

Conserved regulatory motifs at phenylethanolamine *N*-methyltransferase (*PNMT*) are disrupted by common functional genetic variation: an integrated computational/experimental approach

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Received: 6 November 2009 / Accepted: 8 February 2010 / Published online: 5 March 2010
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Abstract The adrenomedullary hormone epinephrine transduces environmental stressors into cardiovascular events (tachycardia and hypertension). Although the epinephrine biosynthetic enzyme *PNMT* genetic locus displays both linkage and association to such traits, genetic variation underlying these quantitative phenotypes is not established. Using an integrated suite of computational and experimental approaches, we elucidate a functional mechanism for common (minor allele frequencies > 30%) genetic variants at *PNMT*. Transcription factor binding motif prediction on mammalian *PNMT* promoter alignments identified two variant regulatory motifs, SP1 and EGR1, disrupted by G-367A (rs3764351), and SOX17 motif created by G-161A (rs876493). Electrophoretic mobility shifts of approximately 30-bp oligonucleotides

containing ancestral versus variant alleles validated the computational hypothesis. Queried against chromaffin cell nuclear protein extracts, only the G-367 and -161A alleles shifted. Specific antibodies applied in electrophoretic gel shift experiments confirmed binding of SP1 and EGR1 to G-367 and SOX17 to -161A. The *in vitro* allele-specific binding was verified *in cella* through promoter reporter assays: lower activity for -367A haplotypes cotransfected by SP1 ($p = 0.002$) and EGR1 ($p = 0.034$); and enhanced inhibition of -161A haplotypes ($p = 0.0003$) cotransfected with SP1 + SOX17. Finally, we probed *cis/trans* regulation with endogenous factors by chromatin immunoprecipitation using SP1/EGR1/SOX17 antibodies. We describe the systematic application of complementary computational and experimental techniques to detect and document functional genetic variation in a trait-associated regulatory region. The results provide insight into *cis* and *trans* transcriptional mechanisms whereby common variation at *PNMT* can give rise to quantitative changes in human physiological and disease traits. Thus, *PNMT* variants in *cis* may interact with nuclear factors in *trans* to govern adrenergic activity.

Electronic supplementary material The online version of this article (doi: 10.1007/s00335-010-9253-y) contains supplementary material which is available to authorized users.

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Introduction

The enzyme *PNMT* (phenylethanolamine *N*-methyltransferase) catalyzes the synthesis of epinephrine (adrenaline), providing the only route to formation of this hormone and neurotransmitter. Variation in the human *PNMT* promoter was first noted by Wu and Comings (1999) and later systematically documented in European-American, African-American, and Japanese populations (Ji et al. 2005; Saito et al. 2001). Two common *PNMT* proximal promoter SNPs are located -367 (rs876493) and -161 (rs3764351) bp

upstream of the 5' UTR (untranslated region) (Kaneda et al. 1988), corresponding to -390 and -184 bp upstream of the translational start (ATG) codon (Sasaoka et al. 1989). Reported linkage disequilibrium (LD) is high ($D' > 0.9$) across the promoter in the HapMap CEU population (Utah residents with Northern and Western European ancestry from the Centre d'Etude du Polymorphisme Humain collection) (Frazer et al. 2007; Thorisson et al. 2005).

The *Pnmt* locus is within the genome-wide confidence interval for linkage (meiotic cosegregation) with several stress traits in rodents (<http://rgd.mcg.edu/>), including salt-loaded SBP QTL-9, urine volume QTL-18, and corticosterone (glucocorticoid) level QTL-5. Several human disease associations to *PNMT* promoter genetic variants have been documented, including hypertension in African-Americans (Cui et al. 2003), early-onset Alzheimer's disease (Mann et al. 2001), multiple sclerosis (Mann et al. 2002), drug-assisted weight loss (Peters et al. 2003), and reward dependence temperament (Yamano et al. 2008). Although two studies failed to find association with hypertension in European (Kepp et al. 2007) and European-American (Cui et al. 2003) subjects, we observed sex-dependent association to diastolic blood pressure (Rana et al. 2007).

Prior *PNMT* genetic studies have not definitively identified molecular mechanisms whereby genetic variation may interact with stressors to change *PNMT* expression. Ji et al. (2005) compared *PNMT* promoter haplotype activity, observing a significant decrease in activity for the A.A (-367.-161) haplotype versus G.G. Extensive work in the PC12 rat pheochromocytoma cell line suggests orthologous regulatory motifs for SP1, EGR1, AP2, MAZ, and GRE in the first 1000 base pairs of proximal promoter (Wong and Tank 2007).

Here we explore regulatory effects of common *PNMT* promoter genetic variants (G-367A, G-161A) in *cis*, as well

as the effects of factors in *trans*, including SP1 (at G-367A) and SOX17 (at G-161A), via orthologous motifs in the human promoter, demonstrating an allele-specific effect for SP1 and EGR1 on the G-allele at position-367. Furthermore, we identify a novel allele-specific inhibitory effect of SOX17 interacting with SP1 to preferentially inhibit the A allele at position -161. Sox17 is a transcription factor involved in development of heart and vascular and lung tissue (Liao et al. 2009; Liu et al. 2007; Matsui et al. 2006; Park et al. 2006); all are important targets of the epinephrine-mediated stress response.

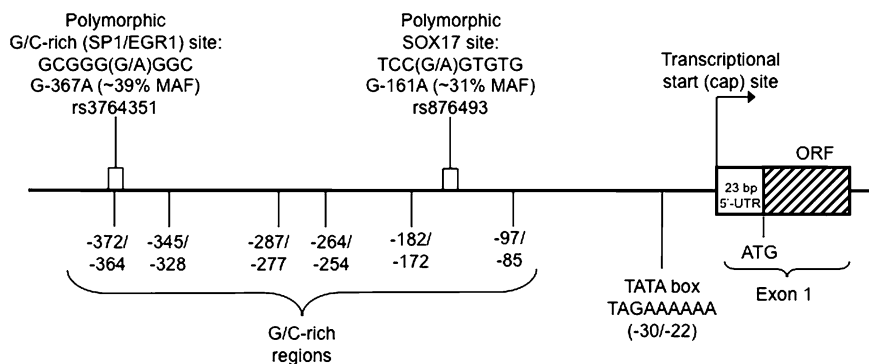
Materials and methods

Genomics and luciferase promoter/reporter plasmids

Human *PNMT* sequence information was obtained at NCBI (<http://www.ncbi.nlm.nih.gov>) or UCSC (<http://genome.ucsc.edu>) using source clone X52730 (JO3727) (Sasaoka et al. 1989). Mammalian promoter sequencing and polymorphism discovery at the *PNMT* locus in genomic DNA were conducted using dideoxy sequencing on an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA). The *PNMT* promoter regions in chimpanzee (NG06939), bonobo (NG05253), and rhesus (NG07109) DNA from Coriell repository samples, as well as rat (strains SHR, WKY, and BN) samples, were resequenced.

At *PNMT*, two promoter variants (G-367A and G-161A) were especially common minor allele frequency $>30\%$ (Ji et al. 2005). The very proximal core promoter (Fig. 1) just upstream of exon 1, including the TATA box, was invariant; there were also six G/C-rich stretches, one of which was disrupted by G-367A. The two common promoter variants (-367.-161) displayed consistent LD, with D' approximately 0.90 in Caucasians. Of the four possible promoter haplotypes

Fig. 1 Common genetic variation in the proximal human *PNMT* promoter. Common core promoter motifs (e.g., TATA box, G/C-rich domains) are illustrated. The two common proximal variants (G-367A and G-161A) are depicted



(G/A): Polymorphic base
MAF: Minor allele frequency
Based on genomic DNA clone X52730=J03727
DTC, 06-01-2009

(Ji et al. 2005) at -367.-161, the A.A (at 50.6%) and G.G (35.1%) were common, while A.G (12.0%) and G.A (1.9%) were unusual. Based on promoter location, high frequencies, and LD of promoter variants G-367A and G-161A, we then focused functional studies on this region.

Human *PNMT* promoter/luciferase reporter plasmids were constructed as previously described (Chen et al. 2008). G.G and A.G haplotype fragments corresponding to *PNMT* -428 to +10 bp [with respect to cap site of 23 bp 5' UTR in NCBI source clone X52730 (J03727): 1327-1793, Chr 17: 35077803-35078242] were amplified from genomic DNA of known (previously resequenced) promoter genotype (G.G, 68-year-old black female [#2154]; A.G, 45-year-old Hispanic male [#4261]); these two individuals did not exhibit other *PNMT* promoter variants. The haplotype fragments were subcloned by sticky-end ligation into the upstream promoter site of the pGL3-Basic vector (Promega Inc., Madison, WI, USA) between *XhoI* and *KpnI* restriction digest sites (New England Biolabs, Ipswich, MA, USA). Successful ligations were verified by electrophoresis on 1% agarose of 1 µg plasmid digested overnight with *KpnI* and *XhoI*; correct sequence and orientation were verified by sequencing with RV3 reverse primer (Promega). G.A and A.A haplotypes (-367.-161) were generated by site-directed mutagenesis per the manufacturer's specifications (QuickChange II; Stratagene, La Jolla, CA, USA) using the aforementioned G.G and A.G haplotype plasmids as template and the same oligonucleotide primers as in the electrophoretic mobility shift assay (EMSA) experiments.

Plasmids were amplified in DH5-alpha cells (Invitrogen, Carlsbad, CA, USA), and supercoiled plasmids were purified by column adsorption/elution (PureLink HiPure Plasmid Maxiprep Kit, Invitrogen). Plasmid concentrations were quantified by SmartSpec (Bio-Rad, Hercules, CA, USA) and adjusted to 1.0 µg/µl before transfection.

Cell biology: transient promoter haplotype transfection and reporter assay

PC12 rat adrenal pheochromocytoma cells were grown in high-glucose Dulbecco's modified Eagle medium supplemented with penicillin G (100 U/ml), streptomycin sulfate (100 mg/ml), 5% fetal bovine serum, and 10% horse serum. PC12 cells were distributed among 12-well plates and allowed to reach 60% confluence before transfection. Plasmid DNA was incubated in 100 µl per well of serum-free media with TransfectIN reagent (Bio-Rad) for 15 min and then added to 400 µl fresh PC12 media. Cells were transiently transfected for 4 h with 1 µg per well haplotype plasmid or pGL3-Basic vector and then washed with serum-free media followed by 1 ml PC12 media. Firefly luciferase activity was measured after 48 h of incubation at 37°C, 6%

CO₂. Luciferase activity was normalized by total protein per well measured by Bradford assay (Protein Assay, Bio-Rad) in a Wallac spectrophotometer. Linear range of protein concentration was verified using serial dilutions of bovine serum albumin (BSA) as a standard. pCMV-driven transcription factor expressing plasmids, including EGR1, AP2, SOX17, and SP1 plasmids (from Open Biosystems, Huntsville, AL, USA), were cotransfected at 0.1 µg per well. Dual cotransfection of SP1 with SOX17 or SP1 with pcDNA (pCMV) was also tested at 0.1 µg per well.

PNMT promoter haplotype/luciferase reporter activity

Haplotype luciferase activity (RLU) was normalized to total protein (Bio-Rad/Bradford Coomassie blue assay at A₅₉₅; also background subtracted and divided by volume). The activity shown in Fig. 3 is the mean activity for four wells ±1 standard error. In order to compare experiments, we normalize all values to the empty vector pGL3-Basic activity. Finally, in order to control for effects of plasmid cotransfection on promoter activity, we subtract the activity of the mock cotransfection of the pcDNA3.1 plasmid, which contains the promoter used to drive transcription factor expression.

Bioinformatics

Mammalian promoter alignment

Mouse, dog, cat, horse, and cow *PNMT* sequences were obtained from <http://genome.ucsc.edu> (Blanchette et al. 2004; Kent et al. 2002; Kuhn et al. 2009). Due to a gap in the *R. norvegicus* reference genome assembly, we also sequenced the promoter of the WKY rat. Promoter orientation for each sequence was determined by ClustalW alignment of the exon 1 coding sequence. Promoter sequences were aligned using ClustalW with a gap penalty of 100. Phylogenetic trees of the mammals were constructed manually using taxonomy information from NCBI.

Prediction of allele-specific conserved regulatory motifs

We predicted transcription factors with allele-specific binding to motifs containing G-367 or G-161A using phylogenetic footprinting (Wasserman and Sandelin 2004). Human and rat *PNMT* promoter sequences were pairwise aligned using the LAGAN algorithm (Brudno et al. 2003), as implemented by the CONREAL web server (Berezikov et al. 2005). Motifs with more than 50% human-rat homology (length > 10 bp) were scored on publicly available position-weight matrices (PWMs) from JASPAR (Vlieghe et al. 2006) and TRANSFAC v7.0 (Wingender et al. 1996) public databases. Motifs scoring greater than

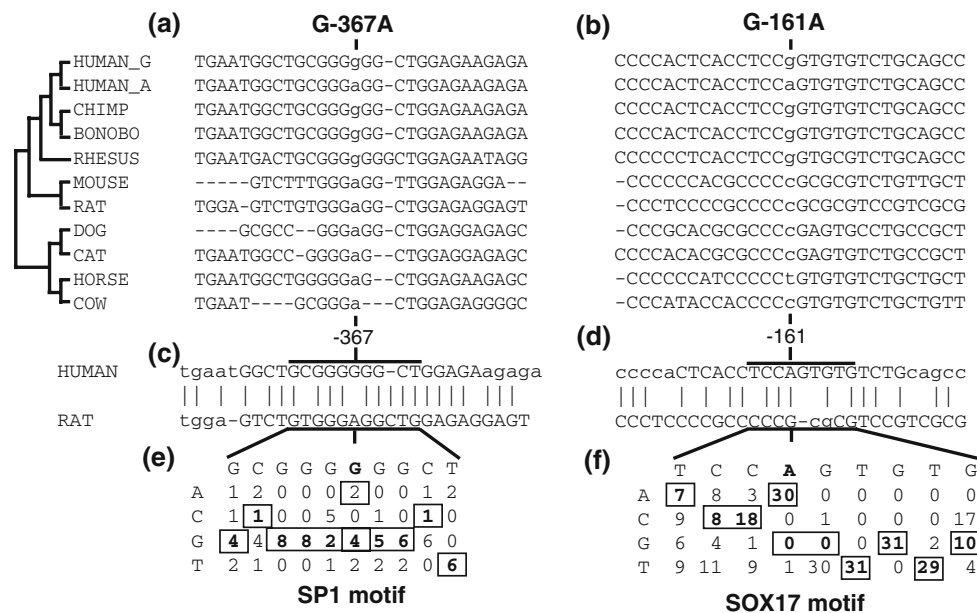


Fig. 2 Allele- and factor-specific transcription enhancement and inhibition in the human *PNMT* promoter. ClustalW alignment of orthologous mammalian *PNMT* promoter motifs, including two human alleles, three primates, and six other mammals, with (a) -367 and (b) -161 positions in lowercase. Rat-human alignments shown with greater than 50% conserved motif in uppercase for (c) -367 and (d) -161. JASPAR position frequency matrix scoring of

human sequence (boxes) using (e) SP1 on -367 and (f) SOX17 on -161. Polymorphic position in boldface type. Using the SOX17 JASPAR motif (YWYTGKB), there are an additional 22 nonpolymorphic SOX17 sequence matches at 7 of 8 or better stringency in the first 500 bp of proximal promoter. Using a conventional G/C-rich motif (S_n) consensus, there are an additional five nonpolymorphic matches spanning 11–18 bp each (see Fig. 1)

80% of maximum in one allele with a difference in score between alleles were considered positive hits (summarized in Fig. 2 using SOX17 and SP1 as examples). Results were cross-verified using the RAVEN web server (Andersen et al. 2008), which uses seven-vertebrate PhastCons (Felsenstein and Churchill 1996) alignments for identification of conserved motifs. Candidate hits were prioritized for experimental validation if known to regulate the well-characterized rat *PNMT* promoter (Wong and Tank 2007), or if adrenal, chromaffin cell, or PC12 expression activity in GEO (Edgar et al. 2002) expression array repository was above 25% of minimum.

Electrophoretic mobility shift assay (EMSA)

Biotinylated double-stranded 29-mer oligonucleotides (ValueGene, Biotin 3' End DNA Labeling Kit, Pierce, Rockford, IL, USA) with SNP at position 15 were incubated 45 min and with nuclear extracts of PC12 rat pheochromocytoma cells for 20 min in the presence of 0.1 $\mu\text{g}/\mu\text{l}$ salmon sperm DNA to minimize nonspecific binding. Nuclear extracts were prepared from PC12 cells grown in 10-cm plates by Qproteome Nuclear Protein Kit following the manufacturer's specifications (Qiagen) (Avery-Kiejda et al. 2008). In separate reactions, either specific (i.e., unlabeled same-allele oligonucleotides) or nonspecific

competitor (i.e., unlabeled opposite-allele oligonucleotides) was added at 1000 \times concentration to assay allele-specific binding. Antibody assays included 2 μl of antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in lieu of competitor oligonucleotide, with nonspecific IgG as a negative control). DNA-protein complexes were electrophoresed in 5% acrylamide gels for 1 h at 100 V in 0.5 \times tris-borate ethylene diamine tetra acetic acid (TBE) and then transferred to nylon membranes at 380 mA in 0.5 \times TBE and cross-linked with 1200 nm UV light for 1 min. Complexes were then visualized on X-ray film using horseradish-peroxidase-conjugated streptavidin per the manufacturer's protocol (Chemiluminescent Nucleic Acid Detection Module, Pierce, Rockford, IL, USA) (Matata and Galinanes 2002; Oltra et al. 2003). Forward/reverse allele-specific oligonucleotide probes were as follows (polymorphism in lowercase):

-367 A fw: 5'-TGAATGGCTGCGGGaGGCTGGAGAA GAGA-3'
 -367 A rv: 5'-TCTCTTCTCCAGCCtCCCCGAGCCAT TCA-3'
 -367 G fw: 5'-TGAATGGCTGCGGGgGGCTGGAGA AGAGA-3'
 -367 G rv: 5'-TCTCTTCTCCAGCCcCCCCGAGCCAT TCA-3'

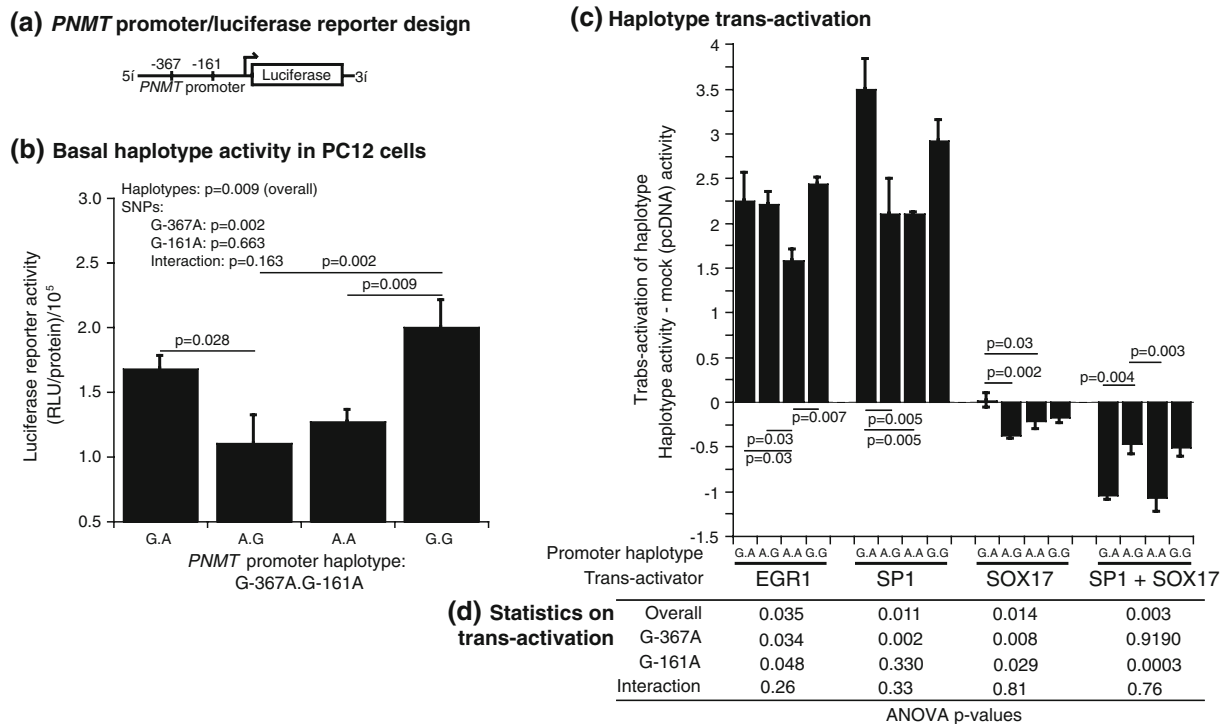


Fig. 3 SNP-interrupted conserved regulatory motifs in the human *PNMT* promoter: functional consequences. **a** Cartoon of *PNMT* promoter/reporter construct, one of four possible promoters based on variation at positions G-367A/G-161A (G.A, A.G, A.A, G.G), which were ligated 5' of firefly luciferase in pGL3-Basic plasmid and transfected into PC12 cells. **b** Basal (unstimulated) promoter activity for transfected *PNMT* promoter haplotypes (G-367A/G-161A; G.A, A.G, A.A, G.G), measured in cell lysates after 48 h of transient transfection, mean of four wells \pm 1 standard error. Statistics: Haplotype effect (1-way ANOVA, overall $p = 0.009$) is shown first. Two-way ANOVA is then shown, factoring by each SNP. Horizontal bars indicate post-hoc t tests between haplotypes. X axis: Promoter haplotype; Y axis: Promoter activity in RLU/protein. **c** Change in

promoter activity for *PNMT* promoter haplotypes, measured in cell lysates after 48 h of transient transfection; mean of four wells \pm 1 standard error. Y axis shows fold change of activity versus pGL3-Basic promoterless empty vector, with mock pcDNA3.1 (pCMV) cotransfection activity subtracted, and normalization by total protein (quantified by the Bradford assay). Along the X axis are the transfected promoter haplotypes (G-367A,G-161A) and the pCMV-driven transcription factors cotransfected (SP1, EGR1, SOX17, and SP1 + SOX17). Horizontal bars indicate post-hoc t tests for haplotype comparisons within a cotransfection state. **d** ANOVA p values for overall effect, individual SNP effects, and SNP \times SNP (G-367A \times G-161A) interactions, during transcriptional activation by cotransfection

-161 G fw: 5'-CCCCACTCACCTCCgGTGTGTCTGC AGCC-3'
 -161 G rv: 5'-GGCTGCAGACACACcGGAGGTGAG TGGGG-3'
 -161 A fw: 5'-CCCCACTCACCTCCaGTGTGTCTGC AGCC-3'
 -161 A rv: 5'-GGCTGCAGACACACTGGAGGTGAGT GGGG-3'

Chromatin ImmunoPrecipitation (ChIP)

PC12 cells at 70% confluence grown on 10-cm poly-L-lysine-coated plates were transiently transfected with 5 μ g promoter haplotype/reporter plasmid DNA 4 h (Bio-Rad transfectin). Cells were washed with serum-free medium (DMEM high-glucose), grown 24 h (5% FBS, 10% HS, 1% PSG), washed with phosphate-buffered saline (PBS), fixed

with 37% formaldehyde, pelleted, and transferred to lysis buffer for fragmentation of chromatin to nucleosomes by controlled sonication (11 cycles in a Branson Sonifier; 20-s pulse at 50% amplitude with 1 min cooling). Nucleosomes were immunoprecipitated with antibodies for EGR1 (Santa Cruz Biotechnologies; sc-110x), SP1 (sc-420x), SOX17 (sc-20099x), or IgG/pre-immune serum ("Mock"; sc-33608) conjugated to Protein A agarose beads (ChIP Assay Kit, USB Corporation, Cleveland, OH, USA). After precipitation, complexes were washed and crosslinking was reversed at 65°C overnight. After column purification (QiaQuick PCR Purification, Qiagen), DNA was amplified for 26–30 cycles (to preclude signal from the negative control) with SNP-flanking primers designed using PRIMER3, and products were electrophoresed on 1.5% agarose gels and photographed with a digital camera. Primers were as follows:

G-367A fw: 5'-CTGGGCAAAAGAGGTGAATG-3'
 G-367A rv: 5'-CCTCACCCCCACAATCTTTA-3'
 G-161A fw: 5'-CTGCGGGAGCAAGTACGG-3'
 G-161A rv: 5'-CCCACCCATTCATCCATCT-3'

Statistics

Changes in promoter haplotype/reporter activity were evaluated by one-way or two-way analysis of variance (ANOVA), as appropriate, using SPSS software (SPSS, Inc., Chicago, IL, USA), using (as the independent variable) either the haplotype or the SNP. Post-hoc corrections to significance were done by the Fisher LSD (least significance difference) method.

Results

SNP-interrupted conserved regulatory motifs in the *PNMT* promoter

Alignment of orthologous 1-kb promoters (upstream of the ATG initiation codon), including three primates, six mammals, and human reference/variant sequences, showed a moderately conserved G/C-rich (GGGGGG) motif in primates, interrupted by the A allele at position -367 in the human promoter (GCGGG[G/A]GGC; Fig. 2a). In chimpanzee, bonobo, and rhesus, G occurs at the polymorphic site; however, in the less conserved region in mouse, rat, dog, cat, horse, and cow, the allele is A.

At human promoter position -161, G is the major primate allele (Fig. 2b); however, the surrounding bases are not well conserved with rodents and nonprimate mammals. This region of the promoter is divergent across mammals but conserved within subgroups as clustered by the phylogenetic tree in Fig. 2 (mouse and rat, dog and cat, horse and cow all have pairwise-conserved motifs).

Phylogenetic footprinting was used to predict which transcription factors are responsible for the allele-specific EMSA shifts observed in Fig. 4. Alignments with rat promoter for human sequences flanking promoter positions -367 (Fig. 2c, human G allele shown) and -161A (Fig. 2d, human A allele shown) identified regions with greater than 50% sequence identity (in uppercase). Position frequency matrix scoring using JASPAR matrices (SP1 on -367 G vs. A, Fig. 2e; SOX17 on -161 G vs. A, Fig. 2f) highlights allelic differences: at G-367A, a two-point difference between alleles, with the -367 G allele scoring higher for SP1 (88% of maximum score), and at G-161A there was a 30-point difference between alleles, with -161 A scoring higher on SOX17 (TCC[G/A]GTGTG, 80% of maximum score). Scoring matrices were selected from the top hits identified using CONREAL, with both SP1 and SOX17

mRNA expression in PC12 cells more than 50% of maximum in GEO profiles of PC12 cells.

Allele-specific transcription: functional enhancement or inhibition of expression

The transcription factors tested in cotransfection assays (Fig. 3) were selected by a combination of *in silico* and *in vitro* predictions of regulatory function; at G-367A, G/C-rich-region candidates SP1 and EGR1 were tested, while at G-161A, factor SOX17 was evaluated. We compared the transcriptional response of four *PNMT* promoter haplotypes (G-367A.G-161A, illustrated in Fig. 3a) to transcription factors EGR1, SP1, and SOX17. Even though haplotype G.A did not occur in human samples, creation of this fourth haplotype (by site-directed mutagenesis) allowed statistical inferences on each of the two contributory SNPs, by two-way ANOVA. Graphical and statistical results are shown in Fig. 3.

Basal haplotype expression

Under basal circumstances, the four haplotypes differed in transcriptional strength (Fig. 3b) overall (ANOVA $p = 0.009$), with a significant effect of G-367A ($p = 0.002$), but without SNP \times SNP interaction (two-way ANOVA $p = 0.163$). As previously reported (Ji et al. 2005), haplotype G.G was more active than haplotype A.A ($p = 0.009$).

G-367A and SP1 or EGR1

We observed a significant difference in promoter stimulation (Fig. 3d) between G-367A alleles upon SP1 cotransfection ($p = 0.002$), with higher activity for the G allele that displayed a superior SP1 match (Fig. 3c). The response of the G.A haplotype to SP1 was greater ($p = 0.005$) than either of the A.X haplotypes (A.G or A.A). EGR1 *trans* activation also differed across *PNMT* promoter haplotypes ($p = 0.035$).

G-161A and SOX17

SOX17 cotransfection produced a modest decline ($p = 0.014$) in the activity of each variant haplotype except G.A (Fig. 3c); there were significant differences between G-161A alleles ($p = 0.029$) and G-367A alleles ($p = 0.008$) (Fig. 3d).

Factor cotransfection

In order to further characterize the transcription inhibition effect of SOX17 on the *PNMT* promoter, we attempted

dual/cotransfection of SP1 and SOX17. Overall, we observed an enhanced, supra-additive inhibitory effect upon transcription ($p = 0.003$) (Fig. 3c, far right), especially on G.A and A.A haplotypic backgrounds. We also observed an allelic difference at G-161A in response to the factors in combination (SOX17 + SP1, $p = 0.0003$), with lower activity for the A allele.

Allele-specific factor binding: EMSAs and specific antibody disruption

The work described in Figs. 2, 3, 4 reflects a cyclical *in silico* → *in vitro* → *in cella* approach to dissecting allele-specific transcription regulation by specific transcription factors. Allele-specific shifts of human oligonucleotides bound to rat chromaffin cell nuclear proteins observed in Fig. 4 prompted the screening for position-weight matrices scored in Fig. 2. The regulatory effect of these factors is confirmed *in vivo* in Fig. 3. However, regulatory stimulation does not guarantee binding of the putative transcription factor to a specific motif. Hence, we select antibodies based on the combined results to confirm allele-specific binding *in vitro* in an EMSA antibody disruption assay (Fig. 5).

During EMSA of G-367A (Fig. 4a), the labeled G allele displayed multiple (2–3) shifts by PC12 nuclear proteins, which could be partially displaced by the unlabeled G probe, although not the unlabeled A probe. The labeled A

allele was not shifted by nuclear proteins, indicating specificity. Based on the phylogenetic footprinting results shown in Fig. 2, we tested the effect of antibodies to SP1 (Fig. 5a, lane 3) or EGR1 (lane 4); in each case, the shifted G-allele bands (lane 2) were disrupted or abolished by coinubation with the antisera, suggesting involvement of these two factors. Involvement of EGR1 at G-367A is further illustrated by Supplementary Fig. 1, pointing out the overlapping EGR1 motif match, which is superior for the G allele.

During EMSA of G-161A, we observed multiple electrophoretic mobility shift bands for both labeled alleles (Fig. 4b, lanes 2 and 6). In each case, the labeled allele shift is best competed for by the unlabeled A allele (Fig. 4b, lanes 4 and 7), suggesting higher affinity of the A allele for the nuclear proteins bound. Based on phylogenetic footprinting results shown in Fig. 2, we tested the effects of an anti-SOX17 antibody on the complex (Fig. 5b, lane 9); this addition also manifested as a disappearance of the previously shifted A-allele band (lane 9), once again suggesting antibody sequestration of nuclear SOX17, freeing the SOX17 binding site on the -367A allele oligonucleotide. We did not observe such effects at G-161A using SP1 or mock/IgG antibodies, confirming specificity (Fig. 5b, lanes 8 and 10). Furthermore, what appears to be a G-allele shift (Fig. 5b, lane 2; Fig. 5b, lane 2) was not displaced by any of the tested antibodies, and hence is of uncertain significance.

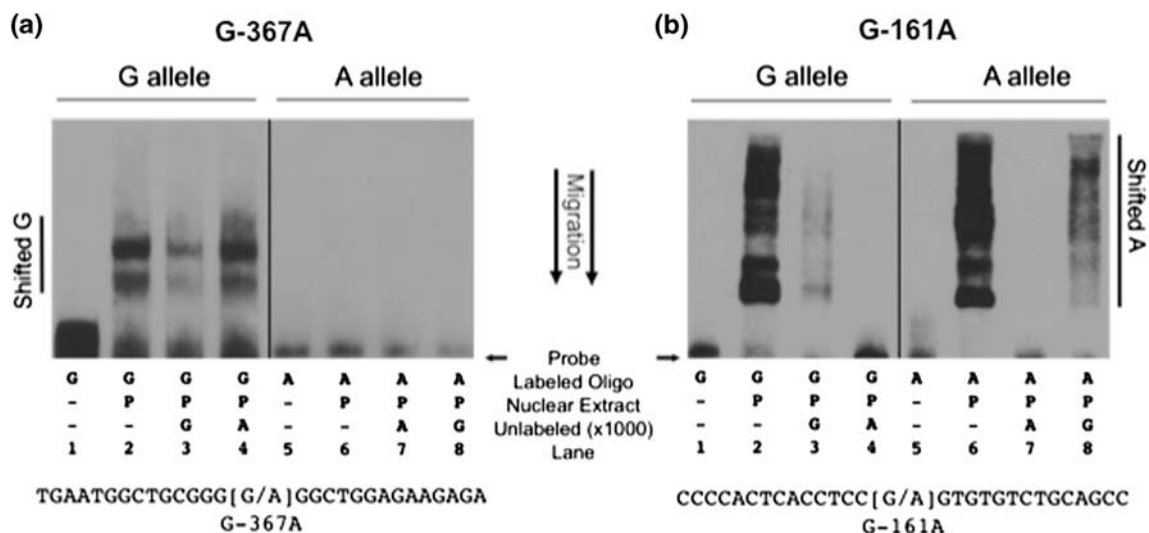


Fig. 4 Allele-specific electrophoretic mobility shifts. Electrophoresis at 90 V of double-stranded biotinylated oligonucleotides incubating in the presence/absence of PC12 nuclear DNA-binding protein extracts (water-soluble) and 1000 × molar excess unlabeled oligonucleotide. Visualization of bands by trans-blot to nylon membrane, streptavidin-horseradish-peroxidase conjugation, and incubation for 5 min with luminol solution, followed by exposure on X-ray film.

Horizontal black bar separates G- (left) from A- (right) allele oligonucleotide experiments (label on top of image). Oligonucleotide sequences designed to match 29-bp motifs at **a** -367 and **b** -161, with polymorphic position in brackets (bottom). Center bar illustrates migration from top to bottom and highlights G-allele-specific shift for -367 oligonucleotide and A-allele-specific shift for -161 oligonucleotide

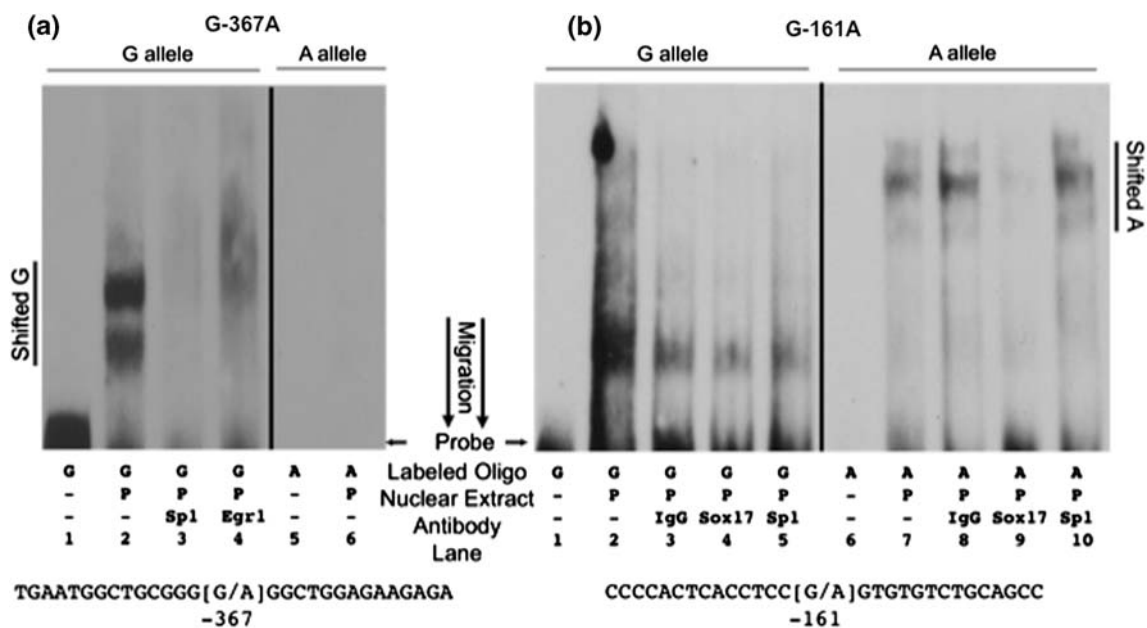


Fig. 5 Electrophoretic mobility shifts: Perturbation by transcription factor-specific antibodies. Electrophoresis at 90 V of double-stranded biotinylated oligonucleotides incubate in the presence/absence of PC12 nuclear DNA-binding protein extracts (water-soluble) and

antibodies to **a** SP1 and EGR1 versus -367 oligonucleotides or **b** anti-SOX17 and anti-SP1 versus -161 oligonucleotides. No-antibody shift same as Fig. 4 used as a positive control. Nonspecific (preimmune) IgG (“Mock”) was a negative control. Same protocol as Fig. 4

Haplotype-specific transcription factor binding to the motifs in chromatin

Formation of oligonucleotide-factor-antibody complexes in vitro is a model but not a substitute for in vivo complex formation in the cell nucleus. Although the results of the cotransfection promoter/reporter assays, phylogenetic footprinting, and electrophoretic mobility shift assays produce a consistent story of SP1 and EGR1 upregulation on the G allele at human promoter position -367 and of SOX17 downregulation on the A allele at position -161, we attempted to extend this result by chromatin immunoprecipitation (ChIP), which may yield insight into *endogenous* transcription factor binding by a motif in nuclei prepared from *living* cells.

Antibodies to both SP1 and EGR1 immunoprecipitated nucleosomes harboring the proximal *PNMT* promoter (Fig. 6b), as evidenced by the PCR amplicon spanning G-367A; specificity was indicated by lack of immunoprecipitation by preimmune serum (“Mock”). Thus, *endogenous* SP1 and EGR1 seem to interact with the *PNMT* proximal promoter within chromatin in vivo, although the results of this semiquantitative experiment did not distinguish differential roles for the G and A alleles.

An antibody to SOX17 also immunoprecipitated nucleosomes spanning the *PNMT* promoter (Fig. 6c), as evidenced by the PCR amplicon spanning G-161A, although the G and A alleles did not differ in band intensity; specificity was indicated by lack of immunoprecipitation by

preimmune serum (“Mock”). Once again, lack of difference for the G and A alleles may simply reflect the semi-quantitative nature of the experiment.

Discussion

Through an integrated in silico → in vitro → in cella approach, we identified functional allele-specific transcription factor motifs in the human *PNMT* promoter, including the human ortholog of a rat SP1/EGR1 motif (reviewed in Wong and Tank 2007) and a novel SOX17 motif.

G-367A motif and factors SP1 and EGR1

The G/C-rich SP1 motif is conserved across mammalian species and disrupted by common human variant G-367A (GCGGG[G/A]GGC, rs3764351; Fig. 2a), resulting in a loss of observed binding in EMSA experiments and a decrease in promoter enhancement in luciferase assays. An EGR1 motif is also likely in this region (Supplementary Fig. 1). We confirm regulatory effects of SP1 and EGR1 on this motif in both antibody disruption (Fig. 5) and ChIP (Fig. 6) experiments. The consistency of the effects across experiments, backed by extensive mammalian conservation of the motif, provides evidence supporting a functional polymorphism. Of note for the potential physiological significance of these findings, SP1 and

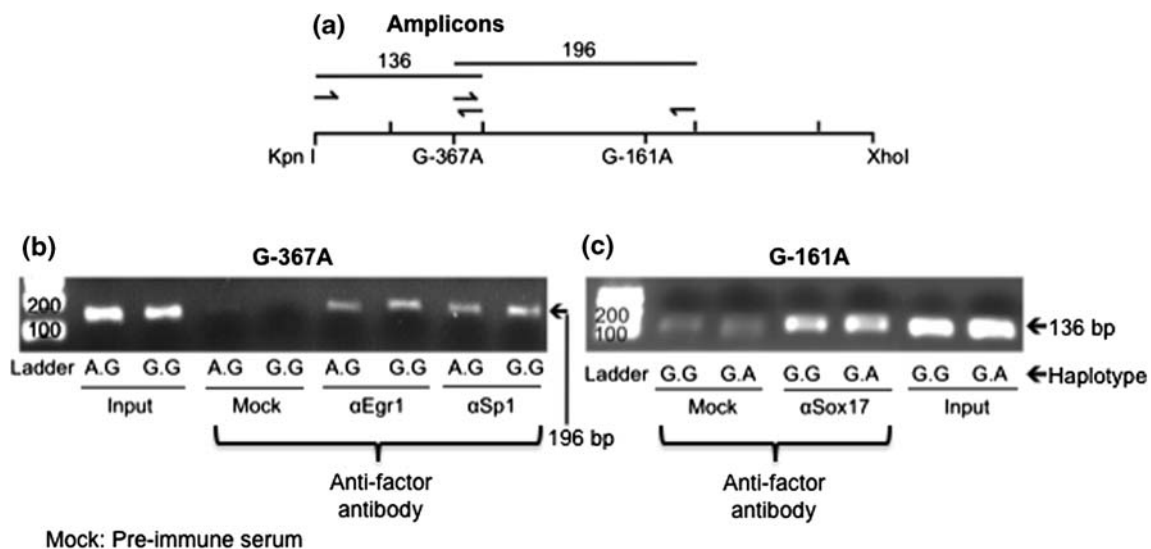


Fig. 6 Haplotype-specific endogenous transcription factor binding in the human *PNMT* promoter. Chromatin Immunoprecipitation (ChIP) assay to determine binding of particular transcription factors to the motifs in vivo. **a** Cartoon of primer design and expected PCR amplification fragment size. Primers specific for amplification of

b -367 were used to identify DNA-protein complexes precipitated with anti-EGR1, anti-SOX17, or nonspecific IgG (“Mock,” pre-immune serum) antibodies, or raw sonicated DNA (“Input”) as a positive control. **c** Identical experiment testing anti-SOX17 with -161 primers

EGR1 are highly expressed in PC12 chromaffin cells (GEO database GDS1038 and GDS1039 at <http://www.ncbi.nlm.nih.gov/geo/>).

G-161A motif and factor SOX17

At G-161A, a SOX17 motif conserved in primates (Fig. 2) is disrupted by the variant allele (TCC[G/A]GTGTG, rs876493), and the downregulatory response of the A allele at this motif was augmented by SOX17 + SP1 co-transactivation. We documented an effect of SOX17 on the G-161 motif in both antibody disruption (Fig. 5) and ChIP (Fig. 6) experiments. SOX17 is also highly expressed in PC12 chromaffin cells (GEO GDS1039).

Perspectives for human trait determination

The significance of the results presented here is best understood in the context of the body of linkage and disease associations already identified for the *PNMT* gene. Indeed, not only is the *PNMT* locus within the genome-wide confidence interval for linkage with multiple stress traits in rodents (<http://rgd.mcw.edu/>), but *PNMT* promoter allelic associations occur for several human traits with likely derangements in adrenergic function, including the common diseases hypertension (Cui et al. 2003; Rana et al. 2007) and obesity (Peters et al. 2003). While published single-marker-on-trait associations for both promoter SNPs exist, the high

degree of linkage disequilibrium ($D' > 0.9$) between the SNPs makes it difficult to definitively ascribe a causal association to one of the two variants. Using the PC12 cell as a model of the human chromaffin cell, we were able to show allele-specific differences of promoter activity in the presence of transcription factors SP1, EGR1, and SOX17.

An earlier report on *PNMT* transcription using a longer (~ 1 kb) transfected human promoter (Ji et al. 2005) found similar rank-order activities of the two most common *PNMT* promoter haplotypes (G.G > A.A; $p < 0.002$), but no significant differences from G.G for the other haplotypes ($p > 0.05$) in PC12 (rat pheochromocytoma) and TE671 (human medulloblastoma) cells; however, that report did not pursue regulatory motifs at -367/-161. In a follow-up study, Ji et al. (2008) also explored the effect of a relatively rare polymorphism in the first *PNMT* intron (G280A) on plasma epinephrine as well as on *PNMT* transcription. Here we focused instead upon the *PNMT* proximal promoter, since the bulk of previous clinical associations implicated this region (Cui et al. 2003; Mann et al. 2001, 2002; Peters et al. 2003), the LD across locus is high, and common functional variation may influence large numbers of individuals.

Acknowledgments Thanks to Manjula Mahata (UCSD Department of Medicine) for PC12 cells. This work was supported by the National Institutes of Health and the Department of Veterans Affairs. J.L.R.F. was funded by a NHGRI pre-doctoral fellowship. This work was supported by grants from the National Institutes of Health: HL58120, MD000220, and DK079337.

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