Human Tyrosine Hydroxylase Natural Genetic Variation
Delineation of Functional Transcriptional Control Motifs Disrupted in the Proximal Promoter

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Background—Tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine biosynthesis. Common genetic variation at the human TH promoter predicts alterations in autonomic activity and blood pressure, but how such variation influences human traits and, specifically, whether such variation affects transcription are not yet known.

Methods and Results—Pairwise linkage disequilibrium across the TH locus indicated that common promoter variants (C-824T, G-801C, A-581G, and G-494A) were located in a single 5’ linkage disequilibrium block in white, black, Hispanic, and Asian populations. Polymorphisms C-824T and A-581G were located in highly conserved regions and were predicted to disrupt known transcriptional control motifs myocyte enhancer factor-2 (MEF2), sex-determining region Y (SRY), and forhead box D1 (FOXD1) at C-824T and G/C-rich binding factors specificity protein 1 (SP1), activating enhancer-binding protein 2 (AP2), early growth response protein 1 (EGR1) at A-581G. At C-824T and A-581G, promoter and luciferase reporter plasmids indicated differential allele strength (T>C at C-824T; G>A at A-581G) under both basal circumstances and secretory stimulation. C-824T and A-581G displayed the most pronounced effects on both transcription in cella and catecholamine secretion in vivo. We further probed the functional significance of C-824T and A-581G by cotransfection of trans-activating factors in cella; MEF2, SRY, and FOXD1 differentially activated C-824T, whereas the G/C-rich binding factors SP1, AP2, and EGR1 differentially activated A-581G. At C-824T, factor MEF2 acted in a directionally coordinate fashion (at T>C) to explain the in vivo trait associations, whereas at A-581G, factors SP1, AP2, and EGR1 displayed similar differential actions (at G>A). Finally, chromatin immunoprecipitation demonstrated that the endogenous factors bound to the motifs in cella.

Conclusion—We conclude that common genetic variants in the proximal TH promoter, especially at C-824T and A-581G, are functional in cella and alter transcription so as to explain promoter marker-on-trait associations in vivo. MEF2, FOXD1, and SRY contribute to functional differences in C-824T expression, whereas SP1, AP2, and EGR1 mediate those of A-581G. The SRY effect on TH transcription suggests a mechanism whereby male and female sex may differ in sympathetic activity and hence blood pressure. These results point to new strategies for diagnostic and therapeutic intervention into disorders of human autonomic function and their cardiovascular consequences. (Circ Cardiovasc Genet. 2010;3:187-198.)

Key Words: tyrosine hydroxylase ■ hypertension ■ gene expression ■ catecholamines ■ genetics ■ autonomic ■ nervous system, sympathetic

The enzyme tyrosine hydroxylase (tyrosine 3-monooxygenase; TH; EC-1.14.16.2) catalyzes the rate-limiting step in catecholamine biosynthesis: conversion of the amino acid L-tyrosine to L-dihydroxyphenylalanine. In previous clinical studies,1,2 we found that common allelic variation within the TH locus exerts a heritable effect on autonomic activity and blood pressure (BP) response to environmental stress suggest consequences for later development of cardiovascular disease traits. Common variants in the promoter region,2 especially C-824T and A-581G, predicted heritable alterations in multiple autonomic traits (biochemical and physiological) and ultimately to the disease trait of hypertension. However, it was not clear whether such variants were in themselves functional or simply exerted a statistical association with autonomic traits by virtue of proximity to an actual causative locus (ie, by linkage disequilibrium [LD]).

Clinical Perspective on p 198

In the present study, we probed the functional consequences of variation in the proximal promoter region of TH,
beginning with promoter haplotype and luciferase reporter plasmids. We went on to computational approaches, site-directed mutagenesis, and characterization of likely trans-acting factors, both endogenous (by chromatin immunoprecipitation [ChIP]) and exogenous (by cotransfection). We thereby developed evidence that variation in the promoter, especially at C-824T and A-581G, disrupts particular motifs (myocyte enhancer factor-2 [MEF2], sex-determining region Y [SRY], and forkhead box D1 [FOXD1]) at C-824T; G/C-rich binding factors at A-582G) to alter transcriptional activity.

Methods

Linkage Disequilibrium

Patterns of LD were analyzed and visualized by using the software Haploviev. Ld blocks were derived by the solid spline criterion in Haploviev from unphased diploid genotypes of subjects from the following 4 diverse biogeographic ancestry groups systematically sequenced across the TH locus: white (European ancestry, 2n=194 chromosomes), black (sub-Saharan African ancestry, 2n=50), Hispanic (Mexican American, 2n=56), and east Asian (2n=36).

Statistical Analyses

Statistical analyses were performed with SPSS version 11.5. Parametric, general linear model (typically, 2-way ANOVA) analyses were used to test the associations of haplotype and luciferase reporter activity for each construct. Interactions of promoter variants (haplotypes or single-nucleotide polymorphisms [SNPs]) with stimuli (eg, nicotine) was performed in the univariate tests of the general linear model, wherein there was a single dependent variable (eg, luciferase activity) and the interactive (multiplicative) effects of the 2 independent variables (eg, haplotype and drug) could be modeled. Each factor (independent variable or the interaction between 2 independent variables) was assessed for significance using F tests, with significance defined as P≤0.05. Effects cotransfected transcription factors also were analyzed by both 2-tailed t test and univariate test of the general linear model.

Biochemical Phenotyping: Catecholamines

Samples for measurement of plasma and urine catecholamines were quickly frozen at −70°C before a sensitive radioenzymatic assay based on catechol-O-methylation. The assay uses a preconcentration step that increases sensitivity by 10-fold over other catechol-O-methyltransferase-based assays and ~20-fold over many high-performance liquid chromatography assays, permitting accurate measurement of basal plasma epinephrine levels, which are at the limit of sensitivity for high-performance liquid chromatography assays. Urine catecholamine values were normalized to creatinine excretion in the same sample.

Computational Prediction of Transcription Factor-Binding Motifs Overlying TH Promoter Common Variants

Multiple sequence alignments were performed by using Clustal-W. Potential transcription factor-binding motifs were predicted beginning with the Web-based phylogenetic footprinting methods at Consite (http://aspi.ii.uib.no:8090/cgi-bin/CONSITE/consite/) and Conreal (http://conreal.niob.knaw.nl/), focusing on areas of conserved sequence across species. We also studied potential motifs with MacVector, evaluating both all alleles of each biallelic variant.

Exogenous/Cotransfected Transcription Factors

Plasmids containing cDNAs encoding transcription factors MEF2 and FOXD1 are from Open Biosystems. Sp1, activating enhancer-binding protein 2α (AP-2α), and EGR-1 were from Stratagene. MEF2 and its dominant-negative mutant mMEF2β were provided by John C. McDermott (York University, Toronto, Ont, Canada). cDNAs were either obtained in pCMV expression plasmids or subcloned into pcDNA3.1 (Invitrogen). The SRY full-length open-reading frame was amplified from human male genomic DNA and subcloned into pcDNA3.1 vector between KpnI and Xhol sites.

TH Promoter Haplotype/Luciferase Reporter Activity Assays

Human TH haplotype-specific promoter fragments, corresponding to TH-944/-1 bp in TH isoform B (source clones NM_000360, NT_009237.17, and NP_000351) were polymerase chain reaction (PCR) amplified from genomic DNA of known homozygotes (after resequencing) and cloned into the promoterless firefly luciferase reporter plasmid pGL3-Basic (Promega). Promoter positions are numbered upstream (−) of the start codon. Common haplotypes (>1% to 5% frequency) of the TH promoter were generated by site-directed mutagenesis from a known haplotype, verified by sequencing, and numbered as previously described. Supercopiled plasmids were purified on columns (Qiagen) before transfection.

Luciferase Reporter Assays of TH Promoter Variants

Pheochromocytoma (PC12) cells were transfected (at 50% to 60% confluence, 1 day after 1:4 splitting) with 1 µg of supercoiled promoter haplotype-firefly luciferase reporter plasmid and 10 ng of the Renilla luciferase expression plasmid pRL-TK (Promega) as an internal control per well by the liposome method (Superfect; Qiagen). The firefly and Renilla luciferase activities in cell lysates were measured 16 to 24 hours after transfection, using the Dual Luciferase reporter assay system (Promega), and the results were expressed as either the ratio of firefly/Renilla luciferase activity or the ratio of firefly activity/total protein in the lysate. Each experiment included 4 replicates. Results were expressed as mean±SEM. Statistical significance was calculated using Student t test or 1-way ANOVA, and significance was established at the P<0.05 level.

Sympathochromaffin Secretory Stimulation: Nicotine and Pituitary Adenylate Cyclase-Activating Peptide Effects on TH Promoter Haplotype/Luciferase Reporter Activity

PC12 cells were treated with nicotine (1 mmol/L) or pituitary adenylate cyclase-activating peptide (PACAP) (200 nM) versus mock in normal growth media after transfection. Luciferase activity was assayed with D-luciferin sodium (Sigma) and normalized to total protein. Results are shown as the fold stimulation by treatment.

Transactivation: Cotransfection of Transcription Factors With TH Promoter Haplotype/Reporters

A total of 50 or 100 ng of each transcription factor plasmid, or pcDNA3.1 empty vector, was cotransfected into PC12 cells, along with 1 µg of TH promoter haplotypes differing by particular variants. Luciferase activities were assayed as described previously and normalized by total protein. Reaction of the TH promoter to trans-activation was expressed as fold change of the mean value of luciferase activity between the transcription factor-transfected group and the mock-transfected (empty vector, pcDNA3.1) group.

Chromatin Immunoprecipitation

PC12 cells were transfected with particular TH promoter haplotype/reporters to get the different allele for each variant. ChIP assays were performed using the kit from Upstate Biotechnology. Cells (5 to 10×10^6) were cross-linked in 1% formaldehyde for 10 minutes at 37°C and washed with ice-cold PBS containing a protease inhibitor mixture (Sigma P8340; 10 µg/mL) then resuspended in cell lysis buffer (0.1% Triton X-100; 10 mmol/L KC1; and 10 mmol/L Tris, pH 8). After sonication (Branson) and centrifugation, samples were precleared with a salmon sperm DNA/protein A agarose 50% slurry to reduce nonspecific background and incubated with factor-specific
antibodies (Santa Cruz Biotechnology or preimmune IgG as a negative control) at 4°C overnight with rotation. Characteristics of the Santa Cruz Biotechnology antibodies were as follows: MEF2, sc-313X, rabbit polyclonal IgG; FOXD1, sc-47585X, goat polyclonal IgG; SRY, sc-8233X, goat polyclonal IgG; SP1, sc-420X, mouse monoclonal IgG; AP2, sc-184X, rabbit polyclonal IgG; and EGR1, sc-110X, rabbit polyclonal IgG. The immune complex was captured with a salmon sperm DNA/protein A slurry after incubation at 4°C for 1 hour. The immune complex was pelleted, washed 4 times, and eluted by 1% sodium dodecyl sulfate/0.1 mol/L NaHCO3, and cross-links were then reversed by heating in 0.2 mol/L NaCl at 65°C for 4 hours, followed by proteinase K digestion at 45°C for 1 hour. The DNA was subsequently extracted and purified with QIAquick PCR kits (Qiagen).

Immunoprecipitated nucleosomal DNA samples were analyzed by PCR using primers forming an amplicon that encompassed the C-824T (~908 to ~754 bp; sense, 5'-CTGGTGGGGTAGAGGAGAAA-3'; antisense, 5'-AACCCACTACGCCCCCTCTAT-3') or A-581G (~671 to ~512 bp; sense, 5'-GGCGGCTCTCCATAGA-3'; antisense, 5'-GAGTGGCATCTGGCCACA-3') sites in the human TH promoter. Extracted DNA from the chromatin fractions before antibody precipitation was used as a positive control (input DNA). To ensure that the PCR amplification was in the linear range, reactions with different amounts of input DNA samples were performed for various (typically 15 to 40) cycle numbers; a linear range of amplification typically occurred at ~25 cycles. After amplification, PCR products were separated on 1.5% agarose gels.

Chromatin Isolation and PCR After the Transfection of TH Promoter Into PC12 Cells
TH promoter haploptypes-1 (6 μg in 6 μL) was transfected into 10-cm plates of PC12 cells (~105 cells/plate) at ~80% to 90% confluence as described previously. Chromatin was isolated by differential centrifugation, as outlined with a protocol available at http://www.lamondlab.com/pdli/chromatinisolation.pdf. In brief, 24 hours after transfection, cells were lysed in an isotonic buffer containing 0.1% Triton X-100 and then centrifuged at low speed (1300 g at 4°C for 5 minutes) to obtain a low-speed pellet (P1) and low-speed supernatant (S1), after which S1 was centrifuged at high speed (20 000 g at 4°C for 5 minutes) to obtain a soluble “cytosolic” fraction (S2). The P1 was then subjected to hypotonic nuclear lysis and centrifuged at low speed (1700 g) for 5 minutes (S3) to obtain a soluble nuclear fraction (S1) and a chromatin-enriched fraction (P3). P3 was resuspended in 100 μL of distilled water. Aliquots of equal amounts (2 μg protein each) of the S2, S3, and P3 fractions were subjected to PCR amplification, with the same primers flanking the C-824T polymorphism as were used for ChIP as described previously. After amplification or 15, 20, or 25 cycles, PCR products were separated on 1.5% agarose and 90 m MTris/64.6 m M boric acid/2.5 mM EDTA, pH 8.3 gels followed by ethidium bromide (0.2 mg/L) staining.

Results
LD Patterns in the TH Promoter Region
We scored 13 common variants (minor allele frequency, >10%) spanning the locus in subjects of European, African, Hispanic, or Asian ancestry. To visualize patterns of SNP associations, pairwise correlations among the 13 common SNPs were quantified as LD parameter r2 by the solid spline criterion in Haploviev across the TH locus. In these subjects, a block of LD in the promoter region was maintained in subjects of European, Hispanic, and Asian ancestry, although in blacks, the promoter block was somewhat truncated, and in Asians, the 5’ block extended into exon 4 (Figure 1A). TH promoter allele and haplotype frequencies differed across the 4 biogeographic ancestry groups (online-only Data Supplement Table I).

Genetic Variation in the Proximal Human TH Promoter: Motifs
Numbering upstream (5’-3’) from the translational start codon (ATG), motifs identified included: −59/−54 TATA box (TTTATAA), −74/−67 cAMP response element (TGACGTCA), and −233/−227 AP1 site (TGAGTCA). Several G/C-rich regions (−284/−276, −249/−240, and −150/−143) also were noted in the proximal promoter. By resequencing across the TH locus, we identified 10 polymorphisms in the promoter, the following 4 of which were common (minor allele frequency, >10%): C-824T (rs10770141, 38.7%), G-801C (rs10840940, 11.6%), A-581G (rs10770140, 38.1%), and G-494A (rs11042962, 43.1%). Of note, the very proximal core promoter (−284/−1 bp) was devoid of common variation. By using prediction algorithms, we identified motifs likely to be disrupted by the promoter variants (Figure 1B). Among these, C-824T may alter the binding potential for factors MEF2, FOXD1, and SRY, whereas A-581G is located in a G/C-rich region, a likely binding site for factors such as SP1, AP2, and EGR1. We did not identify motifs altered by common variants G-801C or G-494A.

Effect of TH Promoter Variants on Transcriptional Activity
Twenty-four hours after transfection into PC12 cells, human TH promoter haplotypes showed substantial differences in luciferase reporter activity (at P<0.001) (Figure 2A, left). Among the common haplotypes, haplotype-2 (at 30% of chromosomes) was more active (P<0.001) than haplotype-1 (at 43%). Information on the haplotypes and their frequencies in different biogeographic ancestry groups is provided in supplemental Table I. By using 2-way ANOVA, 2 of the 4 common variants (C-824T and A-581G) altered transcriptional activity (each at P<0.0001) (Figure 2A, right), although the other 2 had no independent effects. Common variants C-824T and A-581G displayed interactive effects on transcription (P=0.00067), whereas variants A-581G and G-494A also interacted (P=1.17E-05). The interactions indicate that the effect of any particular SNP is highly dependent on genetic background (ie, other SNPs in the same promoter haplotype). Comparison of these in cella results with the effects of the same 4 variants in vivo (Figure 2B) indicates that the same variants (C-824T and A-581G) that exert the greatest effect on TH transcription in cella also have the most pronounced effect on human catecholamine excretion in vivo.14

Effect of Secretory Stimulation (Nicotine and PACAP) on Transcriptional Efficiency of TH Promoter Variants
TH promoter and luciferase reporters exhibited uniformly greater activity when treated by the secretory stimuli nicotine (1 mmol/L) or PACAP (200 nM), with an especially marked effect of PACAP. Haplotype-2 displayed consistently greater responses to both nicotine (≈2.6-fold) and PACAP (≈39.7-fold) than did haplotype-1 (nicotine, ≈2.07-fold; PACAP, ≈21.9-fold) (Figure 2C).
Figure 1. Genetic variation at the human TH locus. A, LD blocks across the locus in 4 biogeographic ancestry groups. Common (minor allele frequency, >10%) SNPs used to demonstrate LD (numbering from the ATG translational start codon) are as follows: C-824T, G-801C, A-581G, G1862A, G2066A, G2426C, G3034A, T3832C, C4581T, G4779A, C5162G, and T6681C. LD blocks were assigned by the solid spline method in Haploview. LD value shown: r²*100 r² color scheme: r²=0, white; 0<r²<1, shades of grey; r²=1: black. Common SNPs: MAF>10%.

B Genetic variation in the proximal human TH promoter: Impact on domains and motifs

**Bold:** Minor allele frequency >10% (Parentheses): SNP variant, e.g. (T/C) [Brackets]: Predicted motif

SNP position numbered upstream (-) of start codon (ATG)

TH core promoter [-284/-1] Transcriptional start (cap) site

TATA box [-59/-54] TTATA

CAMP response element [-74/-67] TGACGTC

G/C-rich domains [-284/-276, -249/-240, -150/-143]

AP1 [-233/-227] TGATTCA

G-494A C-388T G-94T

Exon 1

A-641G T-741C G-814A

C-824T C-833T

G/C-rich domain [-882/-578] C(GA)GCC

G/C-rich site [826/-821] TT(G/T)AAA

SRY site [-826/-821] TT(G/T)AAA

FOXD1 site [-625/-818] T(T/C)AAAGAA

MEF2 site [-327/-818] C(T/C)AAAGAA

White 2n=194

Black 2n=50

Hispanic 2n=56

Asian 2n=36

Figure 1. Genetic variation at the human TH locus. A, LD blocks across the locus in 4 biogeographic ancestry groups. Common (minor allele frequency, >10%) SNPs used to demonstrate LD (numbering from the ATG translational start codon) are as follows: C-824T, G-801C, A-581G, G1862A, G2066A, G2426C, G3034A, T3832C, C4581T, G4779A, C5162G, and T6681C. LD blocks were assigned by the solid spline method in Haploview. The TH exon/intron structure is superimposed above each panel; there were no common variants in the final (most 3') exon. B, Genetic variation in the proximal TH promoter: effect on domains and motifs. The C-824T polymorphism interrupts motifs for transcription factors MEF2, FOXD1, and SRY, whereas A-581G disrupts a G/C-rich domain, likely altering binding of transcription factors such as SP1, AP2, and EGR1. Functional elements in the core TH promoter are derived from previous reports in the literature, largely documenting sites in the rat promoter.11-13
Thyrosine hydroxylase (TH) promoter haplotypes: Differential strength in transfected pheochromocytoma cells

**TH promoter haplotypes:**
Differential strength in transfected chromaffin cells

**TH promoter common variants:**
Effects on transcription

- ANOVA: F=9.707, p<0.001
- Interactions:
  - 824-by-581 p=0.023
  - 581-by-494 p=0.002
- p value (by 2-way ANOVA) p=1.17E-05

**TH promoter: common natural haplotypes**
Positions: -824, -801, -581, -494

**Figure 2.** TH promoter haplotype activities. Statistical analyses were based on results from 8 naturally occurring haplotypes (1, 2, 3, 7, 8, 9, 11, and 13) (supplemental Table I). A, Basal activity. The left side shows haplotype effects and differential luciferase reporter activity in transfected PC12 cells. Here, only the 3 most common haplotypes (frequency ≥10%) are shown based on the 4 common promoter variants: C-824T, G-801C, A-581G, and G-494A. Results of ANOVA are shown for the haplotype as the independent variable and luciferase activity as the dependent variable. The right side shows individual SNP effects. Results of ANOVA are shown for the individual SNP as the independent variable and luciferase activity as the dependent variable. A log10 probability value plot is shown for significance of each of the 4 common variants. B, TH promoter common variant effects on gene expression in cella and in vivo. The common variants C-824T and A-581G that exert the greatest effect on TH transcription (horizontal axis) also have the most pronounced effect on human catecholamine excretion in vivo (vertical axis). The horizontal axis is derived from probability values obtained by ANOVA, with the individual SNP as the independent variable and luciferase activity as the dependent variable (A, right). Results in vivo (vertical axis) were obtained in catecholamine-phenotyped subjects of self-identified European ancestry in analyses wherein the independent variable was the promoter SNP and the dependent variable was renal norepinephrine excretion. Thus, both analyses used individual SNPs (rather than haplotypes) as the independent variables. C, Secretory stimulation effects of nicotine or PACAP on common TH promoter haplotype transcriptional activities. Bars show the fold increase of luciferase reporter activity after stimulation for each common haplotype. D, TH promoter common variant C-824T functional variation under basal and stimulated circumstances. Experiments were conducted on 2 haplotype backgrounds. Bars show mean ± SEM luciferase activity for each state. C-824 (CGAA) is from haplotype-1, -824T (TGAA) from haplotype-13, C-824 (CGGG) from haplotype-8, and -824T (TGGG) from haplotype-2 (supplemental Table II). Results are shown as fold of basal (no drug treatment). E, TH promoter common variant A-581G functional variation under basal and stimulated circumstances. Experiments were conducted on 2 haplotype backgrounds. Bars show mean ± SEM luciferase activity for each state. A-581 (CGAA) is from haplotype-1, -581G (CGGA) from site-directed (artificial) mutation 1 mol/L of haplotype-1, A-581 (TGAG) from haplotype-11, and -581G (TGGG) from haplotype-2 (supplemental Table II). Results are shown as fold of basal (no drug treatment).
We then considered the individual variants on balanced haplotype backgrounds (Figure 2D and 2E). Information on the haplotypes used in this study is given in supplemental Table II.

**C-824T**
Under basal circumstances (Figure 2D, left), greater activity of the T allele was most apparent on the haplotype-2 background. When stimulated by nicotine, C-824T responded differentially, with an increased effect of the drug on the T allele, on either haplotype-1 or haplotype-2 backgrounds (Figure 2D, middle). When stimulated by PACAP, the increased response of the T allele was most apparent on the haplotype-2 background (Figure 2D, right).

**A-581G**
In the basal state (Figure 2E, left), greater activity of the G allele was especially apparent on the haplotype-1 background. During nicotine stimulation, greater activity of the G allele was most apparent on the haplotype-2 background (Figure 2E, middle). In response to PACAP, the G allele was more responsive on either background, although most prominently on haplotype-2 (Figure 2E, right).
TH C-824T Polymorphism: Role of Transcription Factor MEF2

Sequence Conservation and Alignment
C-824T is located in a region highly conserved across sequenced primates (supplemental Figure I), with the C allele ancestral in the primate lineage. In this local region, there is a partial consensus match for an MEF2 site (Figure 3A), with an improved match for the T allele (8/10 bp) over the C allele (7/10 bp).

Endogenous MEF2 (by ChIP)
We probed the potential interaction of endogenous MEF2 with C-824T by ChIP and found that both the C and T alleles were indirectly captured by an antibody directed toward MEF2, suggesting increased binding by the T allele (Figure 3B).

Exogenous (Transfected) MEF2
Cotransfected human MEF2 increased TH promoter expression to 131.3% on the T allele (P<0.001) but had little effect on the C allele (P=0.254). MEF2 dominant-negative mutant mMEF2 decreased TH promoter expression down to 71.3% on the T allele (P=0.033) but had little effect on the C allele (P=0.368) (Figure 3C). Two-way ANOVA documented the substantial effects of MEF2 and its dominant-negative mutant on TH transcription (F=11.931, P=0.001).

TH C-824T Polymorphism: Role of Transcription Factors FOXD1 and SRY

Sequence Homology and Alignment
C-824T also displayed partial consensus matches for transcription factors FOXD1 and SRY (Figure 3A). At FOXD1, the T allele was a better match than C (6/8 bp versus 5/8 bp), whereas at SRY, the C allele displayed a better match than T (5/6 bp versus 4/6 bp) (Figure 3A).

Exogenous (Transfected) Factors
When cotransfected with FOXD1 into PC12 cells, the T allele was substantially stimulated (1.94-fold; P=0.0056), whereas the C allele had little response (1.12-fold; P=0.52). When cotransfected with SRY, the C allele displayed only a slightly greater response (1.57-fold; P=0.019) than the T allele (1.50-fold; P=0.054) (Figure 4A). Two-way ANOVA showed that both FOXD1 (F=11.369; P=0.006) and SRY (F=22.183; P=0.001) had significant effects on TH promoter transcription (Figure 4A).

Endogenous Factors (by ChIP)
During ChIP, both the C and the T alleles could be captured by anti-FOXD1 and anti-SRY antibodies (Figure 4B), with little difference between the 2 alleles in quantity of PCR product.

TH A-581G Polymorphism: Effect of Transcription Factors SP1, AP2, and EGR1

Sequence Homology and Alignment
TH A-581G is located in a conserved G/C-rich region, CCCCAC/G/A[GGGCG, with the G allele ancestral in the primate lineage. Computationally, the G allele is predicted to exhibit enhanced binding to transcription factors SP1, AP2, and EGR1 (Figure 5A).

Endogenous Factors (by ChIP)
During ChIP, each transcription factor (SP1, AP2, and EGR1) on the target promoter could be captured by specific antibodies, with a greater signal than IgG control serum, although the 2 alleles did not differ substantially in binding (Figure 5B).

Exogenous (Transfected) Factors
Each of the 3 transcription factors (SP1, AP2, and EGR1) increased gene expression, with a greater response for the G than for the A allele (Figure 5C).
Presence of the Transfected TH Promoter Plasmid in the Chromatin Fraction of PC12 Cells

To explore whether the ChIP technique is appropriate for transfected plasmids, we studied the subcellular localization of the human TH promoter and luciferase reporter plasmids after transfection and expression in PC12 cells. When different fractions were analyzed by PCR, using primers flanking the C-824T site, the plasmid was localized to the P3 (chromatin) fraction of the cell (Figure 6); the plasmid was not observed in either the cytosolic or the soluble nuclear fractions of the cell.

Discussion

Overview

Human TH represents a rate-limiting point for control of catecholamine biosynthesis and potentially BP. Our previous work showed that TH promoter common haplotypes (across C-824T→G-801C→A-581G→G-494A) exhibited pleiotropic effects on autonomic function, increasing both norepinephrine excretion and BP during stress.2 In those studies, individual variations at C-824T and A-581G were especially predictive of autonomic function, with the (minor) -824T and -581G alleles associated with increased catecholamine secretion and stress BP increments in twin pairs.2 In this study, we explored how common genetic variation in the TH proximal promoter influences gene expression. We present evidence from several approaches (bioinformatic, expression activity, and binding) in which we found that both C-824T and A-581G alleles associated with increased catecholamine secretion and stress BP increments in twin pairs.3 In this study, we explored how common genetic variation in the TH proximal promoter influences gene expression. We present evidence from several approaches (bioinformatic, expression activity, and binding) in which we found that both C-824T and A-581G conferred functional changes in the TH promoter and that particular transcription factors were implicated (at C-824T, MEF2, FOXD1, and SRY; at A-581G, G/C-rich domain recognition factors SP1, AP2, and EGR1).
TH Promoter Variant C-824T

T Allele With Increased Transcriptional Activity

In the previous clinical study, we discovered that the TH promoter C-824T minor (T) allele was associated with both higher urinary catecholamine excretion and greater changes in BP response to cold stress. We used transfected TH promoter haplotype and luciferase reporters to establish that T allele confers increased transcriptional activity in cella (1.36-fold greater than the C allele; P = 0.0082; haplotypes-2 and -8). In addition, the T allele displayed an augmented response to typical chromaffin cell secretory stimuli, such as nicotine or PACAP (T versus C for nicotine, 2.59-fold versus 2.07-fold, haplotypes-13 and -1; T versus C for PACAP, 39.7-fold versus 24.8-fold, haplotypes-2 and -8).

Role of MEF2: Endogenous and Exogenous

The T allele was predicted to have increased binding to transcription factor MEF2, and this increment was confirmed by ChIP, indicating that endogenous MEF2 can interact with the motif. Cotransfection of MEF2 yielded a greater increase in TH promoter and luciferase reporter activity in the T than in the C versions, and cotransfected dominant-negative mMEF2 decreased TH promoter and luciferase reporter activity at the T but not the C version. Each of these in cella results suggests a differential role for MEF2 at the T allele in vivo.

C-824T is located in an A/T-rich region constituting a binding motif for the transcription factor MEF2. The MEF2/MADS family of transcription factors are widespread in expression and involved in the regulation of multiple cellular processes, including muscle differentiation, immune

Figure 4. TH promoter variant C-824T: FOXD1 and SRY motifs. A, Exogenous factor cotransfection of an expression plasmid for FOXD1 or SRY. The C allele (CGAA) is from haplotype-1, and the T allele (TGAA) is from haplotype-13 (supplemental Table II). Results are shown as fold of basal (cotransfection with empty pCMV vector). B, Endogenous FOXD1 or SRY by ChIP. The C allele (CGAA) is from haplotype-1, and the T allele (TGAA) is from haplotype-13 (supplemental Table II).
cell apoptosis, and the survival of neurons. Preferential activation of the T allele by MEF2 in cella may begin to explain the exaggerated in vivo effects of -824T on catecholamine secretion and BP in the population.

**Role of SRY: Endogenous and Exogenous**

SRY, a transcription factor from the high-mobility group family, initiates male sex determination in mammals. Previous reports have indicated that an SRY expression plasmid trans-activated a TH promoter/reporter gene, although it was not clear whether the effect was direct. SRY gene delivery to the rodent adrenal medulla increased not only TH enzymatic activity, but also plasma norepinephrine and ultimately BP. This finding, coupled with interaction of SRY with the TH promoter, implicates the SRY allele as a hypertensive component of the spontaneously hypertensive rat Y chromosome. Our data showing that SRY binds to the C-824T motif and increases TH transcription suggest that SRY may play a role in maintenance of catecholamine synthesis, and hence BP, and thus might contribute to well-documented male and female sex differences in population BP.

**Role of FOXD1: Endogenous and Exogenous**

FOXD1 (also known as FREAC4 or FKHL8) is a member of the forkhead/winged helix family of transcription factors, which are characterized by a conserved monomeric DNA-binding domain called the winged helix. Although FOXD1 has been implicated in neuronal induction and differentiation, to our knowledge this is the initial report about its potential role in control of catecholamine biosynthesis.

**Multiple Factors**

At C-824T, we found that the interaction of multiple factors (MEF2, SRY, and FOXD1) with their target motifs was likely to be disrupted by the polymorphic base; the evidence arose from bioinformatic, functional, and binding analyses. How such factors might interact at the motif in vivo, whether additively or synergistically, remains unexplored.

**TH Promoter A-581G**

**Transcriptional Activity**

In the previous clinical study, we found that the -581G allele predicted increased catecholamine secretion and stress BP changes in twin pairs. We found that the G allele promoted increased basal gene expression (luciferase activity) in cella, with augmented responses to the secretory stimuli nicotine and PACAP.
Role of G/C-Rich Binding Factors

A-581G occurred in a relatively G/C-rich region, which presented the potential for binding by such transcription factors as SP1,23 AP2,24 or EGR1.25 We found that cotransfection of SP1, AP2, or EGR1 preferentially trans-activated the G allele. The effects of endogenous SP1, AP2, and EGR1 in cella were captured by ChIP. How might changes in the effects of such factors influence human catecholaminergic responses? EGR1, a transcription factor with 3 zinc fingers of the Cys2His2 class,26 is rapidly induced by stimuli modeling stress effects in experimental animal models,27,28 thereby altering transcription of genes encoding the catecholamine biosynthetic enzymes TH,29 phenylethanolamine N-methyltransferase,30,31 and dopamine β-hydroxylase.32 To our knowledge, this study represents an initial finding that naturally occurring genetic polymorphism in the catecholaminergic pathway may alter the binding patterns of G/C-rich motif recognition factors, such as EGR1, to affect autonomic responses in human populations.

Conclusions and Perspectives

We conclude that TH promoter common polymorphisms C-824T and A-581G are not only statistical predictors of catecholamine secretory and stress BP traits in vivo,2 but also causally responsible for alterations in transcriptional efficiency of the TH gene. These conclusions emerge from a convergence of computational and experimental approaches in which experiments documented the effects of particular factors, both endogenous and exogenous. The pleiotropic effects of the 2 polymorphisms seem to arise from differential actions of specific transcription factors at the TH promoter: MEF2, SRY, and FOXD1 at C-824T and SP1, AP2, EGR1 at A-581G. The SRY effect on TH transcription suggests a mechanism whereby male and female sex may differ in sympathetic activity and, hence, BP. The results may augment our understanding of molecular events underlying interindividual variations in autonomic function and the genetic predisposition to such cardiovascular disease states as hypertension.

Sources of Funding

This study was supported by the Department of Veterans Affairs, National Institutes of Health.

Disclosures

None.

References


CLINICAL PERSPECTIVE

Stress exposure activates the sympathoneural system, resulting in catecholamine release mediating the fight-or-flight response. Chronic stress is associated with the development of numerous disorders, including cardiovascular diseases such as hypertension. Probing genetic factors underlying stress responses may be important for prevention and treatment of such disorders. Tyrosine hydroxylase is the rate-limiting enzyme in biosynthesis of catecholamines. We studied 2 common genetic variants in the promoter (transcriptional control) region of the tyrosine hydroxylase gene. Changes in gene expression seemed to arise from differential actions of particular transcription factors at the 2 variable sites. The results may augment our understanding of molecular events underlying interindividual variations in autonomic function and the genetic predisposition to such cardiovascular disease states as hypertension.
Human Tyrosine Hydroxylase Natural Genetic Variation: Delineation of Functional Transcriptional Control Motifs Disrupted in the Proximal Promoter
Kuixing Zhang, Lian Zhang, Fangwen Rao, Bhawanjit Brar, Juan L. Rodriguez-Flores, Laurent Taupenot and Daniel T. O’Connor

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SUPPLEMENTAL MATERIAL

On-line supplementary Table1. Haplotype distribution in the tyrosine hydroxylase (TH) promoter region among four human populations. TH promoter haplotypes were imputed by PHASE using all SNPs (2n=586 chromosomes/n=293 individuals). Data are taken from SNP discovery by resequencing unrelated individuals of four ethnicities (east Asian, 2n=60 chromosomes; black, 2n=78; Hispanic, 2n=70; white, 2n=378). The 4 most common (minor allele frequency >10%) SNPs (C-824T, G-801C, A-581G, G-494A) are indicated in bold type. Haplotype 9 possesses the major (most frequent) human allele at each position (though it comprises only ~5.3% of human chromosomes). Haplotype 8 matches the non-human primate alleles at the 4 most common SNPs (CGGG). Promoter bp positions are numbered upstream (-) of the translation initiation codon (ATG). The 3 non-human primates were homozygous at each position. This information is also available from the on-line table of: Rao F, Zhang L, Wessel J, Zhang K, Wen G, Kennedy BP, Rana BK, Das M, Rodriguez-Flores JL, Smith DW, Cadman PE, Salem RM, Mahata SK, Schork NJ, Taupenot L, Ziegler MG, O’Connor DT. Tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis: discovery of common human genetic variants governing transcription, autonomic activity, and blood pressure in vivo. Circulation. 2007;116:993-1006.
<table>
<thead>
<tr>
<th>Promoter haplotype number</th>
<th>Nucleotide at bp position (upstream of ATG):</th>
<th>Frequency (2n=60)</th>
<th>2n=78</th>
<th>2n=70</th>
<th>2n=378</th>
<th>2n=586</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C T G G T A A A C G</td>
<td>0.617</td>
<td>0.468</td>
<td>0.430</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C C G G T A G G C G</td>
<td>0.100 (6)</td>
<td>0.346 (27)</td>
<td>0.300 (21)</td>
<td>(122)</td>
<td>(176)</td>
</tr>
<tr>
<td>3</td>
<td>C C G C T A A G C G</td>
<td>0.117 (7)</td>
<td>0.077 (6)</td>
<td>0.171 (12)</td>
<td>0.116 (44)</td>
<td>0.118 (69)</td>
</tr>
<tr>
<td>4</td>
<td>C C G G T A G A C G</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.008 (3)</td>
<td>0.005 (3)</td>
</tr>
<tr>
<td>5</td>
<td>C T G G T A G G T G</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.003 (1)</td>
<td>0.002 (1)</td>
</tr>
<tr>
<td>6</td>
<td>C C A G T A A A C C G</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.003 (1)</td>
<td>0.002 (1)</td>
</tr>
<tr>
<td>7</td>
<td>T T G G T A G G C G</td>
<td>0.000 (0)</td>
<td>0.090 (7)</td>
<td>0.000 (0)</td>
<td>0.003 (1)</td>
<td>0.014 (8)</td>
</tr>
<tr>
<td>8</td>
<td>C C G G T A A G G C G</td>
<td>0.017 (1)</td>
<td>0.090 (7)</td>
<td>0.000 (0)</td>
<td>0.019 (7)</td>
<td>0.026 (15)</td>
</tr>
<tr>
<td>9</td>
<td>C C G G T A A G C G</td>
<td>0.067 (4)</td>
<td>0.064 (5)</td>
<td>0.086 (6)</td>
<td>0.042 (16)</td>
<td>0.053 (31)</td>
</tr>
<tr>
<td>10</td>
<td>C T G G T G A G C G</td>
<td>0.000 (0)</td>
<td>0.026 (2)</td>
<td>0.029 (2)</td>
<td>0.000 (0)</td>
<td>0.007 (4)</td>
</tr>
<tr>
<td>11</td>
<td>C T G G T A A A C C G</td>
<td>0.000 (0)</td>
<td>0.064 (5)</td>
<td>0.029 (2)</td>
<td>0.008 (3)</td>
<td>0.017 (10)</td>
</tr>
<tr>
<td>12</td>
<td>C T G G T G A G C G</td>
<td>0.000 (0)</td>
<td>0.038 (3)</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.005 (3)</td>
</tr>
<tr>
<td>13</td>
<td>C T G G T A A A C C G</td>
<td>0.083 (5)</td>
<td>0.013 (1)</td>
<td>0.014 (1)</td>
<td>0.005 (2)</td>
<td>0.015 (9)</td>
</tr>
<tr>
<td>14</td>
<td>C T G C T A A A C C G</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.003 (1)</td>
<td>0.002 (1)</td>
</tr>
<tr>
<td>15</td>
<td>C C G C T A A A C G</td>
<td>0.000 (0)</td>
<td>0.013 (1)</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.002 (1)</td>
</tr>
<tr>
<td>16</td>
<td>C T G G C A G C G</td>
<td>0.000 (0)</td>
<td>0.013 (1)</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.002 (1)</td>
</tr>
<tr>
<td>17</td>
<td>C T G G C A G G C T</td>
<td>0.000 (0)</td>
<td>0.013 (1)</td>
<td>0.000 (0)</td>
<td>0.000 (0)</td>
<td>0.002 (1)</td>
</tr>
<tr>
<td>Chimp</td>
<td>C C G G C A G G C G</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gorilla</td>
<td>T C G G C A G G C G</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Orangutan</td>
<td>C C G G C A G G C G</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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On-line supplementary Table 2: Common 4-SNP haplotypes studied in functional characterization of TH promoter common variants C-824T and A-581G. The 4 common SNPs (C-824T, G-801C, A-581G and G-494A) are in bold type. A single base change (underlined) was made to create the artificial haplotype 1M. Red indicates the SNP position to be balanced. Italic is the position of single base change.

2A: C-824T. SNP C-824T is the only difference between haplotypes 1 and 13, and haplotypes 8 and 2.

<table>
<thead>
<tr>
<th>Promoter haplotype number</th>
<th>4-SNP haplotype</th>
<th>Nucleotide at bp position (upstream of ATG):</th>
<th>Frequency (2n=no. of chromosomes) in that population:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-833</td>
<td>-824</td>
</tr>
<tr>
<td>1</td>
<td>CGAA</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>13</td>
<td>TGAA</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>8</td>
<td>CGGG</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>2</td>
<td>TGGG</td>
<td>C</td>
<td>T</td>
</tr>
</tbody>
</table>

2B: A-581G. SNP A-581G is the only difference between haplotypes 1 and 1M (mutant), and haplotypes 11 and 2.

<table>
<thead>
<tr>
<th>Promoter haplotype number</th>
<th>4 SNP haplotype</th>
<th>Nucleotide at bp position (upstream of ATG):</th>
<th>Frequency (2n=no. of chromosomes) in that population:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-833</td>
<td>-824</td>
</tr>
<tr>
<td>1</td>
<td>CGAA</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>1M</td>
<td>CGGA</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>11</td>
<td>TGAG</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>2</td>
<td>TGGG</td>
<td>C</td>
<td>T</td>
</tr>
</tbody>
</table>
**TH** promoter variant C-824T: Primate inter-species sequence homology

Human C-824   CACTGAGGACGTGCCTCTCTTTCAAAAGAAGGGGCCACAGGACCCCGAGGGAAG
Human -824T  CACTGAGGACGTGCCTCTCTTTAAAGAAGGGGCCACAGGACC-CGGAGGGAAG
Chimpanzee   CACTGAGGACGTGCCTCTCTTTCAAAGAAGGGGCCACAGGACCCGGAGGGAAG
Gorilla       CACTGAGGACGTGCCTCTCTTTCAAAGAAGGGGCCACAGGACC-ACCAGGGAAG
Orangutan     CACTGAGGACGTGCCTCTCTTTCAAAGAAGGGGCCACAGGACC-CCGAGGGAAG
Rhesus        CACTGAGGTACGTGCCTCTCTTTCAAAGAAGGGGCCACAGGACCTCCGAGGGAAG

******** * *** ******** ******************** * ********

**Bold:** Human C-824T variant
*: Identity across species (by Clustal-W)

On-line supplementary Figure 1: **TH** promoter variant C-824T: Inter-species primate sequence homology in the region of C-824T.