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Quantitative Tissue and Gene Specific Differences and Developmental Changes in *Nat1*, *Nat2* and *Nat3* mRNA Expression in the Rat

David F. Barker, **Jason M. Walraven**, **Elizabeth H. Ristagno**, **Mark A. Doll**, **J. Christopher States**, and **David W. Hein**
Department of Pharmacology & Toxicology, James Graham Brown Cancer Center and Center for Environmental Genomics and Integrative Biology, University of Louisville School of Medicine, Louisville, Kentucky

Abstract

Human *N*-acetyltransferase 1 (NAT1) and 2 (NAT2) are important phase II enzymes involved in the biotransformation of xenobiotics. In toxicity and carcinogenicity studies, functional polymorphism of rat *N*-acetyltransferase is considered a model for similar human variability. To accurately quantitate expression of the three rat *N*-acetyltransferases, we developed sensitive, specific assays for *Nat1*, *Nat2* and *Nat3* mRNAs. In male F344 rats, tissue-specific expression varied over a limited range for both *Nat1* (~19-fold) and *Nat2* (~30-fold), with highest expression of both genes in colon. *Nat3* mRNA was at least two to three orders of magnitude less than *Nat1* or *Nat2*. Comparison of *Nat1* and *Nat2* mRNA expression in bladder, colon, liver and lung of male and female F344 rats detected no significant gender-specific difference. In Sprague Dawley and F344 rats ranging in age from neonate to mature adult, colon showed a >10-fold increase in *Nat2* during the first postnatal month that did not correlate with changes in *Nat1*. In contrast, *Nat2* showed no developmental change in Sprague Dawley or F344 liver as *Nat1* increased modestly. These measures of rat *Nat* expression confirm that *Nat3* expression is negligible and that *Nat1* and *Nat2* are the primary determinants of arylamine acetylation activity in all tested tissues. The findings demonstrate differential tissue-specific and developmental regulation of the rat *Nat1* and *Nat2* genes and contribute to more complete understanding of tissue-, gender-, and development-specific expression patterns of the cognate *N*-acetyltransferase genes of humans and other species.

Human *N*-acetyltransferase genes *NAT1* and *NAT2* exhibit genetic polymorphisms that modify pharmaceutical drug toxicities and influence individual susceptibility to cancers caused by exposure to environmental arylamine and heterocyclic amine procarcinogens (Hein, 2000; Hein et al., 2000). The *N*-acetyltransferase enzymes participate in the metabolism of these amines by transferring an acetyl group from acetyl-coenzyme A to an exocyclic amine (*N*-acetylation) or to an oxygen (*O*-acetylation) after the exocyclic amine has been oxidized by a cytochrome P450 (Hein, 1988). *N*-acetylation is typically a metabolic detoxification step, whereas *O*-acetylation leads to formation of reactive nitrenium intermediates that can form mutagenic DNA adducts (Hanna, 1994).

The rat is often used as a model for studies of carcinogenicity and toxicity of arylamines, and functional polymorphism of rat *Nat2* is often considered a model for similar polymorphism in humans. Understanding the expression and activity of the rat *N*-acetyltransferases is thus of

Address correspondence to: David W. Hein, Department of Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY 40292; Tel. (502) 852-5141; Fax. (502) 852-7868; E-Mail: d.hein@louisville.edu.

great value for making relevant extrapolations to humans. The substrate specificity and kinetic properties of the rat and human enzymes and the effects of biological differences and environmental exposures on transcription, translation and in vivo enzyme activity are all of potential importance. Previous studies reported similarities of rat *Nat1* and mouse *Nat1* with human *NAT2*, and of rat *Nat2* and mouse *Nat2* with human *NAT1*, with respect to nucleotide and amino acid sequence, structural stability, and substrate selectivity (Estrada-Rodgers et al., 1998; Walraven et al., 2006; Kawamura et al., 2008). The rat and mouse genomes include a third *N*-acetyltransferase gene, *Nat3*, with no known human ortholog (Boukouvala and Fakis, 2005; Walraven et al., 2006). The *Nat3* genes of both mouse and rat appear to be weakly expressed in vivo and produce enzymes with relatively weak activity towards few known substrates when expressed in recombinant systems (Kelly and Sim, 1994; Fretland et al., 1997; Walraven et al., 2006; Sugamori et al., 2007).

Currently, little is known regarding the nature of the rat *Nat* promoters or control of *Nat* mRNA synthesis. The structures of *Nat1* and *Nat2* cDNA clones isolated from rat pineal gland (Ebisawa et al., 1995) indicate the possibility of a common promoter for both *Nat1* and *Nat2* mRNAs and alternative splicing of non-coding 5′ untranslated region (UTR) exons. The rat *Nat* promoter region inferred from the pineal gland cDNA clones shows strong similarity to *Nat* promoters in human, mouse and cow (Husain et al., 2007a), but no other confirming data are available in the current literature or the rat genome database (http://rgd.mcw.edu/). No rat *Nat3* cDNA clone has yet been isolated. Previous semi-quantitative analysis of rat *Nat1*, *Nat2* and *Nat3* mRNAs in the F344 rat showed that *Nat1* and *Nat2* are both widely expressed, but the level of *Nat3* mRNA was below the limit of detection using ethidium staining of RT-PCR products in agarose gels (Walraven et al., 2007).

Quantitative differences in tissue-specific *N*-acetyltransferase gene expression as well as changes during development have been reported for human and mouse, but not for rat. Human *NAT2* mRNA is expressed in liver and gut at levels 100 to 1000-fold higher than other tissues and human adult liver has close to 100-fold higher expression of *NAT2* than fetal liver (Husain et al., 2007b). Human *NAT1* mRNA is more uniformly expressed than *NAT2* in diverse tissues and shows similar levels in adult and fetal liver and brain (Husain et al., 2007a). Both *Nat1* and *Nat2* mRNA levels in mouse adult liver are more than 100-fold higher than in fetal liver (McQueen et al., 2003). Enzyme and mRNA levels of *Nat1* and *Nat2* rise significantly in mouse liver during the first 30 postnatal days (McQueen and Chau, 2003). In adult mice, *Nat1* and *Nat2* mRNAs are expressed at similar levels in diverse tissues (Sugamori et al., 2003; Loehle et al., 2006). Few gender-specific differences in *Nat* expression have been described in any species. Mouse *Nat2* mRNA and enzyme are reported to be 2-fold elevated in adult male vs. female kidney but not different in liver (Estrada et al., 2000).

To provide additional measurements and comparisons of the contributions of *Nat1*, *Nat2* and *Nat3* to the *N*-acetylation phenotype in rats, we have developed specific assays for each mRNA. The assays are quantitative and nearly identical in sensitivity, allowing direct comparisons of the expression of each gene. We measured *Nat* mRNAs in fourteen tissues of the adult F344 rat and also assessed possible gender differences and developmental changes.

METHODS

RNA Samples

Liver, lung, spleen, kidney, heart, esophagus, stomach, bladder, pancreas, prostate, colon, duodenum, jejunum, and ileum were harvested from exsanguinated F344 adult male rats as described (Walraven et al., 2007). For comparison of *Nat* expression in male and female F344 rats, five adult animals of each sex were asphyxiated with CO_2 and bladder, colon, liver and lung were removed and snap frozen. For the assessment of developmental changes in the F344

rat, colon and liver were collected individually from three animals, following asphyxiation with CO_2, at 4, 15, 35 and 130 days of age. Frozen tissues were thawed and immediately homogenized with a Tissue-Tearor homogenizer (BioSpec Products, Bartlesville, OK) in buffer RLT (Qiagen, Valencia, CA) or thawed in RNA*later*-ICE (Qiagen) at -20°C and homogenized in buffer RLT with a Mini Beadbeater and 1 mm zirconia beads (BioSpec Products). RNA extraction was performed with the RNeasy Mini Kit (Qiagen). Additional RNA samples from the livers of Sprague Dawley (SD) rats ranging in age from 1 day to 1 year and unselected for gender were purchased from Zyagen (San Diego, CA). The SD samples representing ages up to 3 weeks were from pools of 2 to 4 animals and samples representing older ages were from single animals. A similar panel of colon RNA was also purchased from Zyagen. To eliminate residual DNA, RNA samples from all sources were treated with RQ1 DNAse (Promega, Madison WI) followed by extraction with 25:24:1 phenol:chloroform:isoamyl alcohol (pH 8.0, Sigma) and precipitation with isopropanol. RNA quality was confirmed by electrophoresis on 1% agarose gels and RNA was quantified with a Nanodrop 1000 spectrophotometer (Thermo Fisher, Waltham, MA).

Quantitative RT-PCR assay design and preparation of calibration and specificity controls

Primers and probes for specific TaqMan quantitative PCR (q-PCR) of rat *Nat1*, *Nat2* and *Nat3* were designed with the aid of Primer Express software from ABI (Applied Biosystems, Foster City, CA). The open reading frame exon and the 3′-UTR regions of the 3 genes were aligned with ClustalW to identify sequence regions with maximum specificity for each gene. Selected forward and reverse primers and probes within or overlapping the 3′-UTR were located upstream of the most 5′ possible polyadenylation recognition sequence (AATAAA or ATTAAA).

Genomic PCR products specific for each of the rat *Nat* genes, for use as q-PCR controls, were amplified with the high fidelity Phusion polymerase (New England Biolabs) using an appropriate specific primer pair and template genomic DNA isolated from an F344 rat colon sample with the QIAamp DNA Mini Kit (Qiagen). The fragments were separated on 0.6% agarose gels and purified with the Qiaquick Gel Extraction kit (Qiagen). DNA concentrations were determined with the Nanodrop 1000.

Quantitative RT-PCR measurements

For preparation of cDNA, 0.5-1 μg of DNAse treated RNA was reverse transcribed with Superscript III (Invitrogen, Carlsbad, CA) or MultiScribe (ABI) using random primers, as recommended by the manufacturer. For quantitative real-time PCR (q-RT-PCR), four μl of diluted cDNA, equivalent to about 40 ng of the initial RNA template, was used in a 20 μl PCR reaction with 1X TaqMan Universal Master Mix, forward and reverse primers at 300 nM and 100 nM probe. A 40 cycle PCR with fluorescent data collection was performed using the ABI 7700 sequence detection system (ABI). Quantitation of the endogenous control 18S rRNA was performed using TaqMan Ribosomal RNA Control Reagents for 18S rRNA (ABI). Each assay plate included reaction mix controls with water in place of template. To verify the absence of contaminating DNA in the RNA preparations, TaqMan quantitation of reactions prepared in parallel without addition of RT were also run at least once for each set of RNA samples. Baselines and threshold levels were selected as recommended by ABI and values of Ct, the cycle number at which the measured fluorescence reached the set threshold value, were recorded. For each real-time run, a ΔCt (*Nat* Ct -18S rRNA Ct) was calculated for each sample and a ΔΔCt value was then derived by subtracting the smallest ΔCt from all ΔCt values of the samples to be compared. The formula $2^{(-\Delta\Delta Ct)}$ was then used to calculate an initial relative concentration and a final relative measure was obtained by normalizing to the average of all the initial concentrations of the compared set. Samples with a Ct of 40 were assigned as zero.

Statistical analysis

The significance of tissue-specific differences in *Nat1* and *Nat2* was assessed by one-way ANOVA. Gender-specific comparisons in the four tested tissues were tested by two-way ANOVA (gender vs. tissue). Differences in the developmental series were also characterized by appropriate one-way or two-way ANOVA and application of the Tukey HSD post-test. Statistics were calculated with utilities implemented at http://faculty.vassar.edu/lowry/VassarStats.html.

RESULTS

Design and calibration of quantitative PCR assays for rat *Nat1, Nat2* and *Nat3*

The design of accurate and specific q-RT-PCR assays for each of the three rat *Nat* genes is challenging because little is known of spliced rat *Nat* mRNA structures and there is at least 71% nucleotide identity between the open reading frames (ORFs) of any pair of these genes. We therefore focused on sequences spanning the 3′ segment of the ORF and the 5′ portion of the 3′-UTR of each gene since these segments must be present in any functional mRNA and also show sufficient sequence diversity to allow the design of highly specific PCR primers and TaqMan probes (Table 1). To verify the specificity and sensitivity of the assay designs, calibration controls consisting of genomic PCR products spanning the 3′ portion of each gene were prepared using a common forward primer, rNat123F, together with a gene-specific reverse primer (Table 1). The major PCR products from duplicate amplifications, which were of the expected and distinct sizes for each gene, were gel-purified and quantified. Test templates were prepared by serial dilution after adjustment of the purified fragments to equimolarity and each of the assays was performed on each type of fragment template (Figure 1). The minimum Ct difference for any non-specific versus specific amplification was 14.7, for the *Nat1* q-PCR assay on the *Nat2* template, corresponding to selectivity in amplification efficiency of greater than 25,000-fold. Each assay also was performed on serial 8-fold dilutions of its specific template over a $>2\times10^6$ fold concentration range, corresponding to Ct values from 13 to 39 (data not shown). For all assays, the slope, *m*, of the line Ct=*m**log2[template], was in the range -1.205 to -1.219, with r^2 for fit >0.99. Thus, the *Nat1, Nat2* and *Nat3* q-PCR assays all have sufficiently similar detection sensitivities to permit accurate quantitative comparisons based directly on differences in Ct measurements.

Quantitation of *Nat* gene expression in diverse tissues of the F344 rat

The results of q-RT-PCR analysis of RNA isolated from 14 anatomically distinct tissues of the F344 rat are shown in Figure 2. Tissue variability was highly significant (ANOVA P<0.0001) for both *Nat1* and *Nat2* with colon showing the highest expression of both genes. The apparent range of expression for *Nat1* was 19.4-fold (colon vs. esophagus) and for *Nat2* was 30.2-fold (colon vs. heart). For most tissues, *Nat1* and *Nat2* expression levels were nearly coordinate. The largest deviation occurred in liver in which *Nat1*/*Nat2* was >7, compared to a range of this ratio among all other tissues from 0.8 in jejunum to 2.7 in spleen. The expression of *Nat3* in any tissue could not be unambiguously confirmed since *Nat3* mRNA was often near or below the limit of detection and was at least 100 to 1000-fold lower than either *Nat1* or *Nat2* for all tissues.

Comparison of *Nat1*, *Nat2* and *Nat3* expression in male and female F344 rats

The possibility of differences in *Nat1, Nat2* or *Nat3* expression between male and female F344 rats was assessed in liver, colon, lung and bladder from five animals of each sex (Figure 3). The *Nat1* and *Nat2* measurements confirmed the observation of tissue specific differences for both genes (ANOVA P<0.0001), but did not provide significant evidence for gender differences (ANOVA P>0.05). In all female and male tissues tested, the *Nat3* level was at least

two to three orders of magnitude lower than *Nat1* or *Nat2* (Figure 3) and was below the limit of detection, Ct=40, in one or more of the individual samples of liver, lung and bladder. There was no significant difference in the *Nat3* measurements in male vs. female colon. The relatively high *Nat1*/*Nat2* ratio observed in liver from exsanguinated male F344 rats (7.4-fold) was confirmed in both the male (6.4-fold) and female (9.8-fold) F344 rats in these experimental groups. *Nat1* expression was also higher than *Nat2* in lung and bladder, but lower than *Nat2* in colon, in agreement with the relative levels found in the exsanguinated male rats.

Developmental changes in rat *Nat1* and *Nat2* expression

RNA was isolated from colon and liver of F344 rats (N=3) representing 4 developmental stages from 4 to 130 days. Developmental panels of RNA from colon and liver of the Sprague Dawley (SD) rat were obtained from Zyagen (San Diego, CA). The SD RNA panels included single samples from rats of 9 distinct ages, ranging from 1 day to 1 year, with single animals as the source of samples for ages over three weeks. Results of q-RT-PCR assays of *Nat1* and *Nat 2* in these samples are presented in Figure 4.

A striking developmental increase of *Nat2* in colon was observed for both the inbred F344 line and the outbred SD rats (Figure 4A and 4B). In both rat strains, *Nat1* and *Nat2* were at indistinguishable levels shortly after birth ($P>0.05$), but *Nat2* increased approximately 10-fold in the following 30 days of life. In the F344 rats, this was confirmed by the significance of increases of *Nat2* at 35 days ($P<0.01$) and 130 days ($P<0.05$) compared to *Nat2* at 4 or 15 days. In the SD rats, the increase in *Nat2* for rats at least 30 days old compared to rats 14 days old and younger was also significant ($P<0.05$). In both F344 and SD rats, the colon *Nat2* level appeared to reach a maximum as the rats approach sexual maturity at 5 weeks, but the apparent decline of *Nat2* in older animals was not statistically significant. The developmental expression pattern of *Nat1* in rat colon was distinct from *Nat2*. In F344 rats a small developmental increase, ~2.5 fold, in colon *Nat1* mRNA levels was detected, however, this difference was only statistically significant ($P<0.01$) for the 130 day old rats compared to 4 or 15 day old rats. No statistically significant developmental change in SD colon *Nat1* was detected in the comparison of the younger, 1 to 14 day old rats to rats 30 days and older ($P>0.05$)

In contrast to colon, liver showed no developmental increase in *Nat2* in either SD or F344 rats (Figure 4C and 4D). There was no significant difference in liver *Nat2* between any of the four F344 age groups (Tukey HSD $P>0.05$) and no significant difference in liver *Nat2* between the younger, 1 to 14 day, or older, 30 to 365 day, SD rats ($P>0.05$). In the SD strain, a 2.1-fold increase in *Nat1* was significant between rats 30 days and older compared to rats 14 days and younger ($P<0.01$). However, no similar trend of *Nat1* increase with age was detected in F344 liver (ANOVA $P=0.31$).

Although RT-dependent amplification was detected in the *Nat3* q-RT-PCR assays of developmental samples from liver and colon, measurements were near the limit of detection and consequently highly variable. The Ct values for *Nat1* and *Nat2* consistently corresponded to a more than 1000-fold higher expression level than *Nat3* (data not shown).

DISCUSSION

The *N*-acetyltransferase isoenzymes encoded by rat *Nat1*, *Nat2* and *Nat3* have been studied individually in recombinant expression systems and shown to have distinctive kinetic properties and substrate selectivities (Hein et al., 1997; Walraven et al., 2006). *N*-acetyltransferase enzymatic activities have been assessed in rat tissue samples with a variety of substrates, including procainamide which is selective for rat NAT1 and *p*-aminobenzoic (PABA) acid which is selective for rat NAT2 (Hein et al., 1991a; Hein et al., 1991b). However, there is some uncertainty in attributing activity observed in any tissue to a particular *N*-

acetyltransferase isozyme because their substrate specificities are not absolute. The assays described here allow specific and accurate assessment of the expression of each rat *Nat* mRNA in tissues expressing more than one isoform. The results are fully consistent with our previous semiquantitive analyses (Walraven et al., 2007); however, the TaqMan assays allow precise quantitation and also have similar sensitivities, permitting direct comparisons of various Nat mRNA levels within and between tissues. The measurements of tissue-specific and developmental expression of the rat *Nat* genes reported here have revealed some similarities to, but also key distinctions from *N*-acetyltransferase gene expression patterns in mice and humans that have been described previously.

Samples of 14 different adult F344 rat tissues showed a limited range, 20- to 30-fold, in the levels of *Nat1* or *Nat2* mRNA. The *Nat2* mRNA measures correlated well with acetylation activities of the rat NAT2 selective substrate, PABA, previously reported for bladder, colon, kidney, liver and prostate (Hein et al., 1991a; Hein et al., 1991b), with colon showing a distinctly higher activity and mRNA level. Overall, the mRNA measurements indicate an expression pattern in adult rats similar to other rodents. Limited variability of *Nat1* and *Nat2* mRNA was detected by q-RT-PCR in five tissues of adult mice (Loehle et al., 2006). In the Syrian hamster, both NAT1 and NAT2 activities were detectable in diverse tissues (Hein et al., 2006), although NAT2 activity was relatively low in heart. Similarly, we found *Nat2* mRNA to be low in the F344 rat heart (Figure 2). In humans, *NAT1* expression in diverse tissues is relatively uniform (Husain et al., 2007a), but human *NAT2* has >1000-fold higher expression in liver and gut than in low expressing tissues, including brain, heart and prostate (Husain et al., 2007b). Consistently high expression of *N*-acetyltransferase genes in diverse tissues may be associated with the presence of an Sp1 transcription factor binding site within the promoter segment and such a site appears to be conserved in the proposed rat *Nat* promoter, the mouse *Nat1* and *Nat2* promoters, and the human *NAT1* promoter but not the human *NAT2* promoter (Boukouvala et al., 2003; Husain et al., 2007b).

In this study, we detected minimal developmental changes in *Nat* expression in rat liver. There was no apparent change in *Nat2* levels from neonate to adult in F344 or SD rats. We saw only a small change in *Nat1* expression, maximally about 3-fold, that was significant in SD, but not F344 rats. In contrast, postnatal increases of close to 100-fold in both *Nat1* and *Nat2* mRNA in liver have been reported in mice, correlating with lesser, but significant increases in *N*-acetyltransferase activity (McQueen and Chau, 2003). Human *NAT2* mRNA is about 100-fold higher in adult liver compared to fetal liver (Husain et al., 2007b), but *NAT1* mRNA levels are similar in human adult and fetal liver and also human adult and fetal brain (Husain et al., 2007a). In rat liver, it appears that small differences in the developmental profiles of *Nat1* and *Nat2* contribute substantially to the relatively high *Nat1*/*Nat2* ratio that was consistently observed in all of the adult rat liver samples analyzed in this study. The trend of an increased *Nat1*/*Nat2* ratio with age is very evident in the SD liver (Figure 4C), and is also discernable in the F344 liver data (Figure 4D).

The largest developmental changes in rat *Nat* gene expression were found in colon, with both SD and F344 rats showing >10-fold increases in *Nat2* during the first 30 days of life. This increase was followed by a downward trend in *Nat2* as both SD and F344 rats aged further (Figure 4), but additional data are needed to test the significance of this apparent reduction. A small developmental increase in *Nat1* in colon, ~2.5 fold, was only significant for the oldest group of F344 rats. Comparable descriptions of possible developmental changes in *Nat* expression in colon are not currently available for mouse or human.

No significant gender specific differences in rat *Nat* expression were detected in bladder, colon, liver or lung. Although not statistically significant by ANOVA, the largest sex-specific differences detected for *Nat1* and *Nat2* mRNAs were a 2.0-folder higher level of *Nat2* mRNA

and a 1.7-fold higher level of *Nat1* mRNA in male rat colon vs. female rat colon. Testing of additional animals in more narrowly matched age groups might provide a more sensitive means of detecting gender-specific differences or the effects of gender on the precise timing of developmental changes.

In this study, the expression of *Nat3* was undetectable or extremely low in samples that included 14 different tissues of the male F344 rat, 4 tissues of the female rat and developmental samples of liver and colon spanning neonatal to adult stages in F344 and SD rats. The *Nat3* mRNA level was consistently at least 100- to 1000-fold lower than *Nat1* or *Nat2* mRNAs. Colon was the only rat tissue that consistently showed non-zero, Ct<40, readings for *Nat3* by q-RT-PCR. This may reflect a low level of *Nat3* expression in colon, but might also reflect inaccuracy of the *Nat3* assay due to the combined presence of *Nat1* and *Nat2*, which are both at their highest levels in colon. Thus, the evidence that rat *Nat3* is expressed in any natural context is still equivocal. It remains possible that *Nat3* is highly expressed in a tissue or a stage of development that is not represented in our present data set. The likelihood of a retained functional relevance is supported by the similar genomic arrangement of *Nat3* in mouse and rat (Boukouvala and Fakis, 2005) and the fact that it retains an open reading frame encoding a typical mammalian *N*-acetyltransferase protein.

Differential developmental regulation of *Nat1* and *Nat2* was apparent for rat colon and also liver. Although most rat tissues express similar levels of *Nat1* and *Nat2* mRNA, differential tissue-specific regulation was also evident, most notably in adult liver, with *Nat1*/*Nat2* ratios in the range of 6.4 to 9.8, consistently higher than other tissues. Better understanding of the basis of differential regulation of *Nat1* and *Nat2* will require additional molecular characterization of the transcription process for these genes. If both rat *Nat1* and *Nat2* are transcribed from a single promoter in all tissues as they appear to be in pineal gland (Ebisawa et al., 1995), then differential regulation is likely to occur at the level of mRNA splicing or stability. Alternatively, distinct tissue-specific promoters for rat *Nat1* and/or *Nat2* may remain to be discovered.

The importance of age-matching of rats in the control and exposure groups for toxicity and carcinogenicity studies is indicated by the rapid changes in *Nat2* in colon during early life and the possibility of an age related decline in colon *Nat2* in adults. The distinct rates of developmental change of *Nat1* and *Nat2* mRNA observed in colon and liver also imply that the relative activity levels of each enzyme will change, although it is not clear whether this reflects a change in intracellular gene regulation or a shift in the relative proportion of differentiated cell types. The results described here are helpful for guiding the design of exposure studies and the specific mRNA assays will be useful for interpretations of studies that monitor drug-and/or chemical-related changes in gene expression.

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Abbreviations

Ct, cycle threshold
NAT, *N*-acetyltransferase
ORF, open reading frame
PABA, *p*-aminobenzoic acid
PCR, polymerase chain reaction
q-PCR, quantitative PCR

q-RT-PCR, quantitative real-time PCR
RT, reverse transcriptase
RT-PCR, reverse transcriptase PCR
SD, Sprague Dawley rat
UTR, untranslated region

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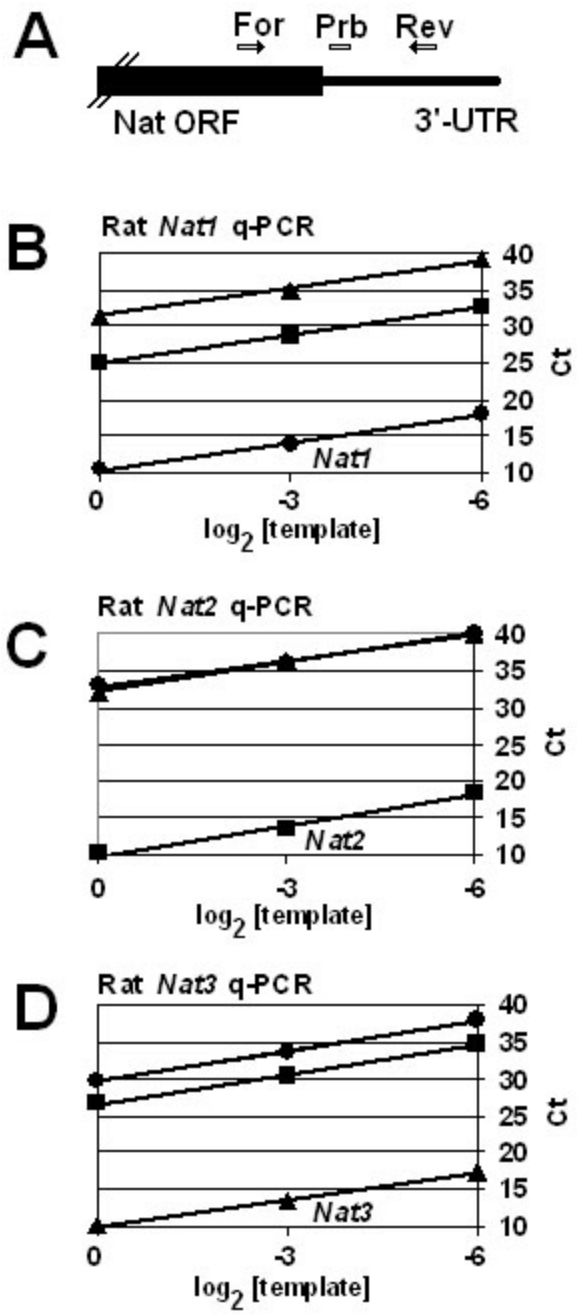
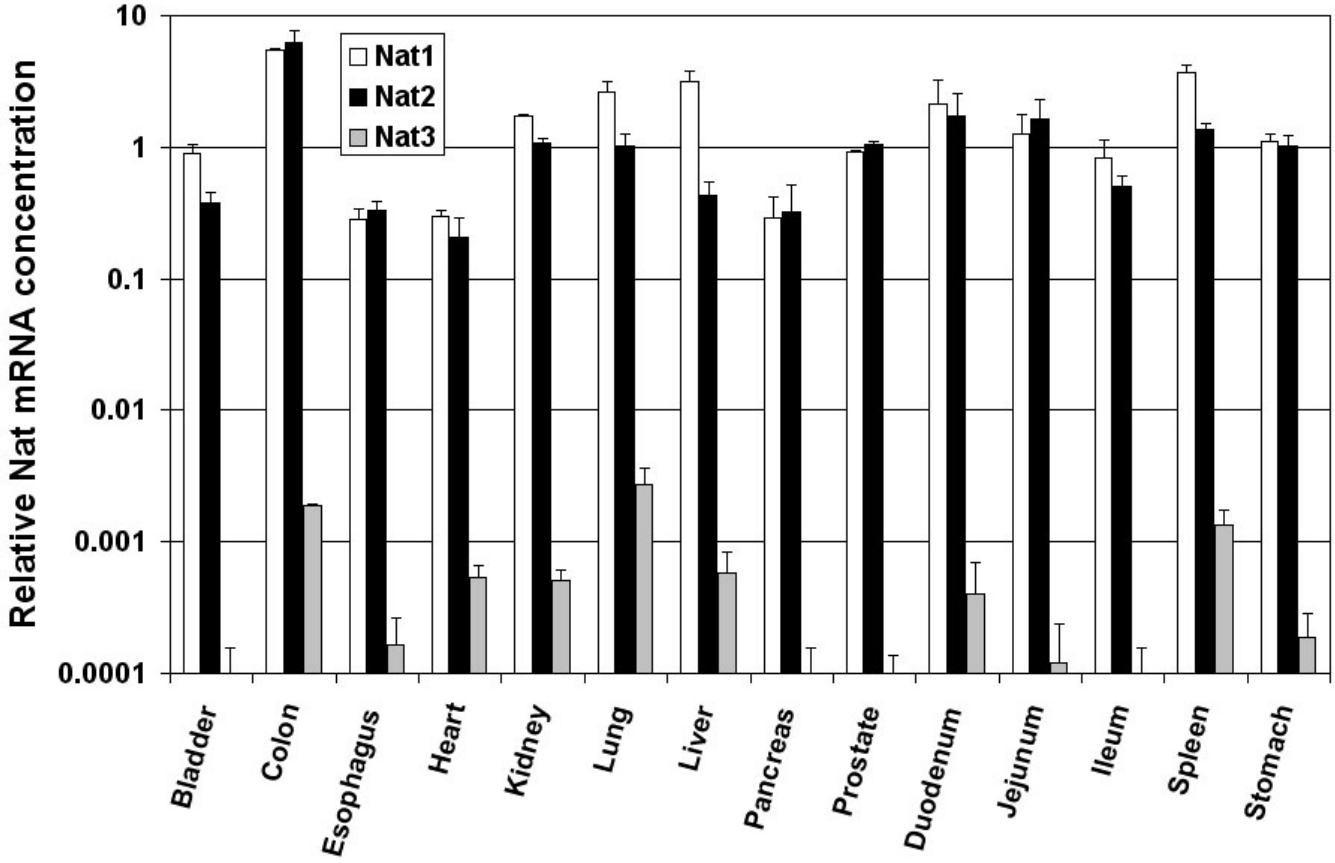


Legend to Figure 1.

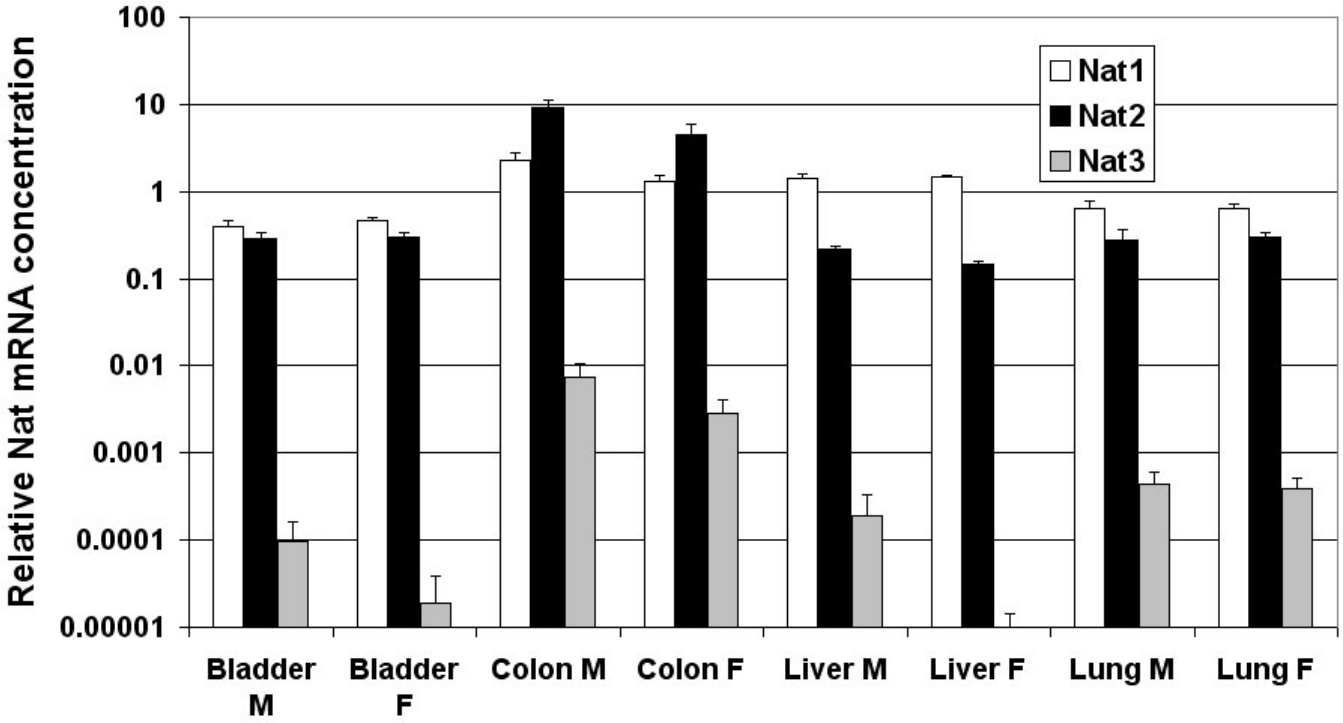
Design of TaqMan q-RT-PCR assays for rat *Nat* mRNAs and verification of assay specificities. The relative locations of forward, reverse and TaqMan probe oligonucleotides were similar for all of the rat *Nat* mRNA assays as shown in panel **A**, except that probe for the *Nat3* assay was also located within the ORF portion of the *Nat3* gene. Panels **B** (*Nat1* assay), **C** (*Nat2* assay) and **D** (*Nat3* assay) illustrate the high specificity of each TaqMan q-RT-PCR with comparisons in each panel of cycle threshold (Ct) values obtained with three equimolar amounts of rat *Nat1* ●, *Nat2* ■, and *Nat3* △ PCR product templates (400, 50 and 6.25 amol). For each assay, the lowest Ct values, indicating the most efficient amplification, are observed with the appropriate gene template (labeled). The least specific cross-combination was the *Nat1* assay

on the *Nat2* template (Panel B) with a Ct difference of 14.7 compared to the *Nat1* assay on the *Nat1* template, representing a selective sensitivity of greater than 25,000-fold for the specific template.



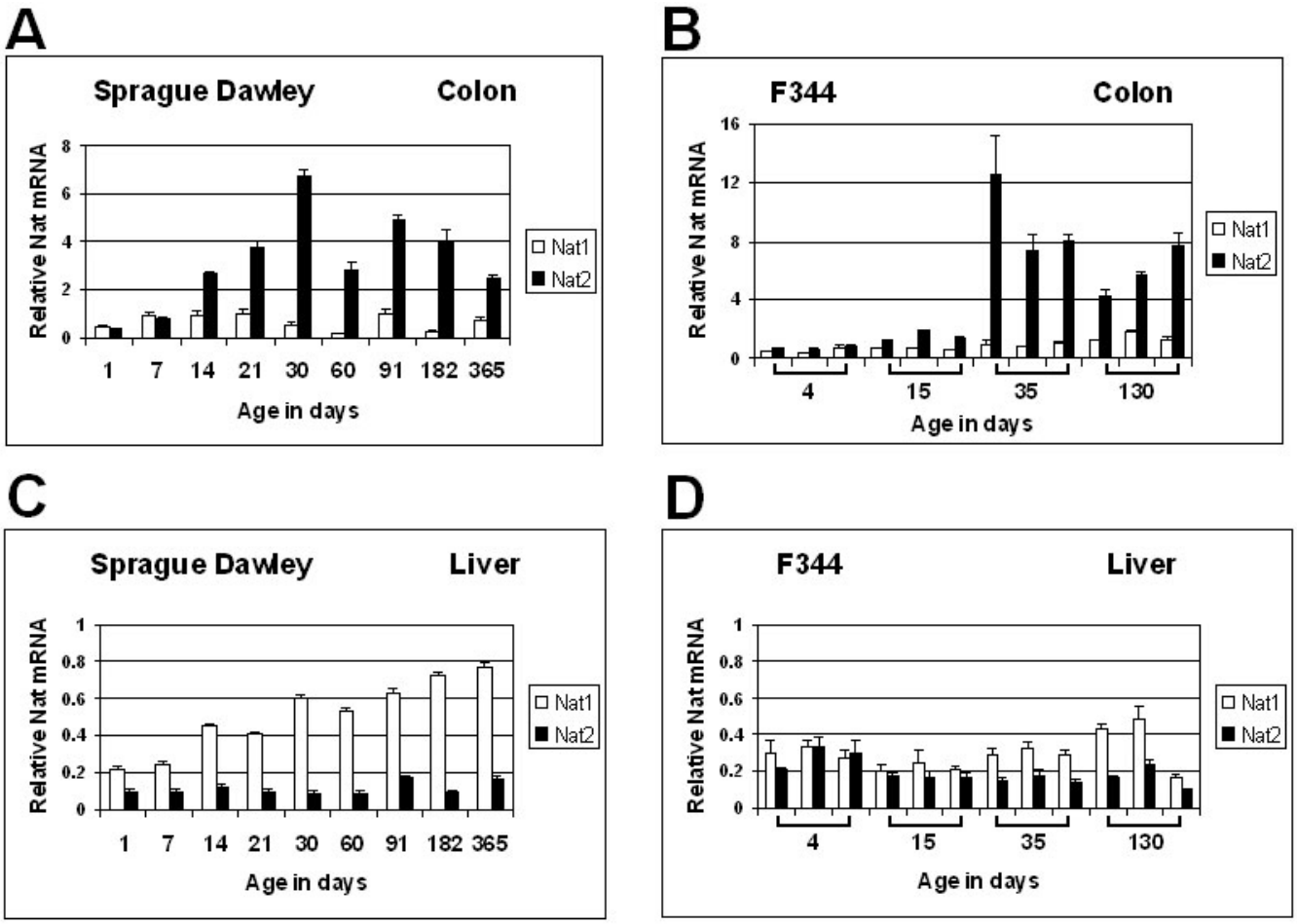
Legend to Figure 2.

Expression of *Nat1, Nat2* and *Nat3* mRNAs in diverse tissues. DNAse treated RNA from the indicated tissues of three male F344 rats were reverse transcribed and two q-RT-PCR measures were performed with each of the *Nat1, Nat2* and *Nat3* assays and the 18S rRNA control. The average of the measures of *Nat1, Nat2* and *Nat3* referenced to 18S rRNA are shown for each tissue on a logarithmic scale, with the error bars indicating the standard error. *Nat1, Nat2* and *Nat3* q-RT-PCR measurements of parallel reactions prepared without the addition of reverse transcriptase showed no detectable amplification after 40 cycles of PCR, demonstrating the absence of contaminating genomic DNA.



Legend to Figure 3.

Test for gender effects on *Nat* gene mRNA levels. Two reverse transcription reactions were prepared and two q-RT-PCR measurements made with each of the *Nat1*, *Nat2* and *Nat3* mRNA assays and the 18S rRNA reference control for DNAse treated RNA from the indicated tissues of 5 male (M) and 5 female (F) rats. The average of the measures of *Nat1*, *Nat2* and *Nat3* are shown for each tissue on a logarithmic scale, with the error bars indicating the standard error. *Nat1*, *Nat2* and *Nat3* q-RT-PCR measurements of parallel reactions prepared without the addition of reverse transcriptase demonstrated the absence of contaminating genomic DNA.



Legend to Figure 4.

Developmental changes in *Nat1* and *Nat2* expression. Liver and colon RNA from SD rats of the indicated ages were obtained from commercial sources (see Methods). F344 colon and liver RNA were isolated from three animals of the ages shown. For each of the SD samples, at least three reverse transcriptions were prepared and assayed for *Nat1*, *Nat2* and 18S rRNA by q-RT-PCR. For each F344 sample, at least two reverse transcription and two q-RT-PCR measures were done for *Nat1*, *Nat2* and 18S rRNA. Panel **A**: Sprague Dawley colon, **B**: F344 colon, **C**: Sprague Dawley liver and **D**: F344 liver. Measurements in all panels are on the same linear relative scale and error bars illustrate standard errors.

Table 1

Summary of names, sequences and utilities of oligonucleotides used in this study

Oligonucleotide	Sequence	Utility
rNat123F	5′-agatgtgggagcctctggaatt-3′	Nat genomic PCR Fwd
grNat1Rev	5′-*ggggg*aagccttctaagccgtgtcatt-3′[a]	Nat1 genomic PCR Rev
grNat2Rev	5′-*ggggg*tgcagtgacatcaatcagca-3′[a]	Nat2 genomic PCR Rev
grNat3Rev	5′-*ggggg*atctttgctggaggaacatg-3′[a]	Nat3 genomic PCR Rev
TQrNat1Fwd	5′-ccaaacatggcgaactcgt-3′	Nat1 q-PCR Fwd
TQrNat1Rev	5′-gaagatacaggtcattagttgatcaatattg-3′	Nat1 q-PCR Rev
TQrNat1 Probe	5′-aattgttctccattattatc-3′MGBNFQ	Nat1 q-PCR Probe
TQrNat2Fwd	5′-gtgcctaaacatggtgatcgatt-3′	Nat2 q-PCR Fwd
TQrnNat2Altrev	5′-acatggtcagaagtatgtccttgtc-3′	Nat2 q-PCR Rev
TQrNat2 Probe	5′-tgtccttccatgtacttg-3′MGBNFQ	Nat2 q-PCR Probe
TQrNat3Fwd	5′-gtagatcttgtggagtttaagactctgaag-3′	Nat3 q-PCR Fwd
TQrNat3Rev	5′-atatagaaacattgaagaccattctccttt-3′	Nat3 q-PCR Rev
TQrNat3 Probe	5′-ccaattaccacatttgg-3′MGBNFQ	Nat3 q-PCR Probe

[a]The italicized sequences represent stabilizing “clamps” added to facilitate the use of high annealing temperatures for specific PCR amplification.