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NATb/NAT1*4 promotes greater arylamine *N*-acetyltransferase 1 mediated DNA adducts and mutations than NATa/NAT1*4 following exposure to 4-aminobiphenyl

Lori M. Millner, Mark A. Doll, Jian Cai, J. Christopher States, and David W. Hein*

Department of Pharmacology and Toxicology, James Graham Brown Cancer Center and Center for Environmental Genomics and Integrative Biology, University of Louisville, Louisville, Kentucky 40202

Abstract

N-acetyltransferase 1 (NAT1) is a phase II metabolic enzyme responsible for the biotransformation of aromatic and heterocyclic amine carcinogens such as 4-aminobiphenyl (ABP). NAT1 catalyzes *N*-acetylation of arylamines as well as the *O*-acetylation of *N*-hydroxylated arylamines. *O*-acetylation leads to the formation of electrophilic intermediates that result in DNA adducts and mutations. NAT1 is transcribed from a major promoter, NATb, and an alternative promoter, NATa, resulting in mRNAs with distinct 5'-untranslated regions (UTR). NATa mRNA is expressed primarily in the kidney, liver, trachea and lung while NATb mRNA has been detected in all tissues studied. To determine if differences in 5'-UTR have functional effect upon NAT1 activity and DNA adducts or mutations following exposure to ABP, pcDNA5/FRT plasmid constructs were prepared for transfection of full length human mRNAs including the 5'-UTR derived from NATa or NATb, the open reading frame, and 888 nucleotides of the 3'-UTR. Following stable transfection of NATb/NAT1*4 or NATa/NAT1*4 into nucleotide excision repair (NER) deficient Chinese hamster ovary cells, *N*-acetyltransferase activity (*in vitro* and *in situ*), mRNA, and protein expression were higher in NATb/NAT1*4 than NATa/NAT1*4 transfected cells ($p < 0.05$). Consistent with NAT1 expression and activity, ABP-induced DNA adducts and *hypoxanthine phosphoribosyl transferase* mutants were significantly higher ($p < 0.05$) in NATb/NAT1*4 than in NATa/NAT1*4 transfected cells following exposure to ABP. These differences observed between NATa and NATb suggest that the 5'-UTRs are differentially regulated.

Keywords

4-aminobiphenyl; *N*-acetyltransferase 1; alternative mRNA isoforms; arylamine DNA adducts; hprt mutants

INTRODUCTION

Human arylamine *N*-acetyltransferase 1 (NAT1) is a phase II cytosolic enzyme responsible for the biotransformation of many arylamine compounds including pharmaceuticals and environmental carcinogens. A common environmental carcinogen found in cigarette smoke is an aromatic amine, 4-aminobiphenyl (ABP) [1]. Arylamines such as ABP can be inactivated via *N*-acetylation [2]. However, if ABP is first hydroxylated by cytochrome

*To whom correspondence should be addressed at University of Louisville Health Sciences Center, 505 South Hancock Street, at Clinical and Translational Research Building Room 303, Louisville, KY 40202-1617. d.hein@louisville.edu; Tel: 502-852-6252; Fax: 502-852-7868.

p4501A1 (CYP1A1), the hydroxyl-ABP then can be further activated by NAT1-catalyzed *O*-acetylation resulting in *N*-acetoxy-ABP [2]. This compound is very unstable and spontaneously degrades to form a nitrenium ion that can react with DNA to produce bulky adducts. If these adducts are not repaired, mutagenesis can occur and result in cancer initiation.

The only known endogenous NAT1 substrate is p-aminobenzoylglutamate (PABG), a catabolite of folate [3]. *NAT1* has been associated with various birth defects [4–5] that may be related to deficiencies in folate metabolism. *NAT1* polymorphisms and maternal smoking have been associated with increased incidence of oral clefts, spina bifida and increased limb deficiency defects [6–7]. *NAT1* polymorphisms have also been associated with increased risk for breast [8–9], pancreatic [10–11], urinary bladder [12–13] and colorectal cancers [14–15], non-Hodgkin lymphoma [16–17], mammary cell growth [18] and breast cancer survival [19]. However, other studies have concluded that NAT1 polymorphism status is not associated with increased risk to bladder, esophageal, prostate or gastric cancers [20–22]. NAT1 has also been implicated in cell growth and survival. Studies have shown that overexpression of NAT1 increased density dependent cell proliferation, and knock-down of NAT1 resulted in marked change in cell morphology, an increase in cell-cell contact inhibition and a loss of cell viability at confluence [18,23]. *NAT1**4 is referred to as the referent allele because it was the most common allele in the population in which it was first identified [24]. To date, 26 human *NAT1* alleles have been identified (<http://louisville.edu/medschool/pharmacology/consensus-human-arylamine-n-acetyltransferase-gene-nomenclature/>). Although the effects of *NAT1* polymorphisms on catalytic activity have been studied, the results are ambiguous. Within single NAT1 genotypes, conflicting phenotypes have been reported, and the relationship between phenotype and genotype remains poorly understood. Since factors other than genotype are likely affecting phenotype, it is important to understand transcriptional and translational control of *NAT1*.

The *NAT1* gene spans 53 kb and contains nine exons (Figure 1a). Several *NAT1* transcripts have been identified containing various combinations of 5'-untranslated region (UTR) exons and are known to originate from two distinct promoters, NATa and NATb. NATb, the major promoter, is located 11.8 kb upstream of the open reading frame (ORF). NATb promotes transcription of Type II transcripts and the major transcript, Type IIA, has been detected in all tissues studied to date [25–26]. An alternative promoter, NATa, originates 51.5 kb upstream of the *NAT1* ORF and promotes transcription of Type I transcripts expressed primarily in kidney, lung, liver, and trachea [25,27]. The NAT1 gene is induced following exposure to androgens and NAT1 protein stability is affected by the presence of substrates [28].

Recent analyses of genome-wide Pol II distribution in *Drosophila* and mammalian systems have reported that regulation of many genes occurs after transcription initiation [29–30] providing evidence for regulatory control in the 5'-UTR that is distinct from promoter regulatory control. Recent studies have shown that between 30–50% of all human genes utilize alternative promoters [31–32] to allow for cell, tissue and disease specific expression. To determine if differences in 5'-UTR have functional effect upon NAT1 activity, DNA adducts or mutations following exposure to ABP, pcDNA5/FRT plasmid constructs were prepared for transfection of full length human mRNAs including the 5'-UTR derived from NATa or NATb, the *NAT1**4 open reading frame, and 888 nucleotides of the 3'-UTR. The constructs were cloned into two expression vectors utilizing two different constitutive promoters, (CMV and the EF1 α promoters) to examine regulatory control located in the 5'-UTR. The cells transfected with NATa/*NAT1**4 and NATb/*NAT1**4 constructs were characterized for NAT1 mRNA and protein expression, *N*- and *O*-acetyltransferase activity

(*in vitro* and *in situ*), ABP-induced DNA adducts and *hypoxanthine phosphoribosyl transferase* (*hprt*) mutations following exposure to ABP.

MATERIALS AND METHODS

Polyadenylation Site Removal

The bovine growth hormone (BGH) polyadenylation site from the pcDNA5/FRT (Invitrogen, Carlsbad, CA) vector was removed to allow the endogenous *NAT1* polyadenylation sites to be active. This was accomplished by digestion at 37°C with restriction endonucleases, *ApaI* and *SphI* (New England Biolabs, Ipswich, MA), followed by overhang digestion with T4 DNA polymerase (Invitrogen) and ligation with T4 Ligase (Invitrogen).

NATb/*NAT1**4 and NATa/*NAT1**4 Constructs

NATb/*NAT1**4 and NATa/*NAT1**4 constructs were created utilizing gene splicing via overlap extension [33] by amplifying the 5'-UTR and the coding region/3'-UTR separately and then fusing the two regions together. Beginning with frequently used transcription start sites, the 5'-UTRs [26–27] were amplified from cDNA prepared from RNA isolated from homozygous *NAT1**4 HepG2 cells. All primer sequences used are shown in Table 1. The primers used to amplify the NATb 5'-UTR region were Lkm40P1 and NAT1 (3') ORF Rev while the primers used to amplify the NATa 5'-UTR region were Lkm41P1 and NAT1 (3') ORF Rev. The coding region and 3'-UTR were amplified as one piece from *NAT1**4 human genomic DNA with *NAT1**4/*NAT1**4 genotype. The forward primer used to amplify the coding region/3'-UTR was NAT1 (3') ORF Forward while the reverse primer was pcDNA5distal Reverse. The two sections, the 5'-UTR and the coding region/3'UTR, were fused together via overlap extension and amplification of the entire product using nested primers. The forward nested primer for NATb was P1 Fwd Inr *NheI* while the forward nested primer for NATa was P3 Fwd Inr *NheI*. The reverse nested primer for both NATa and NATb constructs was NAT1 *Kpn* Rev. Both forward nested primers included the *KpnI* endonuclease restriction site and both reverse nested primers contained the *NheI* endonuclease restriction site to facilitate cloning. The pcDNA5/FRT vector and NATa/*NAT1**4 and NATb/*NAT1**4 allelic segments were digested at 37°C with restriction endonucleases *KpnI* and *NheI* (New England Biolabs). The *NAT1* constructs were then ligated into pcDNA5/FRT using T4 ligase (Invitrogen). These same *NAT1* constructs were also cloned into a second expression vector, pEF1/5V-His (Invitrogen). The NATb/*NAT1**4 construct was amplified using the forward primer, NATb Forward pEF1, while the NATa/*NAT1**4 construct was amplified using the forward primer NATa Forward pEF1. Both forward primers contained the *BamHI* restriction site. Both constructs were amplified using the reverse primer NATa/b Reverse pEF1 which contained the *EcoRV* restriction site. Both NATa/*NAT1**4 and NATb/*NAT1**4 and pEF1/5V-His were digested with the restriction endonucleases, *BamHI* and *EcoRV* (New England Biolabs), followed by ligation into the vector using T4 ligase (Invitrogen). All constructs were sequenced to ensure integrity of allelic segments and junction sites.

Cell Culture

UV5-CHO cells, a nuclease excision repair (NER)-deficient derivative of AA8 which are hypersensitive to bulky DNA lesions, were obtained from the ATCC (catalog number: CRL-1865). Unless otherwise noted, cells were incubated at 37°C in 5% CO₂ in complete alpha-modified minimal essential medium (α-MEM, Lonza, Walkersville, MD) without L-glutamine, ribosides, and deoxyribosides supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/mL penicillin (Lonza), 100 µg/mL streptomycin (Lonza), and 2 mM L-glutamine (Lonza). The UV5/CHO cells used in this study were previously

stably transfected with a single Flp Recombination Target (FRT) integration site [34]. The FRT site allowed stable transfections to utilize the Flp-In System (Invitrogen). When co-transfected with pOG44 (Invitrogen), a Flp recombinase expression plasmid, a site-specific, conserved recombination event of pcDNA5/FRT (containing either NATa/*NAT1**4 or NATb/*NAT1**4) occurs at the FRT site. The FRT site allows recombination to occur immediately downstream of the hygromycin resistance gene, allowing for hygromycin selectivity only after Flp-recombinase mediated integration. The UV5/FRT cells were further modified by stable integration of human *CYP1A1* and NADPH-cytochrome P450 reductase gene (*POR*) [34]. They are referred to in this manuscript as UV5/*1A1* cells.

COS-1 cells, a SV-40 transformed fibroblast cell line derived from African green monkey kidney, were also used for transient transfection. COS-1 cells were obtained from ATCC (catalog number: CRL-1650) and maintained at 37°C in 5% CO₂. COS-1 cells were cultured in complete Dulbecco's Modified Eagle's Medium 4.5 g/L glucose without L-glutamine (DMEM, Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/mL penicillin (Lonza), 100 µg/mL streptomycin (Lonza), and 2 mM L-glutamine (Lonza).

Transient Transfection

UV5/*1A1* and COS-1 cells were transiently transfected with pcDNA5/FRT (Invitrogen) or pEF1/V5-His (Invitrogen) containing NATa/*NAT1**4 and NATb/*NAT1**4 constructs using Lipofectamine reagent (Invitrogen) following the manufacturer's recommendations. UV5/*1A1* and COS-1 cells were co-transfected with pCMV-SPORT-βgal (β-galactosidase transfection control plasmid, Invitrogen). The cells were harvested the next day. Lysate was prepared by centrifuging the cells and resuspending pellet in homogenization buffer (20 mM NaPO₄ pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 2 µg/mL aprotinin and 2 mM pepstatin A). The resuspended cell pellet was subjected to 3 rounds of freezing at -80°C and thawing at 37°C and then centrifuged at 15,000xg for 10 min. The supernatant was used to measure *N*-acetyltransferase activity and β-galactosidase activity.

Stable Transfections

Stable transfections were carried out using the Flp-In System (Invitrogen) into UV5/*1A1* cells that were previously stably transfected with a FRT site (as noted above). The pcDNA5/FRT plasmids containing human NATa/*NAT1**4 or NATb/*NAT1**4 were co-transfected with pOG44 (Invitrogen), a Flp recombinase expression plasmid. UV5/*1A1* cells were stably transfected with pcDNA5/FRT containing NATa/*NAT1**4 and NATb/*NAT1**4 constructs using Effectene transfection reagent (Qiagen, Valencia, CA) following the manufacturer's recommendations. Since the pcDNA5/FRT vector contains a hygromycin resistance cassette, cells were passaged in complete α-MEM containing 600 µg/mL hygromycin (Invitrogen) to select for cells containing the pcDNA5/FRT plasmid. Hygromycin resistant colonies were selected approximately 10 days after transfection and isolated with cloning cylinders.

Measurement of *N*-Acetyltransferase Enzymatic Activity

In vitro assays using the NAT1 specific substrate para-aminobenzoic acid (PABA) or 4-aminobiphenyl (ABP) were conducted and acetylated products were separated utilizing HPLC [35]. Reactions containing 50 µL cell lysate, PABA or ABP (300 µM) and acetyl coenzyme A (1 mM) were incubated at 37°C for 10 min. Reactions were terminated by the addition of 1/10 volume of 1M acetic acid and centrifuged at 15,000Xg for 10 min. Supernatant was injected into a (125 mm × 4 mm; 5 µM pore size) reverse phase C18 column. Reactants and products were eluted using a Beckman System Gold high performance liquid chromatograph (HPLC) system. HPLC separation of *N*-acetyl-PABA was achieved using a gradient of 96:4 sodium perchlorate pH 2.5:acetonitrile (ACN) to

88:12 sodium perchlorate pH 2.5: ACN over 3 min and was quantitated by absorbance at 280 nm. HPLC separation of *N*-acetyl-ABP was achieved using a gradient of 85:15 sodium perchlorate pH 2.5:ACN to 35:65 sodium perchlorate pH 2.5:ACN over 10 min and was quantitated by absorbance at 260 nm. Measurements were adjusted according to baseline measurements using lysates of the UV5/*CYP1A1* cell line. Both stably and transiently transfected cells were normalized by the amount of total protein. Assays involving transiently transfected cells and PABA used β -galactosidase activity to control for transfection efficiency. To correct for transfection efficiency, β -galactosidase plasmids (pCMV-sport- β gal) were co-transfected with pcDNA5/FRT or pEF1/5V-His. β -galactosidase activity was measured in reactions containing 30 μ L cell lysate, 70 μ L of 4 mg/mL *ortho*-nitrophenyl- β -D-galactopyranoside (ONPG), and 200 μ L of cleavage buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, and 1 mM MgSO₄, pH 7.0). The reaction was incubated for 30 min at 37°C. The reaction was terminated by the addition of 500 μ L of 1 M sodium carbonate and absorbance at 420 nm was measured. Protein concentrations were measured using the method of Bradford (Bio-Rad, Hercules, CA). The β -galactosidase activities were normalized to total protein and the resulting values were used to correct for the effect of any differences in transfection efficiency. *In situ* *N*-acetyltransferase activity was studied in a whole cell assay using media spiked with differing concentrations of PABA (10–300 μ M). The cells were incubated at 37°C and media was collected after 5 h, 1/10 volume of 1M acetic acid was added, and the mixture was centrifuged at 13,000xg for 10 min. The supernatant was injected into the reverse phase HPLC column and *N*-acetyl-PABA was separated and quantitated as described above.

Measurement of *O*-Acetyltransferase Enzymatic Activity

N-hydroxy-4-aminobiphenyl (*N*-OH-ABP) *O*-acetyltransferase assays were conducted as previously described [34]. Assays containing 100 μ g total protein, 1 mM acetyl coenzyme A, 1 mg/mL deoxyguanosine (dG), and 100 μ M *N*-OH-ABP were incubated at 37°C for 10 min. Reactions were stopped with the addition of 100 μ L of water saturated ethyl acetate and centrifuged at 13,000xg for 10 min. The organic phase was removed, evaporated to dryness and the residue was dissolved in 100 μ L of 10% ACN. HPLC separation was achieved using a gradient of 80:20 sodium perchlorate pH 2.5:ACN to 50:50 sodium perchlorate pH 2.5:ACN over 3 min and dG-C8-ABP adduct was detected at 300 nm.

Measurement of NAT1 Protein

The amount of NAT1 produced in UV5/*IA1* cells stably transfected with NATa/*NAT1**4 or NATb/*NAT1**4 was determined by western blot. Cell lysates were isolated as described above. Varying amounts of lysate were mixed 1:1 with 5% β -mercaptoethanol in Laemmli buffer (Bio-Rad), boiled for 5 min, and resolved by 12% SDS-PAGE. The proteins were then transferred by semi-dry electroblotting to polyvinylidene fluoride (PVDF) membranes. The membranes were probed with a polyclonal rabbit anti-hNAT1 ES195 (1:1000) kindly provided by Edith Sim [36] and with horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit IgG antibody (1:20,000) (Pierce, Rockford, IL). Supersignal West Pico Chemiluminescent Substrate was used for detection (Pierce) and densitometric analysis was performed using Quantity One Software (Bio-Rad).

Measurement of NAT1 mRNA

Total RNA was isolated from cells using the RNeasy kit (Qiagen) followed by removal of contaminating DNA by treatment with TurboDNase Free (Ambion, Austin, TX). Synthesis of cDNA was performed using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD) using 1 μ g of total RNA in a 20 μ L reaction per the manufacturer's protocol. Quantitative RT-PCR (RT-qPCR) assays were used to assess the relative amount of *NAT1* mRNA in cells stably transfected with NATa/*NAT1**4 compared to cells stably

transfected with NATb/*NAT1**4. The Step One Plus (Applied Biosystems, Foster City, CA) was used to perform qRT-PCR in reactions containing 1× final concentration of qScript One-Step Fast mix (Quanta Biosciences), 300 nM of each primer and 100 nM of probe in a total volume of 20 µL. For qRT-PCR of *NAT1* mRNA, a TaqMan probe was used with NAT1 Total Splice Forward and NAT1 Total Splice Reverse primers (Table 1) designed using Primer Express 1.5 software (Applied Biosystems). An initial incubation at 50°C was carried out for 2 min and at 94°C for 10 min followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. TaqMan® Ribosomal RNA Control Reagents for quantitation of the endogenous control, 18S rRNA, (Applied Biosystems) were used to determine ΔC_t (*NAT1* Ct – 18S rRNA Ct). $\Delta\Delta C_t$ was determined by subtraction of the smallest ΔC_t and relative amounts of *NAT1* mRNA were calculated using $2^{-\Delta\Delta C_t}$ as previously described [27].

Measurement of NAT1 mRNA Stability

Dishes (100 × 20 mm) containing 8×10^6 stably transfected NATa/*NAT1**4 and NATb/*NAT1**4 cells were treated with complete α -MEM media spiked with 10 µg/mL of the transcription inhibitor, Actinomycin D (Sigma, St. Louis, MO). Cells were collected at 0, 2, 4, 6, and 8 hour time points and total RNA was isolated as described above. Relative *NAT1* mRNA levels were determined from cells transfected with NATa/*NAT1**4 or NATb/*NAT1**4 utilizing qRT-PCR assays as described above. The first-order rate decay constant (slope) of *NAT1* mRNA was determined by linear regression.

DNA Isolation and dG-C8-ABP Quantitation

DNA was isolated and dG-C8-ABP adducts were quantitated with modifications to a previously described method [34]. Stably transfected cells grown to approximately 80% confluency in 15 cm dishes were incubated in complete α -MEM media containing 1.56, 3.13, 6.25, 12.5 µM ABP or vehicle alone (0.5% DMSO) at 37°C. The cells were collected following 24 h of treatment, centrifuged for 5 min at 13,000xg, and the pellet was resuspended in 2 volumes of homogenization buffer (20 mM sodium phosphate pH 7.4, 1 mM EDTA), 0.1 volumes of 10% SDS and 0.1 volume of 20 mg/mL Proteinase K and allowed to incubate overnight at 37°C. The DNA was extracted using phenol/chloroform:isoamyl alcohol and precipitated with isopropanol. The pellet was dried and resuspended in 500 µL of DNA adduct buffer (5 mM Tris pH 7.4, 1 mM CaCl₂, 1 mM ZnCl₂, and 10 mM MgCl₂). The DNA was quantitated by spectrophotometry at A₂₆₀. Five hundred pg of internal standard (dG-C8-ABP-d5, Toronto Research Chemicals, North York, Ontario, Canada) was added to 30 µg of sample DNA, treated with 10 units *DNase I* (US Biological, Swampscott, MA) for 1 h at 37°C followed by treatment with 10 units nuclease P1 (Sigma) for 6 h. The reactions were then treated with 10 units of alkaline phosphatase (Sigma) overnight at 37°C. The samples were then loaded onto PepClean C-18 Spin Columns (Thermo Fisher Scientific), washed with 10% acetonitrile (ACN), eluted with 50% ACN by centrifugation at 2000xg and dried. The samples were reconstituted with 25 µL 5% ACN in 2.5 mM NH₄HCO₃ just before analysis and 10 µL of the sample was analyzed by Accela LC System (Thermo Scientific, San Jose, CA) coupled with a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA). Samples were loaded onto a 30 × 1mm × 1.9 µm Hypersil GOLD column (Thermo Scientific, San Jose, CA) and eluted with a 12.5 minute binary solvent gradient (Solvent A: 5% ACN/0.1% formic acid and Solvent B: 95% ACN/0.1% formic acid) at 50 µL/min. The gradient started from 5% Solvent B, increased linearly to 75% Solvent B in 10 min, and then remained at 75% B for 2.5 min. The eluates were ionized by electrospray ionization and dG-C8-ABP and dG-C8-ABP-d5 were detected with linear ion trap and detected by multiple reaction monitoring using the transitions of m/z 435.2 to m/z 319.2 (dG-C8-ABP) and m/z 440.2 to m/z 324.2 (dG-C8-ABP-d5). Concentrations of dG-C8-ABP were calculated from peak areas of dG-C8-ABP

and dG-C8-ABP-d5 with a calibration curve from synthetic dG-C8-ABP and dG-C8-ABP-d5.

Measurement of Cytotoxicity and Mutagenesis

Assays for cell cytotoxicity and mutagenesis were carried as previously described [37] with slight modifications. Cells were grown in HAT medium (30 mM hypoxanthine, 0.1 mM aminopterin, and 30 mM thymidine) for 12 doublings. Cells (1×10^6) were plated, allowed to grow for 24 h and were then treated with 1.56, 3.13, 6.25 or 12.5 μ M ABP (Sigma) or vehicle alone (0.5% DMSO) in media. After 48 h, cells were plated to determine survival and mutagenic response to ABP. To determine cloning efficiency following each dose of ABP, 100 cells were plated in triplicate in 6 well-plates and allowed to grow for 7 days in non-selective media. Colonies were counted and expressed as percent of vehicle control. To determine mutagenic response following ABP exposure, 5×10^5 cells were plated and sub-cultured for 7 days and then seeded with 1×10^5 cells/100 $\times$ 20 mm dish (10 replicates) in complete α MEM containing 40 μ M 6-thioguanine (Sigma). Mutant *hprt* cells were allowed to grow for 7 days and colonies were counted to determine ABP-induced mutants and corrected by cloning efficiency.

Statistical Analysis

Statistical differences were determined using either an unpaired student's t-test or one-way ANOVA using Prism Software by Graphpad (La Jolla, CA).

RESULTS

PABA *N*-Acetylation Following Transfection of NATb/NAT1*4 or NATa/NAT1*4

PABA *N*-acetylation activity was 9- to 12-fold ($p < 0.05$) higher in CHO cells transfected with NATb/NAT1*4 than NATa/NAT1*4 following both transient and stable transfections (Figure 2a,b) utilizing the CMV promoter. Figure 2b shows average PABA *N*-acetylation for 3 stable clones of each NATb/NAT1*4 and NATa/NAT1*4. One clone representative was selected from each group to conduct all further assays. To ensure that the difference was not promoter specific, *N*-acetylation activity was also measured following transfection with constructs utilizing the EF1 α promoter. PABA *N*-acetylation activity was 6-fold ($p < 0.0001$) higher in CHO cells transiently transfected with NATb/NAT1*4 than NATa/NAT1*4 (Figure 2c) utilizing the EF1 α promoter. To more accurately model *in vivo* *N*-acetylation and to confirm the *in vitro* results, an *in situ* assay was performed using PABA as the substrate in a dose response experiment (Figure 2d). The *in situ* assay showed that significantly ($p < 0.05$) more PABA *N*-acetylation activity was observed in cells stably transfected with NATb/NAT1*4 than NATa/NAT1*4 at all concentrations tested (Figure 2d) utilizing the CMV promoter. As shown in figure 3, PABA *N*-acetylation activity also was significantly higher in COS-1 cells transiently transfected with NATb/NAT1*4 than with NATa/NAT1*4 utilizing either the CMV ($p < 0.005$) or EF1 α ($p < 0.0001$) promoters.

ABP *N*-Acetylation and *N*-hydroxy-ABP *O*-acetylation Following Transfection of NATb/NAT1*4 or NATa/NAT1*4

Cells stably transfected with NATb/NAT1*4 were found to have 7-fold ($p < 0.0001$) higher ABP *N*-acetylation activity than cells stably transfected with NATa/NAT1*4 (Figure 4a) utilizing the CMV promoter. *O*-acetyltransferase activity using *N*-OH-ABP as the substrate also was found to be 7-fold ($p < 0.05$) higher in cells stably transfected with NATb/NAT1*4 than NATa/NAT1*4 (Figure 4b) utilizing CMV promoter.

Expression of NAT1 Protein Following Transfection of NATb/NAT1*4 or NATa/NAT1*4

NAT1 expression was determined by western blot in cells stably transfected with NATb/NAT1*4 and NATa/NAT1*4 utilizing the CMV promoter. Four-fold ($p<0.05$) more NAT1 was found in cells stably transfected with NATb/NAT1*4 than cells transfected with NATa/NAT1*4 following densitometric analysis (Figure 5).

Expression of NAT1 mRNA Following Transfection of NATb/NAT1*4 or NATa/NAT1*4

As shown in Figure 6a, 4-fold more NAT1 mRNA was detected in cells stably transfected with NATb/NAT1*4 than in cells transfected with NATa/NAT1*4 ($p<0.05$) utilizing the CMV promoter. To determine the cause of the difference in NAT1 steady-state mRNA between cells stably transfected with NATb/NAT1*4 and in cells transfected with NATa/NAT1*4, an mRNA stability assay was performed in the presence of actinomycin-D. No significant ($p>0.05$) difference in the NAT1 mRNA first-order decay constant was observed between NAT1 mRNA derived from cells stably transfected with NATb/NAT1*4 versus NATa/NAT1*4 (Figure 6b).

Cytotoxicity, dG-C8-ABP Adduct and *hprt* Mutations from ABP in UV5/1A1 Cells Stably Transfected With NATb/NAT1*4 or NATa/NAT1*4

CYP1A1 mediated hydroxylation and NAT1 *O*-acetylation result in DNA adducts and mutations, if not repaired. Significantly ($p<0.05$) greater cytotoxicity (Figure 7a), dG-C8-ABP adducts (Figure 7b) and *hprt* mutants (Figure 7c) were detected in cells stably transfected with NATb/NAT1*4 than NATa/NAT1*4 utilizing the CMV promoter at each ABP concentration tested up to 12.5 μ M.

DISCUSSION

As outlined in the introduction, numerous studies report that *NAT1* genetic polymorphisms increase cancer risk following exposure to heterocyclic and aromatic amines. Due to the large variability in NAT1 activity that has been reported within a single genotype, it is becoming increasingly more apparent that factors other than genetic polymorphisms are affecting gene expression and cancer risk. One such factor is the use of alternative promoters to produce mRNAs with distinct 5'-UTRs. Recent studies have shown that between 30–50% of all human genes utilize alternative promoters [31–32] to allow for cell, tissue and disease specific expression. *NAT1* has two promoters, NATa and NATb, which differ in promoter strength and tissue specificity [27,38]. Transcripts derived from NATa are found primarily in liver, lung, trachea and kidney, while transcripts derived from NATb are found in all tissues studied to date [27,38]. It is possible that NATa transcripts are expressed in a wider range of tissues, but only when the cell is under specific environmental stress or disease states. For example, expression of NATa transcripts has recently been reported in several ER-positive breast cancer cell lines [39]. NATa transcripts may be selectively up-regulated following certain environmental exposures or in specific tissues, such as breast, during certain disease states.

In the current study, two referent *NAT1*4* constructs were cloned to mimic the most common transcripts originating from each of the two alternative *NAT1* promoters, NATa and NATb (Figure 1a). Beginning with frequently used transcription start sites, the constructs include all exons found in the most common *NAT1* transcripts originating at the NATa or NATb promoters and represent Type Ia or Type IIa transcripts [26–27,40]. The NATa/NAT1*4 and NATb/NAT1*4 constructs have identical open reading frames (ORFs) and 3'-UTRs. Both constructs include the entire ORF comprised of 870 nucleotides and 888 nucleotides of the 3'-UTR. The only difference between the two constructs is the 5'-UTR.

The NATb 5'-UTR contains 117 nts and includes exon 4 and exon 8 while the NATa 5'-UTR contains 371 nts and includes exons 1, 2, 3, and 8 (Figure 1b, c).

Two constitutive promoters, the CMV and the EF1 α promoter, were used to drive transcription of either the NATb/*NAT1**4 or NATa/*NAT1**4 full length transcripts to examine regulatory control located in the 5'-UTRs. In this study, we report that cells transfected with NATb/*NAT1**4 had approximately 4-times greater NAT1 expression than cells transfected with NATa/*NAT1**4. A 4-fold difference in NAT1 mRNA expression also was observed, suggesting that transcriptional control is largely responsible for the functional differences observed between NATb/*NAT1**4 and NATa/*NAT1**4. Recent studies have elucidated a large number of tissue- and cell-type specific isoforms of transcription factors and cis-acting factors. Alternative 5'-UTRs contribute to this intricate control of transcription allowing for very specific altered expression in tissues, cells and even disease states [41]. The differences we observed were not caused by a specific interaction between the promoter and one of the 5'-UTRs because results were confirmed using two different constitutive promoters, CMV and EF1 α .

There are many regulatory mechanisms that could be responsible for the observed differences in expression and functional effects including polymerase pausing, microRNA binding, and the presence of upstream open reading frames and stem loops. A recent genome wide study has provided evidence that many genes are controlled after transcription initiation has occurred [29]. Polymerase pausing may be a widespread genetic control of gene expression [42,43]. Elongation of transcription is known to be non-uniform and RNA polymerases are prone to transient pausing that is sequence dependent [44–45]. Polymerase pausing could be examined in the NATa- and NATb-transfected cell lines by nuclear run-on or RIP-chip assays. A second possible mechanism of regulation is microRNA (miRNA) binding which regulates gene expression by catalyzing mRNA cleavage [45–47]. MicroInspector (miRNA target software) predicted only 2 miRNA binding sites in the NATb 5'-UTR located at positions 7 (has-miR-3937) and 46 (has-miR-198) while 54 miRNA binding sites were predicted throughout the NATa 5'-UTR. Regulation by these miRNAs could be analyzed by such methods as northern hybridization or microarray analysis. A third possible mechanism is regulation by upstream open reading frames (uORFs) which have been shown to reduce protein and mRNA expression [48]. Both NATa and NATb 5'-UTRs were examined for uORFs by the NCBI ORF Finder. The NATa 5'-UTR was predicted to have 2 uORFs, while the NATb 5'-UTR was predicted to have none. Studies including a luciferase reporter assay could be conducted to determine the transcriptional effects on the NATa 5'-UTR due to uORFs. Lastly, differential regulation of the NATa and NATb 5'-UTRs could be due to the presence of stem-loops [49]. NATa and NATb 5'-UTRs were both examined for the presence of stem-loops by OligoCalc (Northwestern University, Evanston, IL) with a constraint of 5 base pair minimum. The NATa 5'-UTR has 42 potential stem-loop structures while the NATb 5'-UTR has only 7 potential stem-loop structures. Real time observation of transcription initiation and elongation [50] could be useful to determine the mechanism of the differential regulation observed between NATa and NATb 5'-UTRs.

Significantly more NAT1 activity, protein, mRNA, ABP-induced cytotoxicity, DNA adducts and mutagenesis were detected in cells stably transfected with NATb/*NAT1**4 than in cells transfected with NATa/*NAT1**4 ($p < 0.05$). DNA adduct and mutant levels following exposure to ABP are biological endpoints that are very relevant to cancer risk. The findings that ABP-mediated DNA adduct and mutant levels were significantly higher in cells transfected with elevated NAT1 catalytic activity emphasizes the relative importance of NAT1-catalyzed *O*-acetylation of *N*-hydroxy-ABP in cancer risk. Associations between higher *N*-acetyltransferase 2 catalytic activity with higher ABP-mediated cytotoxicity, DNA

adduct formation, and mutagenesis also were recently reported [51]. The finding that these cancer risk indicators were higher in cells transfected with NATb/*NAT1**4 than cells transfected with NATa/*NAT1**4 suggest that differential regulation in the *NAT1* 5'-UTR also may modify ABP-mediated cancer risk. Because NATb transcripts are expressed ubiquitously, the minor transcript, NATa, may be expressed following environmental exposures or under certain disease states resulting in increased mutagenesis, enhanced tumor growth, and decreased chemotherapeutic sensitivity. For example, expression of NATa transcripts have recently been reported in several ER-positive breast cancer cell lines [39].

The findings of this study are significant due to their relevance to ABP-mediated carcinogenesis. However, translation of our results obtained in cell culture to human subjects will require additional studies to investigate tissue specificity. Although our study focused only on the referent allele, *NAT1**4, future studies should investigate 5'-UTR control with other *NAT1* alleles, particularly those associated with increased cancer risk. Future investigations to determine mechanism(s) and location(s) of the differential regulation in the *NAT1* 5'-UTR also are needed.

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References

1. International Agency for Research on Cancer L. Overall evaluation of carcinogenicity: an update of IARC monographs. Monographs on the evaluation of carcinogenic risk to humans. 1987; (suppl 7): 1–42.
2. Hein DW, Doll MA, Rustan TD, et al. Metabolic activation and deactivation of arylamine carcinogens by recombinant human NAT1 and polymorphic NAT2 acetyltransferases. *Carcinogenesis*. 1993; 14(8):1633–1638. [PubMed: 8353847]
3. Wakefield L, Cornish V, Long H, Griffiths WJ, Sim E. Deletion of a xenobiotic metabolizing gene in mice affects folate metabolism. *Biochem Biophys Res Commun*. 2007; 364(3):556–560. [PubMed: 17961509]
4. Jensen LE, Hoess K, Whitehead AS, Mitchell LE. The NAT1 C1095A polymorphism, maternal multivitamin use and smoking, and the risk of spina bifida. *Birth Defects Res A Clin Mol Teratol*. 2005; 73(7):512–516. [PubMed: 15959877]
5. Lammer EJ, Shaw GM, Iovannisci DM, Van Waes J, Finnell RH. Maternal smoking and the risk of orofacial clefts: Susceptibility with NAT1 and NAT2 polymorphisms. *Epidemiology*. 2004; 15(2): 150–156. [PubMed: 15127906]
6. Carmichael SL, Shaw GM, Yang W, Iovannisci DM, Lammer E. Risk of limb deficiency defects associated with NAT1, NAT2, GSTT1, GSTM1, and NOS3 genetic variants, maternal smoking, and vitamin supplement intake. *Am J Med Genet A*. 2006; 140(18):1915–1922. [PubMed: 16906563]
7. Jensen LE, Hoess K, Mitchell LE, Whitehead AS. Loss of function polymorphisms in NAT1 protect against spina bifida. *Hum Genet*. 2006; 120(1):52–57. [PubMed: 16680433]
8. Millikan RC, Pittman GS, Newman B, et al. Cigarette smoking, N-acetyltransferases 1 and 2, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev*. 1998; 7(5):371–378. [PubMed: 9610785]
9. Zheng W, Deitz AC, Campbell DR, et al. N-acetyltransferase 1 genetic polymorphism, cigarette smoking, well-done meat intake, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev*. 1999; 8(3):233–239. [PubMed: 10090301]

10. Li D, Jiao L, Li Y, et al. Polymorphisms of cytochrome P4501A2 and N-acetyltransferase genes, smoking, and risk of pancreatic cancer. *Carcinogenesis*. 2006; 27(1):103–111. [PubMed: 15987714]
11. Suzuki H, Morris JS, Li Y, et al. Interaction of the cytochrome P4501A2, SULT1A1 and NAT gene polymorphisms with smoking and dietary mutagen intake in modification of the risk of pancreatic cancer. *Carcinogenesis*. 2008; 29(6):1184–1191. [PubMed: 18499698]
12. Sanderson S, Salanti G, Higgins J. Joint effects of the N-acetyltransferase 1 and 2 (NAT1 and NAT2) genes and smoking on bladder carcinogenesis: a literature-based systematic HuGE review and evidence synthesis. *Am J Epidemiol*. 2007; 166(7):741–751. [PubMed: 17675654]
13. Gago-Dominguez M, Bell DA, Watson MA, et al. Permanent hair dyes and bladder cancer: risk modification by cytochrome P4501A2 and N-acetyltransferases 1 and 2. *Carcinogenesis*. 2003; 24(3):483–489. [PubMed: 12663508]
14. Shin A, Shrubsole MJ, Rice JM, et al. Meat intake, heterocyclic amine exposure, and metabolizing enzyme polymorphisms in relation to colorectal polyp risk. *Cancer Epidemiol Biomarkers Prev*. 2008; 17(2):320–329. [PubMed: 18268115]
15. Bell DA, Stephens EA, Castranio title, Umbach DM, Watson M, Deakin M, Elder J, Hendrickse C, Duncan H, Strange RC. Polyadenylation polymorphism in the acetyltransferase 1 gene (NAT1) increases risk of colorectal cancer. *Cancer Res*. 1995; 55:3537–3542. [PubMed: 7627961]
16. Morton LM, Schenk M, Hein DW, et al. Genetic variation in N-acetyltransferase 1 (NAT1) and 2 (NAT2) and risk of non-Hodgkin lymphoma. *Pharmacogenet Genomics*. 2006; 16(8):537–545. [PubMed: 16847422]
17. Kilfoy BA, Zheng T, Lan Q, et al. Genetic variation in N-acetyltransferases 1 and 2, cigarette smoking, and risk of non-Hodgkin lymphoma. *Cancer Causes Control*. 2010; 21(1):127–133. [PubMed: 19809881]
18. Adam PJ, Berry J, Loader JA, et al. Arylamine N-acetyltransferase-1 is highly expressed in breast cancers and conveys enhanced growth and resistance to etoposide in vitro. *Mol Cancer Res*. 2003; 1(11):826–835. [PubMed: 14517345]
19. Ring BZ, Seitz RS, Beck R, et al. Novel prognostic immunohistochemical biomarker panel for estrogen receptor-positive breast cancer. *J Clin Oncol*. 2006; 24(19):3039–3047. [PubMed: 16809728]
20. McGrath M, Michaud D, De Vivo I. Polymorphisms in GSTT1, GSTM1, NAT1 and NAT2 genes and bladder cancer risk in men and women. *BMC Cancer*. 2006; 6:239. [PubMed: 17026750]
21. Wideroff L, Vaughan TL, Farin FM, et al. GST, NAT1, CYP1A1 polymorphisms and risk of esophageal and gastric adenocarcinomas. *Cancer Detect Prev*. 2007; 31(3):233–236. [PubMed: 17646057]
22. Kidd LR, Hein DW, Woodson K, et al. Lack of association of the N-acetyltransferase NAT1*10 allele with prostate cancer incidence, grade, or stage among smokers in Finland. *Biochem Genet*. 2011; 49(1–2):73–82. [PubMed: 20931357]
23. Tiang JM, Butcher NJ, Cullinane C, Humbert PO, Minchin RF. RNAi-Mediated Knock-Down of Arylamine N-acetyltransferase-1 Expression Induces E-cadherin Up-Regulation and Cell-Cell Contact Growth Inhibition. *PLoS One*. 2011; 6(2):e17031. [PubMed: 21347396]
24. Vatsis KP, Weber WW. Structural heterogeneity of Caucasian N- acetyltransferase at the NAT1 gene locus. *Arch Biochem Biophys*. 1993; 301(1):71–76. [PubMed: 8442668]
25. Boukouvala S, Sim E. Structural analysis of the genes for human arylamine N-acetyltransferases and characterisation of alternative transcripts. *Basic Clin Pharmacol Toxicol*. 2005; 96(5):343–351. [PubMed: 15853926]
26. Husain A, Barker DF, States JC, Doll MA, Hein DW. Identification of the major promoter and non-coding exons of the human arylamine N-acetyltransferase 1 gene (NAT1). *Pharmacogenetics*. 2004; 14(7):397–406. [PubMed: 15226672]
27. Barker DF, Husain A, Neale JR, et al. Functional properties of an alternative, tissue-specific promoter for human arylamine N-acetyltransferase 1. *Pharmacogenet Genomics*. 2006; 16(7):515–525. [PubMed: 16788383]
28. Minchin RF, Hanna PE, Dupret JM, Wagner CR, Rodrigues-Lima F, Butcher NJ. Arylamine N-acetyltransferase I. *Int J Biochem Cell Biol*. 2007; 39(11):1999–2005. [PubMed: 17392017]

29. Nechaev S, Fargo DC, dos Santos G, Liu L, Gao Y, Adelman K. Global analysis of short RNAs reveals widespread promoter-proximal stalling and arrest of Pol II in *Drosophila*. *Science*. 2010; 327(5963):335–338. [PubMed: 20007866]
30. Aida M, Chen Y, Nakajima K, Yamaguchi Y, Wada T, Handa H. Transcriptional pausing caused by NELF plays a dual role in regulating immediate-early expression of the *junB* gene. *Mol Cell Biol*. 2006; 26(16):6094–6104. [PubMed: 16880520]
31. Takeda J, Suzuki Y, Nakao M, et al. H-DBAS: alternative splicing database of completely sequenced and manually annotated full-length cDNAs based on H-Invitational. *Nucleic Acids Res*. 2007; 35(Database issue):D104–109. [PubMed: 17130147]
32. Cooper SJ, Trinklein ND, Anton ED, Nguyen L, Myers RM. Comprehensive analysis of transcriptional promoter structure and function in 1% of the human genome. *Genome Res*. 2006; 16(1):1–10. [PubMed: 16344566]
33. Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene*. 1989; 77(1):61–68. [PubMed: 2744488]
34. Metry KJ, Zhao S, Neale JR, et al. 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine-induced DNA adducts and genotoxicity in chinese hamster ovary (CHO) cells expressing human CYP1A2 and rapid or slow acetylator N-acetyltransferase 2. *Mol Carcinog*. 2007; 46(7):553–563. [PubMed: 17295238]
35. Hein DW, Doll MA, Nerland DE, Fretland AJ. Tissue distribution of N-acetyltransferase 1 and 2 catalyzing the N-acetylation of 4-aminobiphenyl and O-acetylation of N-hydroxy-4-aminobiphenyl in the congenic rapid and slow acetylator Syrian hamster. *Mol Carcinog*. 2006; 45(4):230–238. [PubMed: 16482518]
36. Stanley LA, Coroneos E, Cuff R, Hickman D, Ward A, Sim E. Immunochemical detection of arylamine N-acetyltransferase in normal and neoplastic bladder. *J Histochem Cytochem*. 1996; 44(9):1059–1067. [PubMed: 8773572]
37. Wu RW, Tucker JD, Sorensen KJ, Thompson LH, Felton JS. Differential effect of acetyltransferase expression on the genotoxicity of heterocyclic amines in CHO cells. *Mutat Res*. 1997; 390(1–2):93–103. [PubMed: 9150757]
38. Husain A, Zhang X, Doll MA, States JC, Barker DF, Hein DW. Functional analysis of the human N-acetyltransferase 1 major promoter: quantitation of tissue expression and identification of critical sequence elements. *Drug Metab Dispos*. 2007; 35(9):1649–1656. [PubMed: 17591675]
39. Wakefield L, Robinson J, Long H, et al. Arylamine N-acetyltransferase 1 expression in breast cancer cell lines: a potential marker in estrogen receptor-positive tumors. *Genes Chromosomes Cancer*. 2008; 47(2):118–126. [PubMed: 17973251]
40. Butcher NJ, Arulpragasam A, Goh HL, Davey T, Minchin RF. Genomic organization of human arylamine N-acetyltransferase Type I reveals alternative promoters that generate different 5'-UTR splice variants with altered translational activities. *Biochem J*. 2005; 387(Pt 1):119–127. [PubMed: 15487985]
41. Davuluri RV, Suzuki Y, Sugano S, Plass C, Huang TH. The functional consequences of alternative promoter use in mammalian genomes. *Trends Genet*. 2008; 24(4):167–177. [PubMed: 18329129]
42. Core LJ, Waterfall JJ, Lis JT. Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. *Science*. 2008; 322(5909):1845–1848. [PubMed: 19056941]
43. Adelman K, La Porta A, Santangelo TJ, Lis JT, Roberts JW, Wang MD. Single molecule analysis of RNA polymerase elongation reveals uniform kinetic behavior. *Proc Natl Acad Sci U S A*. 2002; 99(21):13538–13543. [PubMed: 12370445]
44. Herbert KM, La Porta A, Wong BJ, et al. Sequence-resolved detection of pausing by single RNA polymerase molecules. *Cell*. 2006; 125(6):1083–1094. [PubMed: 16777599]
45. Ambros V. The functions of animal microRNAs. *Nature*. 2004; 431(7006):350–355. [PubMed: 15372042]
46. Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. *Genes Dev*. 2004; 18(5):504–511. [PubMed: 15014042]
47. Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W. Single processing center models for human Dicer and bacterial RNase III. *Cell*. 2004; 118(1):57–68. [PubMed: 15242644]

48. Calvo SE, Pagliarini DJ, Mootha VK. Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans. *Proc Natl Acad Sci U S A*. 2009; 106(18):7507–7512. [PubMed: 19372376]
49. Malys N, McCarthy JE. Translation initiation: variations in the mechanism can be anticipated. *Cell Mol Life Sci*. 2011; 68(6):991–1003. [PubMed: 21076851]
50. Larson DR, Zenklusen D, Wu B, Chao JA, Singer RH. Real-time observation of transcription initiation and elongation on an endogenous yeast gene. *Science*. 2011; 332(6028):475–478. [PubMed: 21512033]
51. Bendaly J, Doll MA, Millner LM, et al. Differences between human slow N-acetyltransferase 2 alleles in levels of 4-aminobiphenyl-induced DNA adducts and mutations. *Mutat Res*. 2009; 671(1–2):13–19. [PubMed: 19682468]

Figure 1.
(a) Genomic organization of NAT1 gene; (b) Type IIA and Type IA NAT1 RNA (c) and representative NATb/NAT1*4 and NATa/NAT1*4 constructs. (modified from 41).

Figure 2. *N*-acetylation of PABA in UV5/1A1 cells expressing *CYP1A1* and NATb/NAT1*4 (solid bars) or NATa/NAT1*4 (open bars). (a) PABA *N*-acetylation activity following transient transfection with pcDNA5/FRT; (b) PABA NAT1 catalytic activity following stable transfection with pcDNA5/FRT of 3 different clones of each NATb/NAT1*4 and NATa/NAT1*4; (c) PABA *N*-acetylation activity following transient transfection with pEF1/V5-His; (d) PABA *N*-acetylation *in situ* following stable transfection of pcDNA5/FRT. Each bar represents mean $\pm$ S.E.M. for three transient transfections (a, c), 3 separate collections of 3 clones (b) or 3 separate collections of 1 clone (d). Asterisks (*) represent a significant difference ($p < 0.05$) (a, b, d) or ($p < 0.0001$) (c) following a student's *t*-test.

Figure 3. *N*-acetylation of PABA in COS-1 cells transiently transfected with (a) pcDNA5/FRT or (b) pEF1/V5-His containing NATb/*NAT1**4 or NATa/*NAT1**4. Each bar represents mean $\pm$ S.E.M. for three transient transfections. Asterisks represent a significant difference either ($p < .005$) (a) or ($p < .0001$) (b) following a student's *t*-test.

Figure 4. (a) *N*-acetylation of ABP and (b) *O*-acetylation of *N*-hydroxy-ABP in UV5/1A1 cells stably expressing *CYP1A1* and either NATb/*NAT1**4 (solid bars) or NATa/*NAT1**4 (open bars) in pcDNA5/FRT. Each bar represents mean $\pm$ S.E.M. for three separate collections. Asterisks (*) represent a significant difference ($p < 0.0001$) (a) or ($p < 0.05$) (b) following a student's *t*-test.

Figure 5. NAT1 protein expression in UV5/1A1 cells stably expressing *CYP1A1* and NATb/*NAT1**4 (solid bars) or NATa/*NAT1**4 (open bars) in pcDNA5/FRT. (a) Representative western blot of 20 μ g of total protein loaded; (b) Percent intensity units (NATb defined as 100%) of densitometric analysis performed on three independent Western blots. Asterisks (*) represent a significant difference ($p < 0.05$) following a student's t-test.

Figure 6. (a) NAT1 mRNA expression levels; (b) mRNA stability in UV5/1A1 cells stably expressing *CYP1A1* and NATb/*NAT1**4 (solid bars) or NATa/*NAT1**4 (open bars) in pcDNA5/FRT. Each bar represents mean $\pm$ S.E.M. for (a) three or (b) nine determinations. Asterisks (*) represent a significant difference ($p < 0.05$) following a student's t-test.

Figure 7. ABP-induced cytotoxicity, mutagenesis, and DNA adduct formation in CHO cells stably expressing *CYP1A1* only (triangles) and NATb/*NAT1*4* (circles) or NATa/*NAT1*4* (squares) in pcDNA5/FRT. Each data point represents mean ± S.E.M. for three determinations. (a) ABP-induced cytotoxicity; (b) ABP-induced dG-C8-ABP adducts/10⁸ nucleosides; (c) ABP-induced *hprt* mutant levels.

Table 1

Primer Sequences

Primer Name	Use	Sequence
Lkm40P1	NATb 5'-UTR forward specific PCR	5'-GGCCCGCGGCGATTTCAGTCTAGTTCCTGGTTGGCC-3'
P1 Fwd Inr NheI	NATb 5'-UTR forward specific nested PCR	5'-TTTAAAGCTAGCATTCAGTCTAGTCTAGTTCCTGGTTGGCCGGCT-3'
Lkm41P3	NATa 5'-UTR forward specific PCR	5'-GGCCCGCGGGAACACATCTCTGCTCAAAATAAGCCT-3'
P3 Fwd Inr NheI	NATa 5'-UTR forward specific nested PCR	5'-TTAATGCTAGCAACACATTCTGCTCAAAATAAGCCTAGG-3'
NAT (3') ORF Rev	NATa/NATb 5'-UTR reverse PCR	5'-TTCTCTCACTCAGAGTCTTGAACTCTATT-3'
NAT1 (3') ORF For	NAT1 coding region forward PCR	5'-AGACATCTCCATCATCTGTGTTTACTAGT-3'
pcDNA5 FRTdistal Rev	NAT1 3'-UTR reverse PCR	5'-CGTGGGATACCCCTAGA
NAT1 KPN-Rev	NAT1 3'-UTR reverse nested PCR	5'-ATAGTAGGTACCTCTGAATTATATAGATAAGCAAGATTTCAGATTCT-3'
NATb Forward pEF1	NATb 5'-UTR forward specific pEF1a PCR	5'-AGTCGGATCCATTTCAGTCTAGTTCCTGGTT-3'
NATa Forward pEF1	NATa 5'-UTR forward specific pEF1a PCR	5'-AGTCGGATCCCAACACATTCTGCTCAATAAAGCCTAGGCCCAA-3'
NATa/b Reverse pEF1	NAT1 3'-UTR reverse pEF1a PCR	5'-TCGAGATATCAGCTCGGTACCTCTGAAATTATAGA-3'
NAT1 total spliced Forward	NAT1 specific forward q-RT-PCR	5'-GAATTCAAGCCAGGAAAGCA-3'
NAT1 total spliced Reverse	NAT1 specific reverse q-RT-PCR	5'-TCCAAGTCCAAATTGTTCTCTAGACT-3'
NAT1 TAQMAN probe for T.S.	TAQMAN probe for NAT1 total splice	6FAM-5'-CAA TCTGCTCTCTGGATTAA-3' MGBNFQ