes (Sarstedt Inc., Montreal, QC, Canada) at four different time points (15, 30, 60, and 90 min for PAS; 2, 6, 22, and 24 h for ABP and SMZ; 1, 2, 4, and 6 h for AF) and centrifuged to separate plasma. Plasma samples were diluted 1:50 in HPLC mobile phase and analyzed for parent and acetylated products by HPLC as described above for the in vitro assays. For SMZ and N-acetyl-SMZ, a mobile phase consisting of 88% sodium perchlorate buffer/12% acetonitrile and an ultraviolet detector setting of 254 nm were used. Blank plasma samples were spiked with known amounts of parent and acetylated metabolites to quantify the amount of parent and acetylated products present in the samples. Area under the curve values were determined by the trapezoidal rule using the computer program Prism (GraphPad Software Inc., San Diego, CA). Statistical analyses were performed using a Student’s t test with GraphPad Prism.

TABLE 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nat3/Kpn 5’</td>
<td>Sense</td>
<td>GAAAGGAGAACCTGCTTCTCTG</td>
</tr>
<tr>
<td>Nat3/Kpn 5’</td>
<td>Antisense</td>
<td>GGOTGATACCCGTCCTGAATTG</td>
</tr>
<tr>
<td>Nat3 probe 5’</td>
<td>Sense</td>
<td>GCPCAGGGTCCTTCTGCTCATC</td>
</tr>
<tr>
<td>Nat3 probe 3’</td>
<td>Antisense</td>
<td>TGGGAGTCTTCGCTGAGAAAG</td>
</tr>
<tr>
<td>Nat3 5’</td>
<td>Sense</td>
<td>CTACAGGATCCGCAGTCGCAAC</td>
</tr>
<tr>
<td>Nat3 3’</td>
<td>Antisense</td>
<td>GTAGGGCTTCTGCTGTTCGACA</td>
</tr>
<tr>
<td>PGKrev 2</td>
<td>Sense</td>
<td>ATCATTGTCAGTTCTGGCAC</td>
</tr>
<tr>
<td>CASTNat3 5’</td>
<td>Sense</td>
<td>ACCACCCAGCTTCTGAGGAGT</td>
</tr>
<tr>
<td>CASTNat3 3’</td>
<td>Sense</td>
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</tr>
<tr>
<td>β-Actin 5’</td>
<td>Sense</td>
<td>GCTCTTTTGACCTGCTTCTCA</td>
</tr>
<tr>
<td>β-Actin 3’</td>
<td>Antisense</td>
<td>TAATAGGTGCTGGGTGATAGGTC</td>
</tr>
</tbody>
</table>

PCR primers were used to generate the Southern blotting screening probe, for PCR genotyping the Nat3(−/−) mice, and for RT-PCR of Nat3- and β-actin-specific products from the various tissue sources. Conditions used for PCR amplification are described in the text.
Results

Generation of Nat3(-/-) Mice. The targeted disruption of the mouse Nat3 gene was achieved by insertion of the neomycin resistance gene into the KpnI site located within ~480 bp of the Nat3 coding sequence, thereby completely disrupting its function. Homologous recombinant clones with the correct targeting event were identified by Southern blot analysis using a probe designed in the 3' flanking region as shown in Fig. 1A. From 71 double-resistant ES colonies, 4 colonies showing the correct gene targeting event were identified. Six male and four female chimeric mice, distinguished by the ES cell-derived agouti color, were generated by morula aggregation experiments. Germline transmission was observed in two of the six male animals. Mice carrying the targeted allele were genotyped by Southern blot analysis of XbaI-digested genomic DNA (Fig. 1B). The presence of the targeted allele was evident by the presence of a 2.4-kb band, whereas the wild-type allele was identified by a band of 3.1 kb. Alternatively, mice were genotyped by PCR (Fig. 1C).

Chimeric mice showing germline transmission were bred with C57BL/6 mice. Heterozygous mice (+/-) displayed normal viability and fertility and were intercrossed to produce homozygous mutants. Mice homozygous for the disrupted alleles were born normally and appeared indistinguishable (appearance, mortality rate, and reproductive capacity) from their wild-type littermates. In addition, there appeared to be no in utero lethality of the homozygous null mice as determined by their frequency of about 31% in litters from heterozygote crosses (+/+, 21 offspring; +/-, 39 offspring; --/--, 27 offspring), and gross pathological observations revealed no apparent abnormalities. Nat3(+/-) mice were backcrossed 10 times to C57BL/6 mice to generate the knockout on a congenic B6 background.

In Vitro NAT Activity. Nat3(-/-) mice displayed no difference in cytosolic NAT activity compared with wild-type B6 mice for a number of substrates in liver, kidney, or colon (Fig. 2). A previously observed gender difference in PAS, ABP, and AF acetylation was noted in the kidney for both Nat3(-/-) and wild-type mice. Male kidney cytosols for both wild-type and Nat3(-/-) mice exhibited about a 2-fold higher activity (Fig. 2; p < 0.05) than female kidney cytosols of both genotypes. No other tissues displayed a gender difference.

In Vivo NAT Activity. Similar to the in vitro results with the tissue cytosols, Nat3(-/-) mice displayed no difference compared with wild-type mice on the in vivo administration of several arylamine substrates. Area under the curve values for parent and acetylated metabolites were not significantly different between the wild-type and knockout animals (Table 2), indicating that acetylation was not impaired for these substrates in the Nat3(-/-) animals.

Nat3 Expression in Wild-Type, Nat3(-/-), and Nat1/2(-/-) Mice. RNA was isolated from wild-type and Nat3(-/-) mice and subjected to RT-PCR to confirm disruption of the Nat3 gene. The RT-PCR detected only very low levels of Nat3 transcript in wild-type animals and most often only in cerebral cortex and cerebellum (Fig. 3). Nat3 transcript could sometimes be detected in other tissues (spleen, colon, kidney, and bladder) in wild-type animals, but these results were not consistent between animals. However, no Nat3 transcript could be detected in any of the tissues from Nat3(-/-) mice. In contrast, RT-PCR results from Nat1/2(-/-) (Fig. 3) and Nat1/2(+/-) mice (data not shown) clearly and reliably indicated a high level of Nat3 transcript in all tissues. To determine whether this augmented Nat3 transcript in Nat1/2 null mice was caused by a physiologically relevant compensatory mechanism or the possible effect of adjacent insertion of the PGK-neo gene cassette, a transcribed marker polymorphism strategy was used to distinguish between expression of the Nat3 alleles in mice heterozygous for the insertion. 129/SvJ and C57BL/6 mice have wild-type Nat3*1 sequence, whereas CAST/Ei mice possess the Nat3*2 allele, which contains several single nucleotide polymorphisms (Boukouvala et al., 2002). We used RT-PCR and strain- or allele-specific primers to distinguish between transcripts produced from the wild-type Nat3*1 allele and the CAST Nat3*2 allele in F1 129/CAST Nat1/2(+/+) animals (Fig. 4A). RT-PCR analysis indicated that the amplified Nat3 transcript was derived only from the 129/SvJ disrupted allele and not from the allele from the CAST/Ei strain (Fig. 4B). No Nat3 amplification could be detected using the CAST/Ei-specific primer.

Discussion

We have generated a Nat3 knockout mouse model to provide insight into what role, if any, Nat3 plays in the mouse. Previous recombinant protein expression experiments have thus far indicated that only AF (Kelly and Sim, 1994) and 5-AS (Estrada-Rodgers et al., 1998) show any measurable N-acetylation by Nat3. In contrast, the recently cloned rat Nat3 displays both functional N- and O-acetylation activity for a number of substrates when expressed in a bacterial system (Walraven et al., 2006). However, determination of the relative functional contribution of Nat3-mediated acetylation to the whole animal is difficult to assess without subtype-selective substrates because Nat3 activity could be masked by Nat1 or Nat2 activity. A
knockout model completely lacking other endogenous NAT activities or lacking Nat3 may thus help to determine the relative contribution of Nat3 to overall NAT activity.

Nat3(−/−) mice appear normal with respect to growth and reproductive capacity. This is not an unexpected finding given that Nat2(−/−) and Nat1/2(−/−) mice are viable (Cornish et al., 2003; Sugamori et al., 2003), even though Nat2 is implicated in folate catabolism and is expressed early in development in the neural tube and heart (Payton et al., 1999; McQueen et al., 2003; Wakefield et al., 2005). This would indicate that none of the NAT are essential for development or physiological homeostasis, and is in keeping with the theme for many other drug-metabolizing knockout animals in which no deleterious phenotypes have been observed in the absence of chemical challenge (Gonzalez and Kimura, 2003). Nevertheless, possible endogenous roles for NAT may yet be revealed by utilizing microarray approaches, as has been done with Cyp1a2 knockout mice (Smith et al., 2003).

On the other hand, such knockout animals may serve as useful models to study pathways of drug metabolism, chemical toxicity, and carcinogenesis (Gonzalez, 2003) and have revealed some unexpected findings that contradict in vitro studies. For example, Cyp1a2 knockout mice do not display lower ABP-DNA adducts (Tsuneoka et al., 2003) nor protection against ABP-induced carcinogenesis (Kimura et al., 1999), despite efficient in vitro N-oxidation of this chemical to a more highly toxic hydroxylamine by Cyp1a2. Similarly, Cyp1a1 knockout mice exhibit increased rather than decreased toxicity from benzo[a]pyrene exposure (Uno et al., 2001, 2004).

To determine whether N-acetylation activity is impaired in Nat3(−/−) mice, both in vitro activity assays using tissue cytosols and in vivo plasma elimination studies were performed. Our results indicate that there is no decrease in cytosolic NAT activity for a number of substrates in our Nat3(−/−) animals. The gender difference in kidney PAS, ABP, and AF acetylation (Sugamori et al., 2003, 2006) was seen in both the wild-type and Nat3(−/−) mice. In addition, no difference in the in vivo plasma elimination of PAS, ABP, AF, or SMZ was observed between Nat3(−/−) and wild-type mice. This concurs with the in vivo results seen with our Nat1/2(−/−) mice, in which we were unable to detect any acetylated metabolites in the knockout animals at the level of sensitivity of our assays (Sugamori et al., 2003, 2006). The presence of acetylated metabolites in mice lacking both Nat1 and Nat2 would have indicated possible N-acetylation by Nat3. We were also unable to detect any in vitro PAS, AF,
and ABP N-acetylation activity in a number of different tissues (liver, kidney, colon, spleen, and cerebral cortex) from our Nat3 knockout mice (Sugamori et al., 2003, 2006).

Using RT-PCR, no Nat3 transcript was detected in tissues from the Nat3 knockout animals. In wild-type animals, only very low levels of transcript could be seen in some tissues, such as cerebral cortex and cerebellum, and occasionally in a few other tissues (spleen, bladder, kidney, and colon). These results were variable from animal to animal, indicating that the method used may not be sensitive enough to reliably detect low levels of expression, and confirming the difficulty in assessing Nat3 expression in mice. To date, Nat3 transcript has only been identified by other investigators in spleen (Boukouvala et al., 2002). We were unable to detect any NAT activity that could be attributed to this enzyme in the spleens of animals lacking Nat1 and Nat2 function (Sugamori et al., 2003, 2006).

In marked contrast, RT-PCR detected markedly increased levels of Nat3 transcripts in all the tissues from Nat1/2(−/−) and Nat1/2(+/−) animals. To determine whether this represented a compensated change in these animals caused by an impairment of Nat1 and Nat2 activity or an artifact created by the original gene-targeting event, we used a polymorphic marker in the Nat3 gene to distinguish whether only the Nat3 gene downstream of the targeting event was subjected to this augmentation, or whether both alleles were affected. CAST/Ei mice display several single-nucleotide polymorphisms in the coding region of Nat3 (Boukouvala et al., 2002). Thus, by breeding Nat1/2(+/−) mice on a 129/SvJ background to CAST/Ei Nat1/2(+/+) mice to generate F1 heterozygotes, it is possible to distinguish by an allele- or strain-specific PCR reaction whether one or both alleles show increased expression. Increased expression of both the CAST/Ei Nat3*2 allele (with an intact Nat1/2 gene region) and the allele representing the Nat3*1 gene downstream of the disrupted Nat1/2 gene in an F1 heterozygous animal would imply that the effect is a true transcriptional activation of the functional Nat3 promoter, and not merely the presence of the strong constitutive promoter in the PGK-neo cassette targeted to the Nat1/2 gene region that alters transcription of the Nat3 gene, located approximately 45 kb downstream of Nat2 (Boukouvala and Fakis, 2005). Because amplification detected only transcript from the targeted allele, the increase in Nat3 transcript appears to be caused by a “neighborhood” effect, either a result of insertion of the PGK-neo cassette or removal of ~9 kb of genomic sequence.

Such a neighborhood effect has been shown in a number of other knockout mouse models. For example, a reduction was seen in the expression of the Cyp2a5 gene in Cyp2a1 knockout animals (Zhuo et al., 2004), and bidirectional transcriptional activity of PGK-neo resulted in embryonic lethality in heterozygote multiple endocrine neoplasia type 1 chimeric knockout mice (Scacheri et al., 2001). Most often, the PGK-neo cassette was found to affect neighboring genes when inserted within gene clusters or locus control regions, such as the myogenic regulator factor genes (Olson et al., 1996), β-like globin locus control region (Hug et al., 1996), the Hox gene cluster (Ren et al., 2002), and the granzyme B gene cluster (Pham et al., 1996). In most of these cases, downstream gene expression was found to be attenuated with the exception of Hoxa1 expression, which showed ectopic expression as a result of the generation of a neo-Hoxa1 fusion transcript when the PGK-neo cassette was inserted in the 3′-UTR of the Hoxa2 gene (Ren et al., 2002). Removal of the PGK-neo cassette would help determine whether the transcriptional effect is caused by the presence of the selection cassette and not the result of removal of inhibitory regulatory elements or changes in the local chromatin structure as a result of deletion of almost 9 kb of genomic sequence. Regardless, the increase in Nat3 transcript in our Nat1/2(−/−) mice did not result in any functional change because we were unable to detect any Nat3-mediated N-acetylation activity in these animals with prototypical NAT substrates. Our results suggest not only that Nat3 does not play a significant role in acetylating prototypical NAT arylamine substrates in the mouse but also that Nat3 transcript levels are not predictive of enzyme function. In this regard, it is possible that the Nat3 gene is on the evolutionary path to becoming a pseudogene that is incapable of producing a functional product. However, other

**Fig. 4.** Strategy to determine whether the PGK-neo cassette increases Nat3 transcript. A, a transcribed marker polymorphism (single-nucleotide polymorphism) was used to distinguish between Nat3 alleles in mice heterozygous for that marker (Nat3*). 129/SvJ mice have wild-type Nat3*1 sequence; CAST mice have Nat3*2 sequence. A 129 SvJ Nat1/2(+/−) mouse was bred to a CAST/Ei mouse and F1 heterozygotes having one Nat1/2 knocked-out allele (−) carried by the 129 strain and one CAST allele (+) were identified by PCR genotyping. These F1 animals would be heterozygous for Nat3(Nat3*1/Nat3*2). Amplification of only the Nat3*1 sequence carried by the disrupted 129 allele would indicate that the gene-targeting event affected downstream transcription because of a “neighborhood” effect. B, RT-PCR analysis was performed using either allele- or strain-specific primers that distinguishes between the 129 and CAST Nat3 transcripts. Genomic DNA isolated from CAST or 129 strain liver served as a positive control for the primer pairs. The + lane is a PCR reaction after reverse transcription, whereas the − lane is a negative control in which PCR was performed after a mock reverse transcription reaction in which RT was omitted, to control for false-positive signals caused by genomic DNA contamination.
possibilities remain that Nat3 displays activity toward other as-yet-discovered substrates, or that it performs a still unknown role in the mouse.

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