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Functional Analysis of the Human *N-Acetyltransferase 1* Major Promoter: Quantitation of Tissue Expression and Identification of Critical Sequence Elements

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Abstract

Arylamine N-acetyltransferase 1 (NAT1) plays an important role in the biotransformation of xenobiotics and genetic variants have been implicated in susceptibility to cancer and birth defects. A specific and quantitative RT-PCR assay for transcription from the major *NAT1* promoter detected high expression with limited variability in human tissues. A 213 bp minimal promoter was identified by transfection of luciferase reporter constructs into MCF-7 and HepG2 cell lines. Alignment of the 213 bp region with paralogous and orthologous promoters revealed two conserved regions segment, one of which overlaps a 16 bp perfect palindrome. Transfection of luciferase constructs with artificial mutations in the minimal promoter defined two sites important for promoter function. One of these sites included a close match to the Sp1 transcription factor binding consensus sequence. Electrophoretic mobility shift assays (EMSA), followed by competitive and supershift analyses confirmed the Sp1 binding. Mutation of the highly conserved portion of the 16 bp palindrome reduced promoter activity more than 3-fold, and an EMSA shift was detected with an oligonucleotide, 200L29, which spans this segment. The 200L29 EMSA shift could not be competed by consensus Sp1 or AP-2 oligonucleotides, and may represent binding of a transcription factor that is common to *N-acetyltransferase* genes in humans and other species.

Introduction

The human N-acetyltransferase 1 (*NAT1*) and 2 (*NAT2*) genes encode N-acetyltransferases enzymes important in the biotransformation of xenobiotics, including pharmaceuticals and environmental carcinogens. Polymorphisms within and near the single open reading frame exon of *NAT1* define more than 25 distinct haplotypes (Hein et al., 2000). Associations of *NAT1* polymorphisms with susceptibility to various cancers (Hein et al., 2000; Boukouvala and Fakis, 2005) and birth defects (Carmichael et al., 2006; Jensen et al., 2006) have been described, however the effects of the common *NAT1* polymorphisms on enzyme activity have not been well established and their reported associations with cancer or birth defects are inconsistent. The apparent conflicts in epidemiological studies may be due, in part, to incomplete understanding of the effects of uncharacterized genetic and environmental influences on *NAT1* transcription regulation.

Numerous studies have documented interindividual differences in NAT1 protein or enzyme activity in diverse human tissues including erythrocytes (Bruhn et al., 1999), intestine (Hickman et al., 1998), colon (Ilett et al., 1994), skin (Kawakubo et al., 2000), breast (Williams

et al., 2001), placenta (Upton et al., 2000) and prostate (Al-Buheissi et al., 2006) with no known genetic basis. Although the *NAT1* alleles *NAT1*14B*, **15*, **17*, **19* and **22* are known to encode proteins with low enzyme activity (Fretland et al., 2001), these forms are rare in humans and variable activity has also been reported for individuals homozygous or heterozygous for the most frequent *NAT1*4* and **10* alleles. Some of the observed variability is likely due to post-translational effects caused by intracellular redox or metabolic status (Butcher et al., 2000; Butcher et al., 2004; Rodrigues-Lima and Dupret, 2004), but the influence of *NAT1* transcriptional regulation has not yet been determined.

Two alternative *NAT1* promoters, recently renamed NATa and NATb (Minchin et al., 2007), are respectively located 51.5 kb and 11.8 kb upstream of the single translated exon. Transcription of *NAT1* occurs in a wide variety of tissues and cell lines and most mRNAs are initiated at NATb (Husain et al., 2004; Boukouvala and Fakis, 2005; Butcher et al., 2005). The alternative promoter, NATa, is most highly expressed in a few tissues, including kidney, liver, lung and trachea (Barker et al., 2006). The pattern of placental *NAT1* expression during human development (Smelt et al., 2000) and the temporal and spatial expression of mouse *Nat2*, the likely ortholog of human *NAT1*, during cardiogenesis and at neural closure (Wakefield et al., 2005) are probable examples of transcriptional regulation. An androgen analog was recently shown to increase *NAT1* mRNA by stimulation of transcription initiation at NATb in two prostate cancer cell lines (Butcher et al., 2007). The high expression of *NAT1* in many estrogen receptor positive breast tumors is also apparently due to higher mRNA production (Perou et al., 2000; Adam et al., 2003; Sorlie et al., 2003; Tozlu et al., 2006). Further understanding of the increased *NAT1* expression in breast tumors is of interest because of the role of *NAT1* in biotransforming environmental carcinogens and the observed association of higher *NAT1* expression with better patient survival (Bieche et al., 2004; Dolled-Filhart et al., 2006). Further characterization of the NATb promoter is presented here, including quantitation of expression in diverse human tissues and identification of specific functional elements within the minimal promoter sequence.

Materials and Methods

Human RNA samples

Two panels of pooled, purified, tissue-specific, human total RNAs from Ambion (Ambion Inc., Austin, TX) and BD (BD Biosciences Clontech, Palo Alto, CA) and RNAs from the HepG2, HT-29, MCF-7, SW480, Caco-2, HEK293 and A549 human cell lines were as described previously (Barker et al., 2006; Husain et al., 2007). A sample of breast RNA, from one female and a liver RNA sample from one male were also obtained from Ambion. Additional RNAs representing adrenal gland, bladder and liver were obtained from BD.

Quantitative real-time RT-PCR

TaqMan analysis was performed using the ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). The 20 µl PCR reactions were 1X TaqMan Universal Master Mix, with 300 nM forward and reverse primers and 100 nM probe. The sequence of the NATb-specific forward primer (Table 1) is within the first exon of the major *NAT1* mRNA. The sequences of the reverse primer and the probe (Table 1) are within the *NAT1* open reading frame exon and the 79 base 5'-untranslated exon respectively. For PCR, initial incubations at 50°C for 2 min and 94°C for 10 min were followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. Quantitation of the endogenous control 18S rRNA was performed using TaqMan Ribosomal RNA Control Reagents for 18S rRNA (Applied Biosystems). Four µl of diluted cDNA, equivalent to 40 ng of the initial RNA template, was used in each PCR. Two independent RT reactions were prepared for each RNA and TaqMan measurements were carried out in duplicate for each cDNA product, for a total of four readings. Controls and

calculations of relative mRNA quantity were as previously described (Barker et al., 2006). For each RT set, the relative measures obtained from the two real-time runs were averaged and normalized with respect to the average of all samples in the same RT group.

Construction of pGL3-Basic promoter clones and artificial mutant derivatives

For construction of NATb promoter segment clones, DNA from the BAC CTD2547-L16 was used as template for PCR with the high fidelity Phusion polymerase (New England Biolabs, Ipswich, MA) using primers with artificial 5' HindIII sites (Table 1), followed by cloning into the HindIII site of pGL3-Basic (Promega, Madison, WI). Artificial mutant derivatives of the 318 bp promoter fragment were constructed as described (Ho et al., 1989) or with the modification that restriction enzyme digestion and ligation with T4 DNA ligase was used to accomplish fusion of the primary PCR products. Template DNAs for site-directed mutagenesis were either the genomic BAC clone CTD2547L16, the 318 promoter plasmid or, for the construction of the double mutant, a derivative of the 318 promoter plasmid carrying a Dup1-R7 mutation. Primers with mutant sequences and outer nested primers appropriate to each template were used (Table 1). The final inner nested PCR was carried out with primers NATb 318H3 and NAT1TSSZeroRevH3 (Table 1), and the resulting products were cloned into the HindIII site of pGL3-Basic. The presence of mutations was monitored by restriction enzyme digestion and verified by sequencing of plasmid DNAs with the ABI Big Dye Termination Reagents and the 310 Genetic Analyzer (Applied Biosystems).

Selection and retrieval of genomic promoter sequences for cross-species comparisons

Genomic segments containing the human NATb promoter sequence (Husain et al., 2004) and the corresponding promoter sequences from other genes and organisms (Boukouvala and Fakis, 2005) were retrieved at the Genome Browser Gateway (Kent et al., 2002) (<http://genome.ucsc.edu/>) and aligned using ClustalW (<http://www.ebi.ac.uk/services/>). The human NAT2 promoter region was as previously defined (Ebisawa and Deguchi, 1991; Husain et al., 2007). A promoter for mouse *Nat2* has been described (Boukouvala et al., 2003) and the surrounding genomic sequences were retrieved following alignment of the 5' ends of NCBI (<http://www.ncbi.nlm.nih.gov/>) nucleotide database cDNAs CA494799, CN663986 and BF164333 with the mouse genome using BLAT (Kent, 2002) as implemented at the Genome Browser Gateway. A candidate promoter region serving for both rat *Nat1* and *Nat2* was inferred from the structures of previously isolated cDNA clones (Ebisawa et al., 1995). Rat genomic sequence corresponding to the 5' portion of these cDNAs was obtained at the Genome Browser Gateway by BLAT alignment of NCBI cDNAs U01343 for rat *Nat1* and U01347 and U01348 for rat *Nat2*. A promoter region for one bovine *N-acetyltransferase* gene was identified upstream of the most 5' exons of NCBI cDNAs BT025447, CK946692, BE683786, CK960222, DY464402, AW484954 and BG689007, which were retrieved through the Genome Browser Gateway and aligned with the bovine genomic sequence (<http://www.hgsc.bcm.tmc.edu/>) using the BLAT utility. For each promoter, a genomic DNA segment including 1000 nucleotides upstream of the typical promoter exon splice site and 200 nucleotides downstream of this position was selected for alignment. In each gene, this segment includes approximately 800 bp of genomic sequence upstream of the known or suspected transcription start site region. Although no spliced mouse *Nat1* cDNA has been reported, expression of the Nat1 isoenzyme and *Nat1* mRNA in mouse tissues is well documented (Hein et al., 1988; McQueen and Chau, 2003; Loehle et al., 2006). A candidate promoter region for mouse *Nat1* was detected by alignment of the 1200 bp rat promoter segment within a 12.5 kb mouse genomic segment upstream of the mouse *Nat1* coding exon. This alignment identified a mouse DNA segment of 1176 bp that is 9.8 kb upstream of the mouse *Nat1* coding exon and is 82% identical to the rat promoter segment.

Transcription factor database analysis

TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) , MatInspector Release 7.4 (<http://www.genomatix.de/products/MatInspector/index.html>), Alibaba 2.1 (<http://www.gene-regulation.com/pub/programs/alibaba2/index.html>) and CONREAL (<http://conreal.niob.knaw.nl/>) were used to identify candidate transcription factor binding sites. Putative binding sites were considered plausible based on finding at least 75% match with the consensus sequence and on matrix similarity thresholds as defined at each database. TRANSFAC (<http://www.gene-regulation.com/pub/databases.html>) was the source of the Sp1 consensus matrix M00196.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from MCF-7 cells using the Transfactor Extraction Kit (BD Biosciences Clontech) as described by the manufacturer. The crude nuclear pellet was resuspended in 20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, 1 mM DTT containing 1X Complete Protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and disrupted by repeated passaging through a 27-gauge needle. The soluble supernatant was collected after centrifugation at 14,000 g for 10 min at 4°C. The protein concentration, typically about 2 µg/µl, was measured with the Bradford Reagent kit (Bio-Rad, Hercules, CA). Single-stranded oligonucleotides were 5' labeled with γ-[³²P]ATP (3000 Ci/mmol, 150 mCi/mL) (GE Healthcare, Piscataway, NJ) and T4 polynucleotide kinase (New England Biolabs) and purified with Sephadex G-25 spin columns (GE Healthcare). Radiolabeled oligonucleotides were annealed with a 2-fold molar excess of the unlabeled complementary oligonucleotide by incubation at 65°C for 5 min and 37°C for 15 min in 20 mM Tris-HCl (pH 8), 1 mM EDTA, and 50 mM NaCl. Binding reactions for EMSA contained 5 µg nuclear extract in 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol and 400 ng poly(dI-dC):poly(dI-dC) (GE Healthcare) non-specific competitor in 20 µl. Reactions were incubated at room temperature for 10 min prior to addition of 40 fmol probe and continued incubation for 20 min. Competition reactions included a 125-fold molar excess of unlabeled competitor added to the initial incubation. For supershift assays, 1–2 µl of TransCruz gel shift antibody (200 µg/mL) against Sp1 (PEP2, Catalog # sc-59x, Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture and incubated for 20–30 min at room temperature subsequent to incubation of oligonucleotide probes with nuclear extract. DNA-protein complexes were resolved by electrophoresis through 20×15 cm, 4% non-denaturing (29:1) polyacrylamide gels containing 2% glycerol, 22.5 mM Tris base, 22.5 mM boric acid and 0.5 mM EDTA. Gels were pre-run at 250 V, loaded and run at 350 V for 55–65 min, with cooling to 20°C by circulating water bath (Protean2 Xi Cell, Bio-Rad). Gels were exposed for 25–30 min to a phosphorimager screen for analysis by Storm 860 Imager scanner (GE Healthcare).

Results

Quantitative RT-PCR analysis of two panels of diverse human tissue RNAs was performed using an intron-spanning primer pair, with the forward primer specific for mRNA originating at the NATb promoter. Results of testing 44 samples from 29 different tissues, including 15 tissues represented in both panels, are shown in Figure 1. Some variability in NATb mRNA was evident among the different tissues and occasionally between different samples of the same tissue type, but most samples fell within an approximately 10-fold range. The duplicate samples of colon, kidney, lung, prostate, small intestine and testis all fell in the higher end of this range, while the duplicate samples of brain and skeletal muscle had consistently lower levels of NATb mRNA. The relative quantitative differences between tissues were not always consistent however. In series A, for instance, the small intestine sample had more than 3-fold higher mRNA than the prostate sample, but in series B, the prostate level was 4 fold that of small

intestine. Also in series A, mRNA in trachea was 5.5 fold higher than bladder, but in series B, the bladder level was 2.8-fold higher than trachea. Results of quantitative RT-PCR analysis of RNAs prepared from selected human cell lines are shown in Figure 2. The range of NATb expression in most of the cell lines was similar to that found in the human tissue samples (Figure 1). The expression of NATb was highest in the MCF-7 breast tumor line and notably higher than any normal tissue, at more than 120-fold the expression of the reference liver sample.

In a previous report, (Husain et al., 2004), NATb promoter function was shown to reside in a 1437 bp segment that included 940 bp upstream and 495 bp downstream of the region of transcription initiation. Constructs removing the 3' 432 bp and/or the 5' 393 bp of the 1437 bp fragment were subsequently found to retain full promoter function (data not shown). Results of deleting additional sequences in the 5' portion of this segment are shown in Figure 3. The 318, 245, 213, 178 and 150 bp fragments tested respectively include 222, 149, 117, 82, and 54 bp upstream of the most 5' transcription start site detected by 5' RACE (Husain et al., 2004). The 213 bp construct retained full promoter activity in the breast tumor cell line, MCF-7, and in the hepatoma-derived cell line, HepG2. The removal of 35 additional 5' nucleotides to create the 178 bp construct resulted in 4.8 and 2.8-fold reductions in promoter activity in MCF-7 and HepG2 respectively, and further promoter function reductions of 49-fold in MCF-7 and 27-fold in HepG2 were observed with subsequent removal of 28 nucleotides to form the 150 bp construct. These results indicated that important and possibly distinct functional elements of the NATb promoter reside within the 35 and 28 nucleotide segments.

The minimal NATb promoter region was assessed for potentially functional sequence elements by comparison with paralogous and orthologous promoter sequences. The alignment of NATb with promoter sequences from human *NAT2* and orthologous genes from rat, mouse and cow is shown in Figure 4. A high degree of similarity was evident for all of these sequences, but with two particularly strong regions of conservation. At position 37-55 in the NATb segment shown in figure 4, 15/19 of the nucleotides in the sequence CTGCAACTACATTTCCAG were found in all six promoters. A second conserved region, CTTCCCCTGCAG, occurred at position 120-131 in the NATb sequence, with 8/12 of these nucleotides identical in all of the compared promoters. The alignment also showed that of two functional sites previously identified in the mouse *Nat2* promoter (Boukouvala et al., 2003), the Sp1 site at position 81-90 in the mouse *Nat2* promoter was identical to the NATb sequence at 9/10 positions, but the mouse *Nat2* TATA box sequence was not apparent in NATb or any of the other promoters shown in Figure 4.

Additional candidate functional sites in NATb (Figure 5) were identified by scanning for similarities to known transcription factor (TF) recognition consensus sequences in public databases and by reviewing the sequence for other distinctive structures. The database scans identified possible TF recognition sites for AP-1 at nucleotides 79-83 (GTGACT), Sp1 at 87-96 (GGGGCGGGGC) and NF-Y at 106-111 (ATCCAA). Inspection of the sequence also detected a 16 nucleotide perfect palindrome, CCCAGGGGCCCTGGG at position 51-66. Nine of the 16 nucleotides in this palindrome are conserved in all the other promoters shown in Figure 4 and five of these conserved nucleotides occur in the 5' end of the palindrome and overlap with the 19 nucleotide conserved region. Another distinctive feature is a 13 base element, TGAGAGCACTTC, at position 112-124, which overlaps with the 12 nucleotide conserved region, and is exactly duplicated, in the same orientation, at positions 142-154. The elements of this duplication span the 5' end of the region in which NATb transcription start sites have been detected (Husain et al., 2004)

To assess the functionality of sequences of interest within the NATb promoter, artificial mutant derivatives of the NATb 318 luciferase plasmid were constructed with the mutant alterations described in Figure 5. Putative TF binding sites, conserved regions and other structures were

altered to produce novel restriction enzyme sites or other changes that maximized the presence of the least conservative alterations (A vs. C or G vs. T). The results of measuring the promoter activity of these constructs in MCF-7 and HepG2 cells are presented in Figure 6. Effects of the mutations on relative promoter activity levels were similar with both cell lines. The Con1-R7 mutation, ttcccag>gggatcc, at the overlap between the 19 nucleotide conserved region and the 5' portion of the 16 nucleotide palindrome, caused a 3 to 4-fold reduction in promoter activity. The Pal 11/16 mutation, gggcccctggg>tttaaaaattt, which alters the remaining 3' portion of the palindrome, reduced promoter activity by 2- to 3- fold, but the mutation Con1-L9, ctgcaacta>ggatccagc, which alters only the 5' portion of the 19 nucleotide conserved region had a lesser effect on promoter strength. It is notable that the NATb 178 construct which is completely deleted for the sequences corresponding to both the 19 nucleotide conserved region and the palindrome (Figure 5) did not show less promoter activity than any of the individual mutations, suggesting that the absence of all of these sites did not have a compound effect.

A partial deletion combined with two nucleotide changes in the putative Sp1 site, Sp1 del4 +GC->AT (cggggc>at), reduced promoter activity nearly 10-fold in both MCF-7 and HepG2. A second mutation, Sp1 G3G4->AT, changing only the two most conserved positions of the consensus Sp1 sequence, as defined by TRANSFAC M00196, also resulted in a large reduction of promoter activity. The NFY-6 mutation in the putative NF-Y site reduced promoter activity slightly, with a 50% reduction in MCF-7 and a 33% reduction in HepG2. Alteration of a putative AP-1 transcription factor binding site (Figure 5) caused no change in measured promoter activity. No notable effect on promoter activity was observed in the construct with two mutant regions that altered the 5' portion of the 12 nucleotide conserved region and changed both elements of the 13 nucleotide duplicated segment, Dup1-R7+Dup2-L6 (Figure 5).

The two different segments of the NATb promoter in which mutations showed large effects on relative promoter activity were tested by EMSA to investigate the nature and specificity of protein binding. The oligonucleotide 165L26 containing the putative NATb Sp1 site was shifted by MCF-7 nuclear extract with a shift pattern closely related to the shift pattern seen with a consensus Sp1 recognition site oligonucleotide (Figure 7A). The 165L26 shift was also effectively competed by addition of an excess of the consensus Sp1 oligonucleotide (Figure 7A). Figure 7B shows that an anti-Sp1 antibody caused a supershift of the 165L26 complex identical to the supershift seen for the Sp1 consensus oligonucleotide complex. Incubation with nuclear extract from MCF-7 cells also specifically shifted the oligonucleotide 200L29, which spans the sequences altered by the Con1-L9 and Pal 11/16 mutations. Competition with unlabeled oligonucleotides including consensus recognition sequences for AP-2 or Sp1 did not abolish this binding, nor did competition by the 165L26 oligonucleotide that corresponds to the NATb Sp1 site (Figure 7C).

Discussion

A quantitative RT-PCR assay that utilizes intron-spanning primers and is specific for transcripts from the *NAT1* major promoter showed that 44 samples from pooled mRNA preparations representing 29 different human tissues exhibited high expression of the NATb promoter with moderate variation between tissues. These results support the general view that NAT1b mRNA is highly expressed in essentially all tissues, but more extensive analyses with larger numbers of samples from each tissue will be needed to determine the full range of variable expression for each tissue and whether there are consistent trends for inter-tissue differences. Although conclusions would be strengthened by examining additional samples and more types of human tissues, these observations are generally consistent with previously published *NAT1* quantitative RT-PCR data for human tissues, obtained with primers from the 3' terminus of the gene which did not distinguish transcripts from the alternative *NAT1* promoters (Adam et al., 2003). The ubiquitous expression of NATb is in contrast to *NAT2*, as

high-level expression of the *NAT2* promoter is limited to liver, colon and small intestine with up to 1000-fold differences in expression between the highest and lowest expressing tissues (Husain et al., 2007). The alternative *NAT1* promoter, NATa, is most highly expressed in a small number of tissues, including kidney, liver, lung, and trachea (Barker et al., 2006). Thus, the measurements reported here support the view that expression of *NAT1* in diverse tissues is primarily due to transcription of NATb. Future investigations monitoring the relative functionality of the alternative promoters will be facilitated by use of the NATb-specific TaqMan assay utilized here together with the previously described NATa-specific TaqMan assay (Barker et al., 2006) which differs only in the sequence of the forward primer.

Transfection of luciferase expression constructs with progressive deletions defined a minimal NATb promoter with high transcription strength, comparable to the CMV promoter in the pGL3-Control plasmid, in both the MCF-7 and HepG2 cell lines. The 213 bp minimal promoter construct eliminates an additional 173 bp from the 5' end of the pGL-257 minimal promoter described previously (Butcher et al., 2005). Two important functional sites were localized in this minimal region by further deletion and mutation analyses. The presence of a functional Sp1 site was strongly implicated by a conserved alignment with an Sp1 site previously described for the mouse *Nat2* promoter (Boukouvla et al., 2003) and maximal similarity to the TRANSFAC Sp1 consensus matrix M00196. Mutations in the putative Sp1 site caused sharp reductions in the promoter activity of luciferase reporter constructs. The results of EMSA binding, competition and supershift assays provided direct evidence for binding of Sp1 to this segment of the NATb promoter. Sp1 binding is also likely to be an important feature of the promoter activity of the rat, mouse and bovine promoters, since most of these have a highly similar sequence that aligns with the NATb Sp1 site (Figure 4) and matches well with the M00196 Sp1 consensus matrix. Mouse *Nat1* and *Nat2* are both expressed in a wide range of tissues (Sugamori et al., 2003; Loehle et al., 2006), but little is known concerning tissue-specific expression of the bovine or rat genes. The human *NAT2* promoter is notably divergent at the Sp1 site location, however, and shows multiple sequence discordancies with highly conserved positions in the M00196 matrix. Since Sp1 is ubiquitously expressed and usually stimulates transcription (Philipsen and Suske, 1999), the absence of a functional Sp1 site in the *NAT2* promoter is likely relevant to the limited tissue specificity of high level expression of *NAT2* in humans.

A second critical region of the NATb promoter was initially identified by the presence of a highly conserved sequence and an overlapping 16 nucleotide palindrome. It has previously been suggested that transcription factors AP-2 and/or OLF-1 may bind at this site based on a MatInspector analysis (Butcher et al., 2005). Two non-overlapping mutations in this region, Con1-R7 and Pal 11/16, both caused substantial reductions in promoter activity, but absence of the entire segment in the NATb 178 deletion construct did not cause any greater reduction, suggesting that a single functional component of the transcription complex may be affected, although the region may contain binding sites for multiple proteins. EMSA analysis with the 200L29 oligonucleotide spanning this segment demonstrated a specific shift which was not competed by consensus Sp1 or AP-2 oligonucleotides. The exact 16 bp palindrome in NATb was not found in the other promoters shown in Figure 4, but 9/16 of the nucleotides in this segment did show complete conservation, consistent with the presence of a functional element. Further evidence of an important functional site at this location was provided by review of the conservation analysis of the chr8:18,110,586-18,113,015 (March 2006 Assembly) segment in the Human genome Browser Database. The presence of similar sequences in human, mouse, rat, elephant, cow, armadillo, chimpanzee, rhesus monkey, and rabbit is indicated by an isolated, sharp peak of conservation that corresponds to nucleotides 42 to 57 of the NATb promoter. Transcription factor binding at this site is likely to be important for the expression of *N-acetyltransferase* genes across several species.

Although mutations in several of the candidate sites with conserved or distinctive sequence structures (Figure 5) had little or no effect on promoter activity in this assay system, it remains possible that they may have functional significance during embryonic development, in the mediation of specific physiological responses *in vivo* or in the context of normal chromatin and chromosomal structures. It is also possible that sequences outside of the 213 bp segment have important functions that are not detectable under our specific experimental conditions, as exemplified by the recent identification of a region that lies 5' to the 213 bp minimal promoter and mediates a transcriptional response to androgens in two prostate cancer cell lines (Butcher et al., 2007).

Knowledge of the location of critical sequences within the NATb promoter is also important for assessing the possible functional significance of single nucleotide polymorphisms (SNPs) located in the promoter region. Based on information available at the Human Genome Browser Gateway (<http://www.ncbi.nlm.nih.gov/SNP/index.html>), and the NIEHS Environmental Genome Project (<http://egp.gs.washington.edu/>), two SNPs, rs28359482 and rs28359483 can be identified within the sequence of the minimal 213 promoter segment, at positions 85 and 228 of the sequence shown in Figure 5. The rs28359482 SNP is adjacent to the NATb Sp1 site, but the T->C change does not affect the match to the Sp1 consensus matrix M00196. The rs28394583 G>A SNP is an intronic SNP which lies just 3' to the consensus splice donor site of the NATb start exon. The locations of these SNPs indicate little likelihood of large functional effects. Further characterization of *NAT1* transcription is required to address the possibility of functional polymorphisms in control regions that are not part of the NATb minimal promoter or in intronic sequences which affect transcription or splicing of the *NAT1* mRNA.

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ABBREVIATIONS

5'-RACE, 5' rapid amplification of cDNA ends; BAC, bacterial artificial chromosome; bp, basepair; EMSA, electrophoretic mobility shift assay; kb, kilobase pair; mRNA, messenger RNA; NAT1, N-acetyltransferase 1; NAT2, N-acetyltransferase 2; rRNA, ribosomal RNA; RT, reverse transcription; RT-PCR, reverse transcription polymerase chain reaction; SNP, single nucleotide polymorphism; SEM, standard error of the mean; TF, transcription factor; TSS, transcription start site.

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FIG. 1.

Relative expression of NATb mRNA in 29 different human tissues. Series A includes human tissue RNAs primarily from the Ambion test panel and series B includes human tissue RNAs primarily from the BD test panel. An additional liver sample from BD, labeled Liver (BD Ref), was included as a point of reference in the measurements of both series. Fifteen of the 29 tissues are represented, from independent sources, in both series A and B. Error bars indicate the full range of measured values (N=2).

FIG. 2. Relative expression of NATb mRNA in human cell lines. The sample labeled Liver (BD Ref), was included to provide a point of reference with the human tissue measurements. Error bars indicate the full range of measured values (N=2).

FIG. 3.

Definition of the NATb minimal promoter by transient transfection of MCF-7 and HepG2 cell lines. Promoter strengths were assessed by dual luciferase assay and measurements $\pm$ SEM are presented. Lines at the left indicate the approximate boundaries of the segments tested with respect to the diagrammed genomic DNA, including the minimal promoter, indicated by the open box, and the region containing transcription start sites, indicated by the bracket labeled TSS.

Rn <i>Nat</i>	ATTC--GTGG-----CCCAAAGACAAGCAG-GACGCTGCAACTACAGTTCCCGAGAAAGC	51
Mm <i>Nat1</i>	ATTAAGGTGG-----CCCACAGACAAACAG-GATCCTGCAACTACAGTTCCCGAGAGAGC	53
Hs <i>NAT2</i>	ATTTGC GTAAGAGATTACACAGAGGCAACCTGAGGCCCTGCAACTACATTTCCCGAGAGATC	60
Hs <i>NAT1</i>	ATATGAATAAGGGGTCCACAGGAGCAACCTGAGTCCCTGCAACTACATTTCCCGAGGGGCC	60
Mm <i>Nat2</i>	AGTTC-----ACCAGGGTTAAGTGAAG-CCCTACAACCTACATTTCCCGAGAGATC	48
Bt <i>Nat</i>	AGGGG-----CACAGGGTCAACCCAAGTCCCCACGACTACATTTCCCGAGAGCTC	49
	----------*-----*-----*-----*-----*-----*	
Consensus	A-----C-CA--G-CAA-C--G-CCCT-CAACTACA-TTCCCGAG--C	
Rn <i>Nat</i>	CAGGGGATTATTC-----CAGCCAGCCT--TGGGGCAGGGCAATTTGGACA-GCCAATG	102
Mm <i>Nat1</i>	CAGGGGAATATTC-----TAACGAACCT--TGGGGCAGGGCAATTTGGGCA-GCCAATG	104
Hs <i>NAT2</i>	CCTGAGGTGATCC-----TAAGTAGACTCTGGTGTGACAGGTGATACGGAAT-TCCAGTG	113
Hs <i>NAT1</i>	CCTGGGGTAACCC-----TAATGTGACTCTGGGGGCGGGGCCAGTTGGACA-TCCAATG	113
Mm <i>Nat2</i>	CTTGAGATGGTTTAAATAAATAAAGGAGCAACTTGAACA-GCCAATA	107
Bt <i>Nat</i>	C--GGGGTGAC-----GGTAAGTAGAC-TAGGGGGTGGAGCACTTTGCAGACAGGAAGG	100
	----*-----*-----*-----*--*--*-----*-----*	
Consensus	C--G-G-T-A-----AAC-A--C---GGGGC-G-GC-A-TTG-A-A--CCAATG	
Rn <i>Nat</i>	AGCT--CTTCCTCTGCAGACTTCGGCTGGGAGTGGACAGCCTCCACCAGCTTCCTGGTC	160
Mm <i>Nat1</i>	AGCT--CTTCCTCTGCAGAGCTCGGC-----CTCCTACCAGCTTCAGGTC	148
Hs <i>NAT2</i>	AGATCACTTCCCTTGAGACTTTGGAAGGGAGAG--CACTTTATTACAGACCTTGGAAGC	171
Hs <i>NAT1</i>	AGAGCACTTCCCTTGAGACTTTGGAAGTGAGAG--CACTTCCTCATAGACCTTGGAATGT	171
Mm <i>Nat2</i>	AGAGCTCTTCCCTTGAGACCAAGGAACTGAAAC---TCACTCAGCTTACTTCCTGGTC	163
Bt <i>Nat</i>	AGAGCTCTTCCCTGGGCTGGGCCAGGAAGTGAA--GGGTGGCCGTCGCCCTGATT	153
	-----***--**--*-----**-----*-----*	
Consensus	AG----CTTCC--TGCAGA---GG---G-----C---C-T-----	
Rn <i>Nat</i>	TCAGGCCTGCTACACCGG <i>GTAAGT</i> AGACTCCCCGGCT-----GTCAGGCCGA	209
Mm <i>Nat1</i>	ACAGGCCTGCTACAC--GGGAGGCATAGCTTGCTGGCT-----GCCAGACCGA	195
Hs <i>NAT2</i>	AAGAGGATTGC-ATTGAGCCTAGTTCTGGTTGCTGGCCAAA----- <i>GTAAGT</i> GAA	222
Hs <i>NAT1</i>	GGGAGGATTGC-ATTGAGTCTAGTTCTGGTTGCCGGCTGAAATAACCTG <i>GTAAGT</i> GAA	230
Mm <i>Nat2</i>	CGCCCGGTTGCAGTCC---TGGTAGCGG <i>GTA</i> ----- <i>GGGGGG</i> CTC	202
Bt <i>Nat</i>	--CCTGTCGTGGGTC---CCAGAG-AGCTCG----- <i>GTAAGT</i> ACC	189
	-----*-----*-----*--*-----*-----*	
Consensus	-----TGC---C-----G-T-----GT-AG-----	

FIG. 4.

Alignment of promoter regions of *N-acetyltransferase* genes from human *NAT1* NATb (Hs *NAT1*), human *NAT2* (Hs *NAT2*), mouse *Nat1* (Mm *Nat1*), mouse *Nat2* (Mm *Nat2*), rat (Rn *Nat*) and cow (Bt *Nat*). Nucleotides conserved in all six sequences are indicated by an asterisk. On the consensus sequence line, nucleotides present in all six promoters are shown in bold and nucleotides present in 5/6 of the sequences are shown in plain font. A putative TATA box and an Sp1 site in the mouse *Nat2* promoter are italicized. Two regions of high sequence conservation are indicated by thick underlining in the NATb promoter sequence. The bold italicized sequence near the 3' end of Rn *Nat* is the splice donor site defined by cDNAs U01343, U01347 and U01348. The bold italicized sequences near the 3' ends of the Hs *NAT1* and Hs *NAT2* sequences are the splice sites detected in 5'-RACE clones and database cDNAs (Husain et al., 2004; Husain et al., 2007). The bold italicized sequence near the 3' end of the Mm *Nat2* sequence is the donor splice site defined by all three of the mouse *Nat2* cDNAs CA494799, CN663986 and BF164333. The bold italicized sequence near the 3' end of Bt *Nat* is the splice donor site defined by the splicing pattern of the BT025447, CK946692, BE683786, CK960222,

DY464402, AW484954 and BG689007 cDNAs. Genomic sequences were retrieved at the Genome Browser Gateway and aligned using ClustalW as detailed in Materials and Methods.

```

c213      CONSERVED1      Palindrome
|          |
1  ATATGAATAAGGGGTCCACAGGAGCAACCTGAGTCCCTGCAACTACATTTCCTCCAGGGGCCCTGGG 66
      (Con1-L9)  ggatccagc
                                gggatcc (Con1-R7)
                                tttaaaaattt (Pal 11/16)
                                200L29 5'ACATTTCCTCCAGGGGCCCTGGG

c178      AP-1?      Sp-1?      c150      NF-Y?
|          |          |          |
67  GTAACCCTAATGTGACTCTGGGGCGGGGCAGTTGGACATCCAAATGAGAGCACTTCCCTGCAGA 132
      (AP1-5)  aatcc      (NF-Y-6)  gaattc      acaggaa (Dup1-R7)
              ----at (Sp1 del4+GC->AT)
              at (Sp1 G3G4->AT)
GTAACCC3'   5' GACTCTGGGGCGGGGCCAGTTGGAC3' 165L26

5'-TSS      Dup copy 2      3'-TSS
|          |          |
133 CTTTGGAAAGTGAGAGCACTTCCTCATAGACCTTGATGTGGGAGGATTGCATTCACTCTAGTTCT 198
      gtctct (Dup2-L6)

Splice
199 GGTTCGCCGGCTGAAATAACCTGGTAAGTGGAAGTCTGTAAGGGGCT 244

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FIG. 5.

Summary of candidate functional elements in the NATb promoter and the nine different sequence alterations present in artificially mutated constructs. The sequences of EMSA oligonucleotides 200L29 and 165L26 are also shown. Nucleotides in bold on the genomic sequence line are conserved in all six of the promoters shown in Figure 4. The labels c213, c178 and c150 mark the positions of the 5' ends of the genomic segments present in the NATb 213, 178 and 150 bp promoter luciferase constructs respectively. The nucleotides labeled 5'-TSS and 3'-TSS delimit the region including previously identified NATb transcription start sites (Husain et al., 2004).

FIG. 6. Results of promoter strength assays $\pm$ SEM, with deleted and artificially mutated NATb promoter constructs. Duplicate test constructs were assessed by transfections of MCF-7 and HepG2 cell lines and dual luciferase assay ($N \geq 3$). Details of the artificial mutations are shown in Figure 5.

FIG. 7.

Summary of EMSA analysis of oligonucleotides 165L26 and 200L29. **A**, Comparison of binding of MCF-7 nuclear extract to Sp1 consensus and 165L26 oligonucleotides and competition with 165L26 binding. *Lane 1*, Sp1 probe+extract; *Lane 2*, Sp1 probe only; *Lane 3*, 165L26 probe+extract; *Lane 4*, 165L26 probe+extract+Sp1 competitor; *Lane 5*, 165L26 probe+extract+Ap-2 competitor; *Lane 6*, 165L26 probe+extract+Ap-1 competitor; *Lane 7*, 165L26 probe only. **B**, Supershift of Sp1 consensus and 165L26 complexes with Sp1 antibody. *Lane 1*, Sp1 consensus probe+extract; *Lane 2*, Sp1 consensus probe+extract +anti-Sp1 Ab; *Lane 3*, Sp1 consensus probe+extract +anti-Ap-2 antibody; *Lane 4*, Sp1 consensus probe only; *Lane 5*, 165L26 probe+extract; *Lane 6*, 165L26 probe+extract +anti-Sp1 Ab; *Lane 7*, 165L26 probe+extract +anti-Ap-2 Ab; *Lane 8*, 165L26 probe only. **C**, Binding and competition of 200L29 oligonucleotide. *Lane 1*, 200L29 probe+extract; *Lane 2*, 200L29 probe+extract+Ap-2 consensus competitor; *Lane 3*, 200L29 probe+extract+unlabeled 200L29 competitor; *Lane 4*, 200L29 probe+extract+Sp1 consensus competitor; *Lane 5*, 200L29 probe+extract+165L26 competitor; *Lane 6*, 200L29 probe only.

Table 1

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

Oligonucleotide	Sequence	Utility
NATbqF	5'-CTGGTTGCCGCTGAAATAAC-3'	NATb q-RT-PCR For
NATa/bqR	5'-TCCAAGTCCAATTTGTTCTAGACT-3'	NAT1 q-RT-PCR Rev
NATa/bpr	6FAM5'-CAATCTGTCTTCTGGATTAA-3'MGBNFQ	NAT1 TaqMan Probe
NATb 318H3	5'-CCCCCAAGCTTAAGAGCACAGTCCTCCCAGC-3'	318 Forward
NATb 245H3	5'-CCCCCAAGCTTGATATGAATAAGGGGTCCACAG-3'	245 Forward
NATb 213H3	5'-CCCCCAAGCTTAGTCCCTGCAACTACATTTC-3'	213 Forward
NATb 178H3	5'-CCCCCAAGCTTGTAACCTAATGTGACTCTGGG-3'	178 Forward
NATb 150H3	5'-CCCCCAAGCTTGCCAGTTGGACATCCAATG-3'	150 Forward
NAT1TSSZeroRevH3	5'-CCCCCAAGCTTAGCCCTTACAGAGTTCCACTTAC-3'	Promoter clone reverse
Con1-L9BamFor	5'-CCCCGGATCCAGCCATTCCCAGGGGC-3'	Mutant Con1-L9
Con1-L9BamRev	5'-CCCCGGATCCGGACTCAGGTTGCTCCT-3'	Mutant Con1-L9
Con1-R7BamFor	5'-CCCCGGATCCGGGCCCCCTGGGGTA-3'	Mutant Con1-R7
Con1-R7BamRev	5'-CCCCGGATCCCATGTGTCAGGGACTCAG-3'	Mutant Con1-R7
Pal 11/16ApoFor	5'-GGATCCAAATTTGTAAACCTAATGTGACTCTGGG-3'	Mutant Pal 11/16
Pal 11/16ApoRev	5'-GGATCCAAATTTTAACTGGGAAATGTAGTTGCAGG-3'	Mutant Pal 11/16
AP1-5EcoFor	5'-CCCCCAAGCTTCTGGGGCGGGGCCAGT-3'	Mutant AP1-5
AP1-5EcoRev	5'-CCCCCAAGCTTCAATAGGGTTACCCAGG-3'	Mutant AP1-5
Sp1del4, GC >ATBamFor	5'-CCCCGGATCCAGTTGGACATCCAATGA-3'	Mutant Sp1del4, GC >AT
Sp1del4, GC >ATBamRev	5'-CCCCGGATCCCGAGAGTCACATTAGGGT-3'	Mutant Sp1del4, GC >AT
Sp1G3G4 >ATFor	5'-GACTCTGGGATCGGGGCCAG-3'	Mutant Sp1G3G4 >AT
Sp1G3G4 >ATRev	5'-CTGGCCCCGATCCCAGAGTC-3'	Mutant Sp1G3G4 >AT
NF-Y-6EcoFor	5'-CCCCCGAATTCTGAGAGCACTTCCCCTG-3'	Mutant NF-Y-6
NF-Y-6EcoRev	5'-CCCCCGAATTCGTCCAACCTGGCCCCG-3'	Mutant NF-Y-6
Dup1-R7PstFor	5'-CTTCCCCTGCAGACTTTGGA-3'	Mutant Dup1-R7
Dup1-R7PstRev	5'-CCCCCCTGCAGTTCTCTCTCATTGGATGTCCAAC-3'	Mutant Dup1-R7
Dup2-L6For	5'-GGAAGGTCTCTCACTTCCTCATAGACC-3'	Mutant Dup2-L6
Dup2-L6Rev	5'-AAGTGAGAGACCTTCCAAAGTCTGCA-3'	Mutant Dup2-L6
NATb Genomic-For	5'-TCTAGCCTGCCTGAGGACAT-3'	BAC Outer nest For
NATb Genomic-Rev	5'-CTTTGGGACACACACCACTG-3'	BAC Outer nest Rev
RV-3 (pGL3 Basic)	5'-CTAGCAAAATAGGCTGTCCC-3'	Plasmid Outer nest For
GL-2 (pGL3 Basic)	5'-CTTTATGTTTTTGGCGTCTTCC-3'	Plasmid Outer nest Rev
200L29 sense	5'-ACATTTCCCAGGGGCCCTGGGGTAACCC-3'	EMSA
200L29 antisense	5'-GGGTTACCCAGGGGCCCTGGGAAATGT-3'	EMSA
165L26 sense	5'-GACTCTGGGGCGGGGCCAGTTGGAC-3'	EMSA
165L26 antisense	5'-GTCCAACCTGGCCCCGCCCCAGAGTC-3'	EMSA
Sp1 consensus (sense)	5'-ATTCGATCGGGCGGGGCGGAGC-3'	EMSA control/competitor
Sp1 consensus (antisense)	5'-GCTCGCCCCGCCCGATCGAAT-3'	EMSA control/competitor
AP-1 consensus (sense)	5'-CGCTTGATGAGTCAGCCGGA-3'	EMSA competitor
AP-1 consensus (antisense)	5'-TTCGGGTGACTCATCAAGCG-3'	EMSA competitor
AP-2 consensus (sense)	5'-GATCGAACTGACCGCCGCGGCCGT-3'	EMSA competitor
AP-2 consensus (antisense)	5'-ACGGGCCGCGGGCGGTCTCGATC-3'	EMSA competitor