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A Method for Genotyping Murine Arylamine *N*-Acetyltransferase Type 2 (*NAT2*): A Gene Expressed in Preimplantation Embryonic Stem Cells Encoding an Enzyme Acetylating the Folate Catabolite *p*-Aminobenzoylglutamate

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ABSTRACT. Mice have three arylamine *N*-acetyltransferase (NAT) isoenzymes (NAT1, NAT2, and NAT3) of which NAT2 is known to be polymorphic. Humans have two polymorphic isoenzymes, NAT1 and NAT2. The isoenzymes mouse NAT1 and human NAT2 are expressed predominantly in the liver and intestine and are involved in drug and xenobiotic metabolism. Mouse NAT2 and human NAT1 have a widespread tissue distribution and the folate catabolite *p*-aminobenzoylglutamate (*p*AB-Glu) has been proposed as a candidate endogenous substrate. All mice have detectable NAT2 activity, although inbred mouse strains have either a fast or slow acetylator phenotype conferred by the presence of either *NAT2**8 (fast) or *NAT2**9 (slow) alleles at the *NAT2* locus. In this report, we describe a simple method for distinguishing these murine alleles by polymerase chain reaction followed by restriction fragment length polymorphism analysis. We compared the tissue distribution of the acetylation activity found in both fast (C57BL/6J) and slow (A/J) acetylating strains of mice using *p*AB-Glu and *p*-aminobenzoic acid as probe substrates. It has previously been demonstrated that murine NAT2 is expressed in the neural tube prior to closure (Stanley L, Copp A, Rolls S, Smelt V, Perry VH and Sim E, *Teratology* **58:** 174–182, 1998). We demonstrate here that murine *NAT2* is expressed in preimplantation embryonic stem cells. Murine *NAT2* is likely to be expressed prior to neurulation and this may be important in view of the protective role of folate in neural tube development. BIOCHEM PHARMACOL **58**;5:779–785, 1999.

KEY WORDS. mouse; NAT; genotyping; folate; embryonic stem cells

Acetylation of aromatic amines and hydrazines by NAT† (EC 2.3.1.5.) has been shown to be an important route in their metabolism (for review, see [1]). NATs catalyse the acetyl-CoA-dependent *N*-acetylation of these compounds, resulting in their detoxification. NAT can also activate potential DNA adduct-forming compounds by catalysing the *O*-acetylation of arylhydroxylamines [2, 3]. Three functional murine NAT isoenzymes (NAT1, NAT2, and NAT3) [4, 5], and two human isoenzymes (NAT1 and NAT2) [6, 7] have been identified. Murine NAT2 [8, 9] and both human isoenzymes are known to be polymorphic [10–13]. It has been demonstrated that polymorphisms in the human *NAT2* gene affect susceptibility to environmentally induced bladder cancer, such that the slow acetylator phenotype confers a greater risk [14, 15]. It has also been suggested that polymorphisms at human *NAT1* may affect cancer susceptibility [16], though this has been disputed [17]. Furthermore, it has been shown that mice of the slow acetylator type are more susceptible to carcinogenesis [18].

Together with the ability to catalyse the acetylation of carcinogens, NAT isoenzymes also act on a range of non-carcinogenic compounds, including hydrazines and arylamines [19–22]. Mouse isoenzymes NAT1 and NAT2 demonstrate distinct substrate profiles, metabolising isoniazid and *p*ABA, respectively; they have shared specificity for 2-aminofluorene [23, 24]. These substrate profiles have been exploited to identify the tissue distribution of murine NAT1 and NAT2 [25]. Studies have also been supplemented by immunohistochemical analysis using antipeptide antibodies which specifically recognise murine NAT2 [26]. As yet, no probe substrate has been identified for murine NAT3 [4, 24, 27]. Mouse NAT1 and human NAT2 are found predominantly in the liver, whereas mouse NAT2, like human NAT1, is found in most tissues [26]. On the basis of the similarity of their tissue distribution and substrate specificities, human NAT1 and mouse NAT2

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† *Abbreviations:* A*p*AB-Glu, acetyl *para*-aminobenzoylglutamate; gDNA, genomic DNA; NAT, arylamine *N*-acetyltransferase; NTD, neural tube defects; *p*ABA, *para*-aminobenzoic acid; *p*AB-Glu, *para*-aminobenzoylglutamate; and PCR, polymerase chain reaction.

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TABLE 1. Amino acid comparison of human and mouse NAT isoenzymes

	Human (NAT1)	Mouse (NAT1)	Mouse (NAT2)	Mouse (NAT3)
Human (NAT1)	—	[85.8]	[90.3]	[80.7]
Mouse (NAT1)	74.8	—	[90]	[82]
Mouse (NAT2)	82	81.7	—	[83.4]
Mouse (NAT3)	68.2	67.6	73.4	—

Sequence similarity is depicted in square brackets and identity without brackets. Comparisons between human NAT1 and mouse NAT2 are underlined. NAT enzyme isoform is shown in curved brackets below species name.

have been proposed to have a similar role in both species. A comparison at the amino acid level of human NAT1 with the mouse NAT isoenzymes demonstrates a higher level of similarity to mouse NAT2 than to the two other mouse isoenzymes (Table 1). There is an identity of 82% and a similarity of 90.3% between human NAT1 and mouse NAT2. In addition, studies of heterologously expressed murine enzymes have demonstrated that recombinant mouse NAT2 could catalyse the acetylation of the human NAT1 probe substrate *p*ABA [8, 23, 26]. It has been proposed [26] and subsequently demonstrated [24] that the folate catabolite *p*AB-Glu is a substrate of murine NAT2. The compound *p*AB-Glu has previously been described as a likely substrate of human NAT1 [21, 28].

Folate catabolism in humans and rodents involves the enzymatic reduction of the folate molecule to tetrahydrofolate followed by cleavage, which may be spontaneous, at the C9-N10 position to yield the pteridine moiety and *p*AB-Glu [29]. Radiolabelled folate has been used to show that the main metabolite excreted in the urine of rodents is the acetylated form of *p*AB-Glu, A*p*AB-Glu [30]. Human NAT1 can *N*-acetylate *p*AB-Glu [21, 28] and may play a role in removing biologically inactive metabolites from the folate cycle. This is an important finding in the light of recent evidence on human *NAT1* polymorphisms [10–12]. It has been shown that during pregnancy there is an increased catabolism of folate resulting in an elevated excretion of A*p*AB-Glu in rodents [31] and humans [32]. This may explain the requirement for folate supplementation during pregnancy which is known to protect against NTD in humans [33]. Studies have linked certain developmental disorders associated with neural tube closure, such as cleft lip and cleft lip and palate, to the *NAT2* locus in the mouse [34, 35].

To investigate the potential role of NAT in folate metabolism, it is essential to have a useful model. Mice provide a good model in that there are inbred strains of known NAT phenotype [23]. We have established a simple method for genotyping *NAT2* in mouse strains and have also investigated the relationship between mouse NAT2 activity, determined using *p*ABA as substrate, and the ability to metabolise the folate catabolite *p*AB-Glu. In view of the protective role of folate in neural tube defects, we have investigated whether *NAT* might be expressed prior to neurulation. It has been shown previously using immunohistochemistry that NAT2 is present in the neural tube in 9.5-day mouse embryos [36], and we demonstrate here that the *NAT2* gene is transcribed in embryonic stem cells, indicating that it is present prior to implantation.

MATERIALS AND METHODS

Chemicals and Animals

All chemicals were obtained from Sigma Chemical Company and molecular biology reagents were from Promega, unless otherwise stated. All mouse strains were obtained from Park Farm, Oxford except for CBA/6J, which were obtained from the Oxford University Biomedical Unit.

Genomic DNA Isolation

gDNA was extracted from mouse tissue homogenates as previously described for preparation of gDNA from human blood [15]. Mouse embryonic stem cells (ES 129 ola) were kindly donated by Dr. Frances Brook (Department of Zoology, University of Oxford) and were also used for the preparation of gDNA, as before [15].

PCR and PCR–Restriction Fragment Length Polymorphism (RFLP) Analysis

As the open reading frame of *NAT* is intronless, all PCR amplifications were from gDNA. PCR amplification was carried out using high-fidelity pfu DNA polymerase (error rate of 1.3×10^{-6}; Stratagene) for sequence analysis. The open reading frame of murine *NAT2* was amplified using the primers 5′MNAT2/1 (5′-ATGGACATCGAAGCGTACTTTG-3′) and 3′MNAT2/910 (5′-TTCCAAGTACATGGAAGGACACC-3′), in which the underlined number relates to the point of primer attachment (1 represents the adenosine of the start codon ATG). Primers were annealed at 58° for 30 sec and product elongation was at 72° for 2 min to yield a 910 bp cDNA product. PCR reactions were cycled for 15 times to minimise the incorporation of errors. Specific murine *NAT2* primers were used for PCR–RFLP analysis and amplification was performed

using Taq DNA polymerase (Promega). cDNA was amplified by PCR using the murine *NAT2* specific primers 5′MNAT2/278 (5′-GTCTTTAACACTCCAGCC-3′) and 3′MNAT2/409 (5′-ATTCCAGAGGCTCCCAC-3′), in which the underlined number relates to the point of primer attachment (1 represents the adenosine of the start codon ATG). Primers were annealed at 54° for 30 sec and product elongation was at 72° for 30 sec to yield a 128 bp cDNA product. The PCR product was digested with *Tru*91 (*Mse*I) at 65° for 90 min. Products were analysed by electrophoresis on a 3% MetaPhor (Flowgen)/1% agarose (Boehringer) gel. The gene coding for murine β-actin [37] was amplified by PCR across introns II, III, and IV, using Taq polymerase and the primers actin-sense 5′-GGAT-GACGATATCGCTGC-3′ and actin-antisense 5′-GTG-GTACCACCAGACAGCA-3′. Primers were annealed at 58° for 30 sec and product elongation was for 2 min. Product sizes from gDNA and cDNA were predicted to be 1580 bp and 910 bp, respectively.

Sequencing of Murine NAT2

PCR amplification from gDNA was performed using high-fidelity proofreading pfu DNA polymerase (Stratagene). Products were 3′ tagged with dATP, cloned into the vector pGEMT (Promega), and analysed with M13 forward and reverse primers using an ABI 377 automated sequencer (The Advanced Biotechnology Centre, London).

Detection of mRNA in Embryonic Stem Cells

Total RNA was extracted from embryonic stem cells, using Tri-Reagent™ (Sigma), and was used as a template for cDNA synthesis by reverse transcriptase-PCR. Amplification by PCR of murine *NAT2* and the gene encoding for murine β-actin was then performed on cDNA using the specific primers described above.

Enzymic Assays

Mice were killed by cervical dislocation. Blood was collected in 10 mM potassium phosphate pH 7.5, 1.15% (w/v) potassium chloride, 5 mM EDTA after decapitation. Isolated tissues were either used directly for cytosol preparation or snap-frozen in liquid N_2. For cytosol preparation, tissues were prepared in three times their mass of potassium–EDTA buffer (10 mM potassium phosphate pH 7.5, 1.15% (w/v) potassium chloride, 1 mM EDTA, 1 mM dithiothreitol and 0.5 mM pefabloc (protease inhibitor; S. Black) and homogenised on ice with three bursts of 2 min using a tissue grinder (Janke and Kunkel™, labortechnik Ultra-Turrax T25). The homogenate was centrifuged (14,500 *g* for 20 min at 4°) and the supernatant collected. This supernatant was subjected to a further centrifugation (100,000 *g* for 1 hr at 4°) to pellet the microsomal fraction. The cytosol in the supernatant was snap-frozen and stored in liquid N_2. Murine NAT2 activities were determined using *p*ABA as a substrate [26]. *N*-Acetylation of *p*AB-Glu was determined by HPLC (Waters 484/600E) with UV detection at 266 nm using a modified method to that previously described [38]. Arylamine substrates were used at a concentration of 0.22 mM, with acetyl-CoA at 0.44 mM.

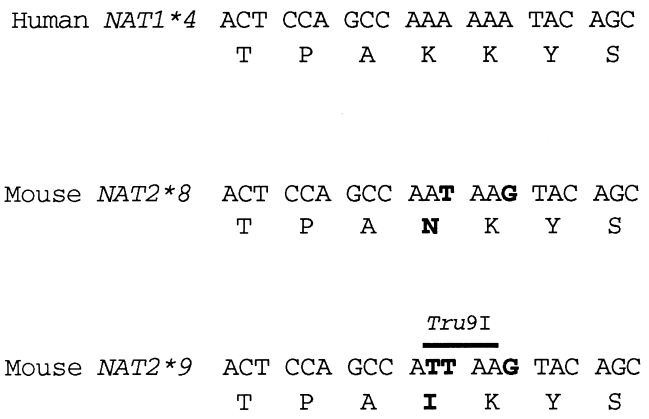

FIG. 1. Known polymorphisms within the murine NAT2 open reading frame compared to wild-type human NAT1. A unique *Tru*91 restriction site is shown (T/TAA) which distinguishes murine NAT2*8 from NAT2*9. Nucleotides and amino acids are represented as single letter codes and are in bold face if different from the most common human allotype, NAT1*4.

RESULTS

Murine NAT2 *Genotype Determination and Acetylation Phenotype*

Open reading frames representing murine *NAT2* were sequenced from mouse strains A/J, C57BL/6J, 129 ola embryonic stem cells, CBA/B6, and Balb/c. The mouse sequences were identical except at nucleotide position 296. At this point, an $A^{296}T$ mutation results in the creation of a *Tru*91 restriction site and an amino acid substitution of $ASN^{99}ILE$ (Fig. 1). This point mutation creates a slow acetylation phenotype, *NAT2**9, from a fast acetylation phenotype, *NAT2**8 [8]. The sequence surrounding this point mutation is highly conserved when compared to the most common allele of human *NAT1* [6, 7], which has a LYS in place of the murine ASN/ILE (*NAT2**8/*NAT2**9). A 128 bp fragment was PCR-amplified from murine gDNA using specific primers for murine *NAT2* (5′MNAT2/1 and 3′MNAT2/910). Digestion with *Tru*91 revealed the presence of a smaller band of 112 bp, characteristic for *NAT2**9, in the A/J mouse (Fig. 2, lane 7). All other products remained the same size as the undigested product, 128 bp, characteristic of *NAT2**8 (Fig. 2, lanes 2–6 and 8–11). Genotypes as described in Fig. 2 in comparison with phenotypes for different mouse strains are shown (Table 2). There is a good correlation of genotype and phenotype, and it is proposed that the 129 ola strain of embryonic stem cells is derived from a fast acetylating strain of mice.

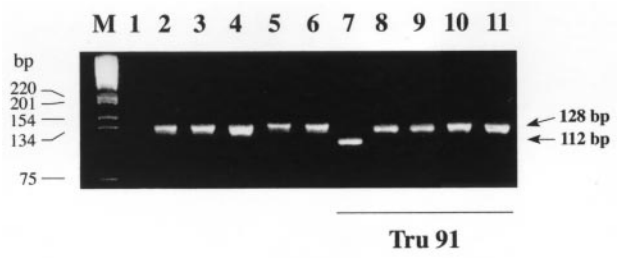

FIG. 2. Gel electrophoresis of murine *NAT2* PCR-amplified products before and after restriction digestion with *Tru*91. Amplification of gDNA results in a fragment of 128 bp which is digested to 112 bp for *NAT29, and remains at 128 bp for *NAT2**8. Lane M represents a 1 kb marker (GIBCO BRL). Lane 1 is a blank containing no gDNA, lanes 2 to 6 are PCR products, prior to digestion, amplified from gDNA isolated from A/J, C57BL/6J, 129 ola embryonic stem cells, CBA/B6, and Balb/c respectively. Lanes 7 to 11 are PCR products digested with *Tru*91.**

N-*Acetylation of* pAB-Glu *and* pABA *Using Mouse Tissue Cytosols*

Acetylation of *p*AB-Glu (Fig. 3a) in both fast and slow acetylating mouse strains followed a similar pattern to that found when using *p*ABA as substrate (Fig. 3b). With both substrates, a difference in the specific activity of acetylation was observed between the two strains of mice for the tissues liver, kidney and spleen. There was a less marked difference between the specific activities of the two strains for heart tissue. Two areas of the brain were investigated and were found to have a lower, but measurable, specific activity compared with the other tissues investigated using *p*ABA and *p*AB-Glu as substrates. These results indicate a low level of murine NAT2 in the brain and are in agreement with other studies [25], including immunohistochemical analysis which demonstrated specific recognition of murine NAT2 within the Purkinje cells of the cerebellum of mice [36].

Transcription in Embryonic Stem Cells

In order to determine whether murine *NAT2* was being transcribed in embryonic stem cells, mRNA was prepared and cDNA was made by reverse transcription. PCR amplification was performed using two sets of primers for murine *NAT2* and β-actin, as described in Methods. The primers for murine *NAT2* will generate a product of 910 nucleo-

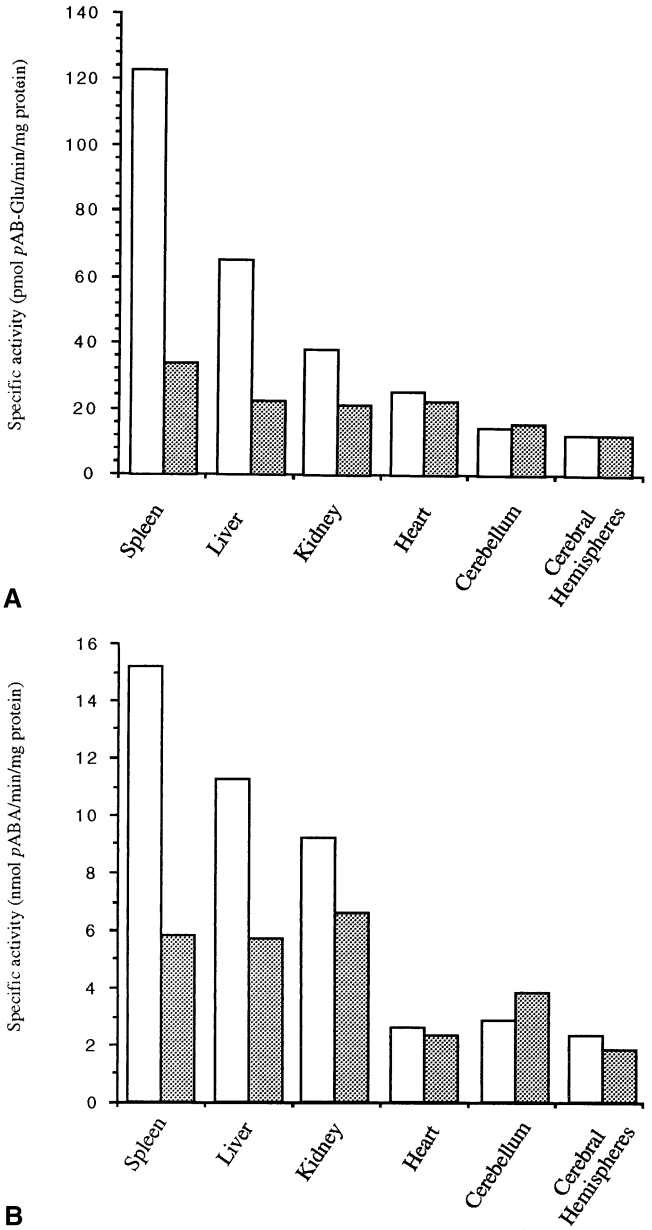

FIG. 3. Comparison of acetylation of (A) *p*-aminobenzoylglutamate and (B) *p*-aminobenzoic acid as substrates with cytosols of different tissues isolated from fast (C57BL/6J) and slow (A/J) acetylating strains of mice. The white columns represent C57BL/6J mice and the shaded columns represent A/J mice. An average of two independent determinants are shown, these values being within a 5 percent error.

TABLE 2. Determined genotype and phenotype for different mouse strains

Mouse strain	Genotype	Phenotype	Reference
A/J	*NAT2**9	Slow	Tannen & Weber, 1980
C57BL/6J	*NAT2**8	Fast	Tannen & Weber, 1980
129 ola embryonic stem cell	*NAT2**8	Fast	*
CBA/B6	*NAT2**8	Fast	†
Balb/c	*NAT2**8	Fast	Kelly and Sim, 1994

* Determined from genotype.

† Determined by comparison of specific activity of *p*ABA acetylation in blood. A/J; 0.071 nmol. min^{-1} mg $protein^{-1}$. CBA/B6; 2.17 nmol. min^{-1} mg $protein^{-1}$.

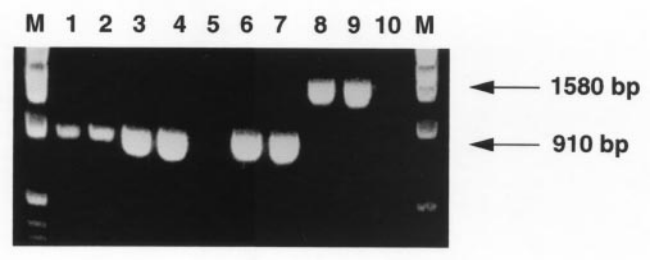

FIG. 4. PCR amplification from cDNA and genomic DNA isolated from embryonic stem cells using specific primers for murine *NAT2* and the gene coding for murine β-actin. PCR products using primers specific for murine *NAT2* (lanes 1–5) and part of the gene encoding for β-actin (lanes 6–10) are shown. Lane M represents a DNA ladder (GIBCO BRL). Lanes 1, 2, 6, and 7 represent amplification from cDNA. Lanes 3, 4, 8, and 9 represent amplification from gDNA. Lanes 5 and 10 are blanks which contain no template DNA.

tides from both cDNA and gDNA. The primers specific for the mouse β-actin gene will generate products of 1580 and 910 nucleotides from mouse gDNA and cDNA, respectively. The results shown (Fig. 4) clearly demonstrate that the cDNA preparation contains no gDNA as indicated by the presence of a 910 bp product only when primers specific for the murine β-actin gene were used. There is no detectable contamination of cDNA by gDNA, since a control amplification from gDNA using the same primers indicates the presence of a product at 1580 bp, a result of the introns within the β-actin gene [37]. Thus, it can be concluded that under the conditions used, amplification by PCR of murine *NAT2* is due to the presence of transcribed RNA (cDNA).

DISCUSSION

This study demonstrates an effective and simple method for the determination of murine *NAT2* genotype and shows a clear association between genotype and phenotype. The use of probe substrates to define NAT isotype activity has been used in many previous studies for humans and mice [1]. The compound *p*ABA is a recognised specific substrate for both human NAT1 and mouse NAT2. The folate catabolite *p*AB-Glu has been demonstrated to be a substrate for human NAT1 [21, 28]. This report provides supporting evidence that *p*AB-Glu is also a substrate for mouse NAT2, an observation recently made by others studying recombinant enzymes [24]. A scheme of folate metabolism (based on [32]), on which the role of NAT in acetylation of *p*AB-Glu is superimposed, can be seen in Fig. 5.

The tissue distribution for murine NAT2 specific activity was similar for both *p*ABA and *p*AB-Glu as substrates investigated with spleen, liver, and kidney from C57BL/6J mice. The differences in *p*ABA and *p*AB-Glu acetylation activities observed between the fast and slow mouse strains in this study (Fig. 3) is at the high end of the ranges which have been observed previously [25, 26], but is within the threefold range predicted for liver NAT activity from *in vivo* studies [39]. The heart and the two regions of the brain investigated demonstrated less difference between the fast and slow acetylating mouse strains with *p*ABA and *p*AB-Glu (Fig. 3, a and b). Previous studies have indicated the presence of murine NAT in the brain [25, 36]. The demonstration of mouse NAT2 activity in the cytosol of the cerebellum agrees with earlier immunohistochemical observations that there is NAT2 in specific neurones within the brain, including the Purkinje cells of the cerebellum [36]. The same cellular distribution of *NAT* in rate cerebellum has also been demonstrated using *in situ* hybridisation [40]. It may be significant that the tissues which show less of a difference between the two species of mice are those in which murine *NAT2* is expressed earliest in embryonic development, namely the heart and neural tissues, Fig. 3 and [36].

Our results support the suggestion that murine NAT2, like human NAT1, could be involved in folate catabolism (Fig. 5). We have observed previously that murine *NAT2* is expressed in the neural tube of developing embryos and show here clear evidence that the gene is transcribed in preimplantation embryonic stem cells (Fig. 4). The acetylated form of *p*AB-Glu is a major excretory product from the folate cycle (Fig. 5). Factors affecting the acetylation of *p*AB-Glu might be expected to influence this cycle. Previous studies have implied a link between acetylation and susceptibility to NTD. Differences in the incidence of cleft lip and cleft lip and palate have been linked to the acetylator locus in congenic mouse strains [34, 35]. The incidence of these congenital defects associated with anterior neural tube closure increases after the addition of the androgen glucocorticoid [41]. A recent study has demonstrated the presence of a hormone response element upstream of murine *NAT2* sensitive to glucocorticoid [42]. Others have observed increased *N*-acetylation in the presence of glucocorticoid in rats [43] and rabbits [44]. These observations forge a link between glucocorticoid administration, murine NAT2 expression, and congenital defects in mice.

Genetic disorders such as mutations in 5,10-methylenetetrahydrofolate reductase (MTHFR) [45], a deficiency in dietary folate, or an imbalance in the removal of folate catabolites could upset the finely tuned folate cycle. Mutations within the MTHFR gene account for approximately 13% of NTDs in humans [46]. However, folate supplementation can result in a 70% decrease in NTDs [33]. Therefore, additional factors must be involved in dysfunction of the folate cycle and defective pyrimidine biosynthesis has been proposed as one factor [47]. We suggest that acetylation polymorphisms at the murine *NAT2*, or human *NAT1*, locus should also be considered as a candidate factor. The observation that murine *NAT2* is transcribed in preimplantation embryonic stem cells, prior to neurulation, strengthens the case for investigation of NAT in relation to defects of neural tube closure in which folate has a protective role.

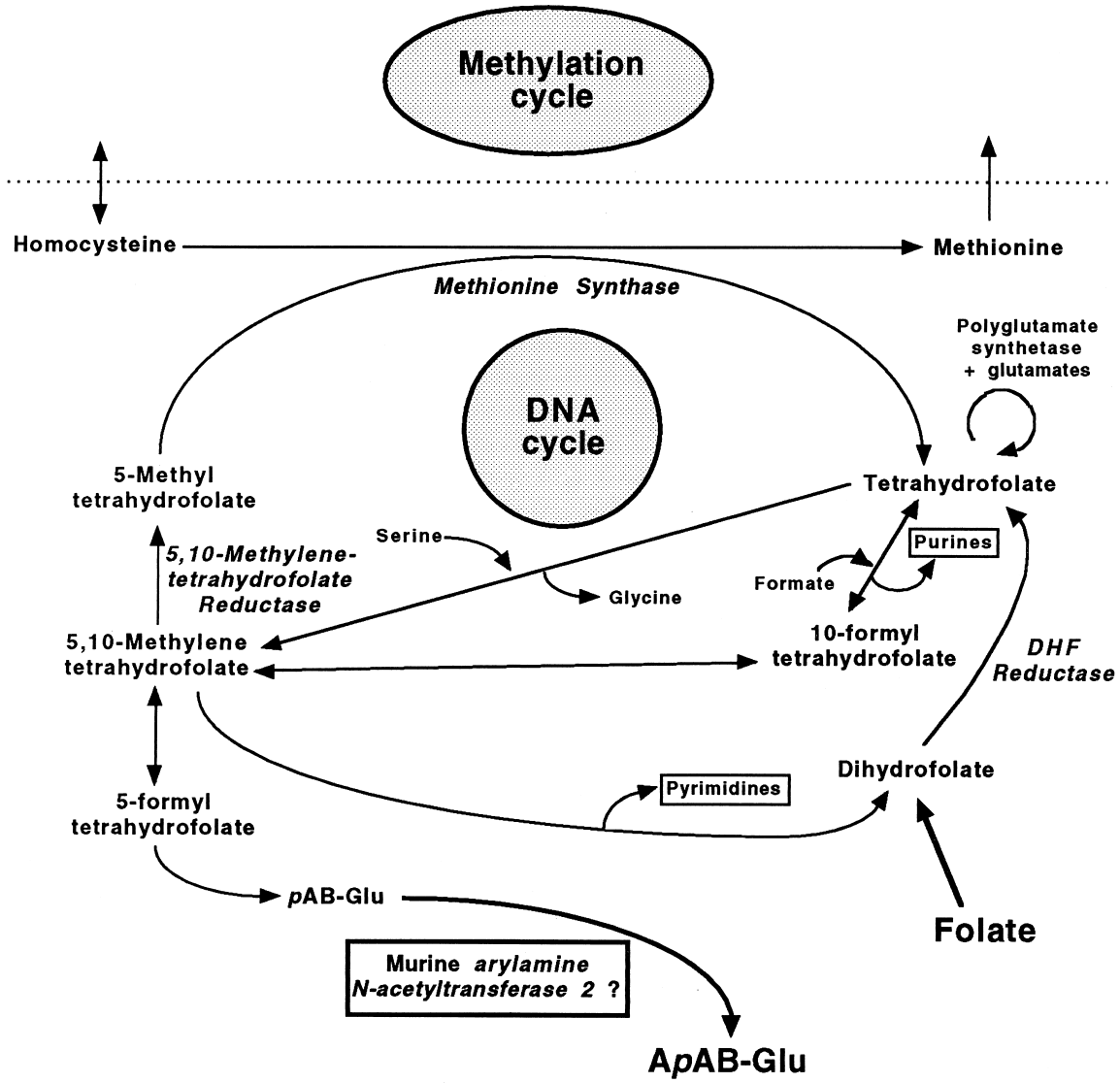

FIG. 5. Simplified scheme of the folate cycle depicting the proposed involvement of murine *N*-acetyltransferase 2 in the metabolism of *p*AB-Glu (based on [32]).

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