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Human dopamine *beta*-hydroxylase (*DBH*) regulatory polymorphism that influences enzymatic activity, autonomic function, and blood pressure

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Abstract

Rationale—Dopamine *beta*-hydroxylase (*DBH*) plays an essential role in catecholamine synthesis by converting dopamine into norepinephrine. Here we systematically investigated *DBH* polymorphisms associated with enzymatic activity as well as autonomic and BP/disease phenotypes *in vivo*.

Methods and Results—70 genetic variants were discovered at the locus; across ethnicities, much of the promoter was spanned by a 5' haplotype block, with a larger block spanning the promoter in whites than blacks. *DBH* secretion was predicted by genetic variants in the *DBH* promoter, rather than the amino acid coding region. The C allele of common promoter variant C-970T increased plasma *DBH* activity, epinephrine excretion, the heritable change in BP during environmental stress in twin pairs, and also predicted higher basal BP in three independent populations. Mutagenesis and expression studies with isolated/transfected *DBH* promoter/luciferase reporters in chromaffin cells indicated that variant C-970T was functional. C-970T partially disrupted consensus transcriptional motifs for *n-MYC* and *MEF-2*, and this variant affected not only basal expression, but also the response to *exogenous*/co-transfected *n-MYC* or *MEF-2*; during ChIP, these two *endogenous* factors interacted with the motif.

Conclusions—These results suggest that common *DBH* promoter variant C-970T plays a role in the pathogenesis of human essential hypertension: common genetic variation in the *DBH* promoter region seems to initiate a cascade of biochemical and physiological changes eventuating in alterations of basal BP. These observations suggest new molecular strategies for probing the pathophysiology, risk, and rational treatment of systemic hypertension.

Keywords

Dopamine *beta*-hydroxylase; polymorphism; hypertension

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INTRODUCTION

Dopamine beta-hydroxylase (DBH) catalyzes the oxidative hydroxylation of dopamine to norepinephrine. In the periphery, DBH is preferentially located in the adrenal medulla and synaptic vesicles of postganglionic sympathetic neurons(1). As a result of its secretory vesicular localization, DBH is released together with norepinephrine during synaptic transmitter release (2,3). Because of its secretion into the extracellular space, DBH occurs in the cerebrospinal fluid (CSF) and plasma. CSF DBH is released mainly from central noradrenergic neurons, while plasma DBH emerges from the sympathochromaffin system. The enzymatic activity of DBH in plasma or CSF corresponds to the level of DBH protein (4,5); such activity is relatively stable over time in same person, and family studies indicate that the effects of environmental factors such as stress and medications on DBH activity are small compared to genetic factors (6): variation in DBH activity in both plasma and CSF is highly heritable, and heredity accounts for over ~90% of human plasma DBH activity variation, or ~80% in central DBH activity variation(7). The ABO blood-group locus adjacent to *DBH* on chromosome 9q34 cosegregates with DBH activity(8,9), indicating that genetic variation in the local *DBH* region regulates DBH expression. Indeed, several polymorphisms at *DBH* associate with either plasma or CSF DBH activity (10-12); for example, a polymorphism in the promoter region of DBH (C-970T) statistically accounts for ~35-52% of variation in plasma DBH activity(12).

Norepinephrine is the pivotal transmitter in the sympathetic nervous system, maintaining such functions as heart rate and BP. Excessive sympathoadrenal activity is implicated in the pathogenesis of hypertension, both primary (genetic, essential) and secondary (acquired), in both humans and experimental animals (13,14). Given its necessary role in converting dopamine to norepinephrine, as well as its hereditary determination, the potential role of DBH in hypertension attracts substantial attention. Several lines of evidence suggest that DBH may play a role in the pathogenesis of hypertension. Genetic deficiency of DBH manifests as hypotension in both humans and knockout mice(15,16). Of note for more common varieties of BP elevation, DBH inhibition attenuates development of hypertension in the spontaneously hypertensive rat (17,18).

However it is not established whether common human genetic variation at *DBH* can be translated into physiological significance, in particular for susceptibility to hypertension. We therefore conducted systematic polymorphism discovery at human *DBH*, and not only tested associations with human autonomic traits but also probed the functional consequences of key genetic variants *in cella*.

METHODS

Populations and phenotypic characterization

UCSD twin pairs—432 white (European ancestry) twin subjects, aged 15-84 years (median, 40 years), were studied: 69% monozygotic (MZ) and 31% dizygotic (DZ). Twin zygosity was confirmed by single nucleotide and microsatellite polymorphisms, as previously described (19-21). 9.9% of the twins are hypertensive (8.8% treated with antihypertensive medications). Subject characteristics (Suppl. Table 1) are defined as in previous reports (22). In the twin subjects, BP (in mmHg) and pulse interval (R-R interval or heart period, in ms/beat) were recorded continuously and non-invasively for 5 min in seated subjects with a radial artery applanation device and dedicated sensor hardware (Colin Pilot; Colin Instruments, San Antonio, TX) and software [ATLAS from WR Medical, Stillwater, MN; and Autonomic Nervous System/Tonometric Data Analysis (ANS/TDA) from Colin Instruments], calibrated every 5 min against ipsilateral brachial arterial pressure with a cuff sphygmomanometer. Heart rate was recorded continuously with thoracic EKG electrodes to

the Colin Pilot. Vital signs were also recorded with the same devices during environmental stress, in the form of the cold pressor test (CPT; immersion of the left hand in ice water for 60 sec after a preceding 10-min rest), as previously described (23). We identified at least three beats with stable BP and heart rate (each beat within 10% of the mean value) just before and at the end of the cold stress. Baroreceptor slope in the time domain was measured in response to both low pressure and high pressure spontaneous stimuli, as described (24).

Black BP extremes (Nigeria)—Samples from African subjects with the most extreme (upper and lower quartile) BPs in the population were kindly provided by Richard Cooper from Loyola University, Chicago. The subjects are from Nigeria, Africa, one of the cohorts in the International Collaborative Study on Hypertension in Blacks, as described (Suppl. Table 2) (25). The samples consist of men and women in the most extreme (upper and lower) quartiles (25th %iles) for systolic BP (SBP), adjusted by age and sex. SBP in the higher BP group was 177.2 ± 1.7 , and 101.2 ± 0.7 in the lower BP group.

San Diego BP unrelated individuals—978 individuals of several biogeographic ancestries were studied: 611 normotensive and 367 hypertensive. 62% of individuals with hypertension took antihypertensive drugs: 43.6% on one antihypertensive medicine, with the remaining 18.4% on 2-6 types of antihypertensive medicines. Since epidemiologic data on blood pressure (BP) may be compromised by the effects of antihypertensive medications (26,27), we accounted for the number of such drugs in the analysis; stepped increments of 8/4 mmHg, 14/10 mm Hg, or 20/16 mm Hg were added to measured SBP/DBP of treated subjects taking 1, 2, or 3 drug classes, respectively (26). Subject characteristics are defined in Suppl. Table 3.

Molecular genetics

Polymorphism discovery across the *DBH* locus in 4 population samples—We resequenced the *DBH* locus (exons, intron/exon borders, UTRs, and proximal promoter) for exhaustive variant discovery in $n=88$ subjects ($2n=176$ chromosomes). These individuals' ancestries were $n=25$ white (European ancestry), $n=25$ black (sub-Saharan African ancestry), $n=22$ east Asian and $n=16$ Hispanic (Mexican-American). Reference (RefSeq) sequences were obtained from the UCSC Genome Browser (<http://genome.ucsc.edu>). Promoter positions were numbered with respect to the mRNA cap (transcriptional initiation) site. Segments of *DBH* were amplified by PCR using primers designed by Primer 3.0(28) and the nucleotide sequence determined using standard reagents and a capillary sequencing instrument as described(29,30). Sequence was determined on an ABI 3100 automated sequencer and analyzed by Phred/Phrap/Consed (31,32). Polymorphism and heterozygosity were identified using PolyPhred(33) and manually confirmed as described (30). Rare variants were confirmed by re-sequencing in multiple individuals and from the reverse direction.

SNPs—Genotypes for twin samples and two cohorts with extreme subjects were scored on amplified DNA by extension based methods: mass spectrometry (Sequenom, La Jolla, CA), or Pyrosequencing (Uppsala, Sweden). To ensure accurate assignment, genotypes were verified by visual inspection and artifacts were excluded from further analysis.

Biochemical assays—Blood and urine samples were obtained from individuals after at least 3 hours of fasting. Blood was collected from seated individuals, with a heparin-lock IV in place, into either heparin tubes (to prepare plasma for DBH assay) or EDTA tubes (to prepare plasma for catecholamine assay). Anticoagulated blood was promptly chilled on ice (at 0°C) prior to centrifugation within one hour for preparation of plasma. In untimed urine specimens, analytes were normalized to endogenous creatinine concentration in the same

sample. Plasma DBH activity was measured by the spectrophotometric method in heparinized plasma (34). The principle of the method is as follows: the synthetic DBH substrate tyramine is converted by DBH (in the presence of Cu^{2+} , N-ethylmaleimide, and fumarate) to octopamine, which is then oxidized to parahydroxybenzaldehyde by sodium periodate. The oxidation is terminated by sodium metabisulfite, and parahydroxybenzaldehyde is then quantified by its absorbance at 330 nm in the ultraviolet. The DBH activity inter-assay coefficient of variation was 4.5%. Plasma and urine catecholamines were assayed by the radioenzymatic method (35). The catecholamine assay involved transfer of a ^3H label to catecholamines from S-adenosylmethionine during O-methylation, mediated by the enzyme catechol-O-methyltransferase (COMT). Prior to O-methylation, plasma catecholamines were extracted into dilute acetic acid to remove COMT inhibitors in plasma. Assay sensitivities (lower limits of detection) were 10 pg for norepinephrine and 6 pg for epinephrine. Intra-assay coefficients of variation were 4% for norepinephrine and 13% for epinephrine. Inter-assay coefficients of variation were 10% for norepinephrine and 13% for epinephrine.

Function of *DBH* promoter variants

Promoter/luciferase reporter activity assays—Human *DBH* promoter/reporter plasmids were constructed essentially as described (30). We obtained a human BAC genomic clone (RP11-317B10) spanning the entire *DBH* gene sequence from CHORI (<http://bacpac.chori.org>), from which we excised 3,051 bp of *DBH* promoter (from -3000 to +51 bp), containing common polymorphic sites, for insertion into the upstream region polylinker of the firefly luciferase reporter plasmid pGL3-Basic (Promega; Madison, WI). Synthetic replacements (at polymorphic sites) were made by site-directed mutagenesis (QuikChange; Stratagene) to produce the common haplotypes showed in Table 1. All inserts were sequence-verified before use. PC12 pheochromocytoma cells were transfected (at ~50-60% 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 (Herpes simplex virus thymidine kinase promoter driving Renilla luciferase; Promega; Madison, WI) as an internal control per well, by the liposome method (Superfect; Qiagen, Valencia, CA). The firefly and Renilla luciferase activities in the cell lysates were measured 16 hours after transfection, and results were expressed as the ratio of firefly/Renilla luciferase activities (“Stop & Glo®”; Promega, Madison, WI). Each experiment was repeated a minimum of three times.

Trans-activation of the *DBH* promoter by co-transfected MEF2, or n-MYC—The pCMV (pcDNA3.1) eukaryotic expression plasmid for human MEF2 (Myocyte Enhancer Factor 2) were obtained from Open Biosystems (<http://www.openbiosystems.com>), while the expression plasmid for n-MYC (oncogene) was obtained from Charles L. Sawyers (UCLA). 50 ng of each expression plasmid were co-transfected with *DBH* promoter/luciferase reporter plasmids (at 1 μg). In control (“Mock”) experiments, the empty expression vector was co-transfected with *DBH* promoter/luciferase reporter plasmids. Firefly luciferase activities in the cell lysates were measured 16 hours after transfection, and the results were expressed as firefly luciferase activity/cell protein, rather than to Renilla luciferase, since the co-transfected trans-activator plasmids differentially activated the *DBH* and TK promoters.

Chromatin ImmunoPrecipitation (ChIP) assays—ChIP assays were performed as described (36) with modifications, using a Millipore ChIP assay kit (catalog #17-295; Millipore, Temecula, CA). Nuclei from PC12 cells ($5\text{-}10 \times 10^6$) transfected with wild-type (C-970) versus variant (-970T) *DBH* promoter/reporter plasmids were crosslinked after 24 hours in 1% formaldehyde for 10 min and resuspended in lysis buffer. Chromatin was

sonicated to nucleosomes in a Branson Digital Sonifier (Branson Ultrasonics; Danbury, CT), with 15 repeated cycles of 20 sec sonication (on power setting 6/10), separated by 1 min on ice. At the conclusion of sonication, a 1% agarose-TBE (tris-borate-EDTA) gel, stained by ethidium bromide, documented fragmentation of genomic DNA into the 300-1000 bp range. After chromatin sonication and centrifugation, samples were pre-cleared with salmon sperm DNA and protein A/agarose slurry, then incubated with anti-MEF2, anti-n-MYC or control (IgG) antibodies (Santa Cruz Biotechnology) at 4°C overnight. Immune complexes were captured with 40 µl of protein A and salmon sperm DNA slurry, washed extensively and eluted with 100 µl of buffer at room temperature. Eluates were pooled with 20 µl of 5 M NaCl and heated at 65°C overnight to reverse cross-links. DNA fragments were purified with QIAquick PCR purification kits (Qiagen). Human-specific *DBH* promoter primers surrounding C-970T were used for PCR amplification with 25-30 cycles (linear range); amplicons did not plateau in yield.

Statistical analyses—Descriptive statistics are reported as mean value \pm one SEM, unless otherwise noted. Haplotypes were estimated from unphased diploid genotypes by PHASE (37) or HAP (38), and coded in each individual by copy number of a particular haplotype per diploid genome (0, 1, or 2 copies). Pair-wise linkage disequilibrium (LD) between each common SNP and polymorphism was quantified as D' or r^2 by the GOLD (39) or Haploview (40) algorithms. One-way ANOVA (using post-hoc correction for multiple comparisons) was performed in SPSS to test the significance of *in vivo* association between SNPs or haplotypes with plasma DBH activity, BP, or *in vitro* reporter activity assays. Generalized estimating equations (GEE) in SAS (Statistical Analysis System) evaluated genetic effects on autonomic biochemistry and physiology traits in the twin study, taking into account intra-pair correlations (22). Genotypes and haplotypes were grouped by the number of copies of the particular allele or haplotype (0,1,2 copies). Univariate analysis within general linear models (i.e., ANOVA) in SPSS were conducted to evaluate the effects of genotype or haplotype on plasma DBH activity, BP or disease status. All analyses were adjusted by age and sex (as covariates). In studies including subjects with hypertension, analyses were adjusted for the effects of antihypertensive medications, as previously described (26). In the single study of subjects with multiple biogeographic ancestries (San Diego blood pressure unrelated individuals), analyses were adjusted by ethnicity (gauged by self-report). Stepwise linear regression was performed in SPSS (version 17.0) on individuals with both promoter sequence and plasma DBH information. Within a haplotype block, SNPSpD (SNP SPectral Decomposition) (41) was used to estimate the experiment-wide significance threshold required to maintain the Type I error rate at 5% or less.

Bioinformatics of variant promoter motifs: Phylogenetic footprinting—Human and other mammalian promoter reference sequences were obtained from ENSEMBL and aligned pair-wise using the Smith-Waterman Algorithm (42). Regions of the alignment with >50% human-rat conservation were scored on position-weight-matrices (PWMs) from JASPAR (43) and TRANSFAC (44) databases, using the ConSite interface <www.phylofoot.org/consite> (45). Motifs from the consensus sequences scoring >80% of maximum for the PWM were considered binding candidates. Details of the scoring function are described elsewhere (46). We then ranked binding candidates in decreasing order, at the top those with maximum difference in PWM score between wild-type and variant human motifs (with at least one allele-score meeting the above criteria). Top hits represent transcription factors that bind to motifs containing at least one allele, are conserved between human and rat, and whose predicted binding motif is altered by mutation and hence likely of functional consequence.

RESULTS

***DBH* systematic polymorphism discovery**

We resequenced 8,971 base pairs of *DBH*, including all coding and evolutionarily conserved regions, as well as ~3 kbp of proximal promoter, from 80 human subjects (2n=160 chromosomes) of diverse biogeographic ancestries (Suppl. Figure 1). To infer the ancestral alleles for each polymorphism, we also resequenced *DBH* from two chimpanzees and one bonobo. We identified 71 polymorphisms across the locus: 68 single nucleotide polymorphisms (SNPs), 2 insertion/deletions and 1 repeat (Suppl. Table 4). Eighteen *DBH* SNPs had minor allele frequencies greater than 5%. In the coding region, we found 8 non-synonymous SNPs (cSNPs), but only one (Ala172Thr) was common (minor allele frequency >5%), and none were in likely functional domains, e.g., Cys residues (that mediate inter-subunit disulfide bonds) (47), or Cu²⁺ cofactor-binding His-His or His-Xxx-His domains (48) (Suppl. Figure 2).

Minor alleles of the non-coding Ins/Del and repeat polymorphisms were also quite rare. We used all 18 common variants (minor allele frequency >5%) to construct haplotype blocks in Haploview, separately for resequenced white and black subjects. Haplotype “block 1” (containing the promoter) spanned a far longer range in whites than blacks, extending into exon-3 in whites (Figure 1A).

Figure 1B positions *DBH* variants in the promoter, with respect to known promoter elements; of note, the core proximal promoter (-210/-1 bp), containing several elements of documented importance (e.g., TATA, CRE, TPA response, API, E-boxes, homeodomains), was devoid of common variation.

***DBH* common genetic variants: Effects on plasma *DBH* enzymatic activity**

(Suppl. Figure 3). To determine which polymorphisms in *DBH* predict release of the enzyme into plasma, we studied 95 healthy subjects from two populations (41 African American [A-A] and 54 European American [E-A]), coupled with genotypes at 18 common polymorphic sites by direct sequencing. *DBH* activity in E-A was nearly two-fold higher than in A-A ($P<0.001$), consistent with previous reports stretching back for nearly 3 decades (34, 49). Several polymorphisms, including C-970T (suppl. Figure 3), showed significant association with *DBH* activity in both A-A and E-A by ANOVA. These SNPs are located in promoter region, except one in intron-1 (G+457C) and one in exon-2 (A+3628G) in strong linkage disequilibrium (LD) with promoter SNPs T-2734C, C-2073T and G+457C in E-A. All promoter SNPs lay within the most 5' haplotype block, both in Whites and Blacks (Figure 1A).

We tested whether *DBH* genetic variants can account for *DBH* activity differences between E-A and A-A; by linear regression, a model containing C-2073T and C-970T accounted for ~59.5% of trait variance, while addition of ethnicity increased R² only to ~67.6%.

***DBH* common promoter variants: Prediction of BP in several populations**

DBH resequencing identified common variants in the promoter region that formed a tightly linked block extending into intron-3 in white individuals, but ending within the promoter in blacks. Thus we began *DBH* marker-on-trait studies with a 7-SNP haplotype (6 in the promoter, one in intron-1) in white twins, while in blacks only 4 SNPs in the promoter region were used (Suppl. Table 5).

DBH C-970T in San Diego twin pairs (white)

We tested the association of promoter SNPs with biochemical and physiological autonomic phenotypes in a series of twins for which we have previously shown high heritability of such traits (22). Within the promoter haplotype block, C-970T was significantly associated with both pre- and post-SBP: the minor allele (T) that predicts lower DBH activity (Suppl. Figure 3) is associated with lower SBP both before and after environmental (cold) stress (Figure 2B). The T allele at C-970T also predicted decreased catecholamine excretion (Figure 2C). By SNPSpD (41) within the promoter block, the experiment-wide significance threshold required to maintain the Type I error rate at <5% remained 0.05 (reflecting the high degree of LD within this block); this threshold was exceeded by C-970T.

DBH C-970T in black (Nigeria) population BP extremes: Replication #1

Since the promoter haplotype block is shorter in blacks than whites (Figure 1A), we genotyped four common promoter SNPs to identify common haplotypes in this sample. In line with other populations, four common haplotypes are present in this extreme sample (Suppl. Table 5). We did not find an overall haplotype effect on BP, but when each SNP was analyzed separately, the T allele at C-970T predicted lower SBP and DBP (Suppl. Figure 4).

DBH C-970T in San Diego unrelated individuals: Replication #2

We typed C-970T in a mixed population of hypertensives and normotensives from San Diego (Suppl. Table 3), and once again found that the T allele predicted lower BP. Since subjects of 5 different biogeographic ancestries were included in this sample, we performed analyses with or without ethnicity as covariate; in each case, the T allele of C-970T predicted lower BP (Suppl. Figure 5).

Baroreceptor sensitivity as a function of DBH genotype

Since biochemical/physiological counter-regulatory responses to hypertension are mediated by baroreceptor/mechanoreceptor systems, we tested the effectiveness of the response to both low pressure and high-pressure stimuli as a function of *DBH* promoter genotype at C-970T (Suppl. Figure 6). Baroreceptor function was intact (at ~12-15 msec/mmHg), but did not differ as a function of *DBH* promoter genotype.

Function of DBH promoter variants (common haplotypes and C-970T)

Basal activity in chromaffin cells—We constructed luciferase reporter plasmids for the 4 most common naturally occurring *DBH* haplotypes (Suppl. Table 5), and then created 4 mutant haplotypes by altering position -970, from C→T or T→C, forming 4 pairs of haplotypes in which only difference occurs at -970. Since variant C-970T appeared to be crucial in influencing BP (Figure 2) and plasma DBH activity (Suppl. Figure 3), we then took advantage of mutant haplotypes to isolate the function of individual variants *in cella*. Higher gene expression resulted from the T allele at C-970T (Figure 3A), when tested upon balanced/matched haplotype backgrounds.

Role of promoter variant C-970T: Differential *trans*-activation—Analysis of the sequence immediately surrounding C-970T revealed partial matches to consensus *n-MYC* (4/6 bases; Figures 1B & 3B) and *MEF2* (5/10 bases; Figures 1B & 3C) motifs. Other sequenced mammals (chimpanzee, cow, rat, mouse) had the C-970 allele at the equivalent position of human C-970T, while a subset of humans displayed transition to -970T. To probe the significance of the putative *n-MYC* and *MEF2* motifs, we tested the effects of *n-MYC* or *MEF2* by co-transfection with *DBH* promoter/reporters bearing either the C-970 or the -970T allele. In each case, the effects were dependent upon haplotype context, with significant factor-by-haplotype interactions. As expected from the *n-MYC* motif match,

transfected n-MYC preferentially activated the T allele on 6H-1, 6H-3, and 6H-4 backgrounds, though not the 6H-2 background. As expected from the *MEF2* motif match, transfected/expressed MEF2 preferentially activated the T allele on a 6H-1 background, but not on 6H-3 or 6H-4 backgrounds (Figure 3C).

Endogenous transcription factor binding to C-970T: ChIP (Chromatin ImmunoPrecipitation)—After nucleosomal immunoprecipitation (Figure 3D), both anti-MEF2 and anti-n-MYC yielded specific DNA amplicons spanning C-970T; at MEF2, the semiquantitative pattern (T allele > C allele) was consistent with the functional results for the most common *DBH* haplotype (6H-1; Figure 3C), while for n-MYC the C>T pattern was consistent with luciferase results for *DBH* common haplotype 6H-2 (Figure 3B).

DISCUSSION

Overview

DBH encodes an indispensable enzyme in the catecholamine biosynthetic chain, resulting in the formation of norepinephrine. In this report we approach the effect of common variation in the *DBH* promoter region for *DBH* expression and activity, catecholamine secretion, autonomic physiology, and finally for cardiovascular disease (hypertension) risk.

We began with systematic variant discovery across the locus in 4 biogeographic ancestry groups, and found that *DBH* promoter variants lie within a linkage disequilibrium (LD) block, whose extent varied by ethnicity. Promoter variants predicted the very proximate trait of plasma *DBH* activity. In twin pairs, *DBH* promoter variant C-970T influenced heritable “intermediate phenotypes” for later development of hypertension: the proximate biochemical trait of urine epinephrine excretion, and the more distant physiological risk trait of BP response to environmental (cold) stress. Finally, *DBH* promoter variant C-970T associated with BP in three different populations.

Transfected *DBH* promoter haplotypes indicated a functional role for variant C-970T, which disrupted known transcriptional regulatory motifs, and *trans*-activation of the variant by the known factors resulted in differential activation of the two alleles.

***DBH* C-970T contribution to blood pressure: Multiple lines of evidence**—First, we used both haplotypes and individual variants in marker-on-trait association analyses. Haplotypes are a useful tool for scanning large genomic regions in the search for disease-predisposition variants (50). Once a trait-contributory genetic locus (or block) has been identified, systematic variant discovery may then yield the responsible polymorphism. During the initial hypertension associations, the haplotypes as well as single SNP C-970T were associated with basal systolic BP in the twin group. However in the replicate population groups, only SNP C-970T (though not more extended promoter haplotypes) predicted BP, perhaps reflecting “dilution” of the effects of the functional variant (C-970T) by the larger genomic region captured by LD.

Second, we investigated the hemodynamic response to environmental (cold) stress as an “intermediate phenotype” in the analyses. In a complex trait (51) such as hypertension, where there are heritable effects but the genetic architecture of the trait is largely unknown, genetic approaches focused only on the ultimate disease trait (either family/pedigree linkage or case/control association) might have limited statistical power. In a setting of late/delayed penetrance of the ultimate disease trait (such as hypertension), as well as likely genetic heterogeneity, the “intermediate” phenotype (52) strategy may be a useful in the search for disease predisposition loci, where such traits may be “intermediate” in both a temporal and a mechanistic sense. The hemodynamic response to environmental (cold) stress is a predictor

of the development of later cardiovascular events, such as hypertension (53-57), rendering it useful even prior to the onset of overt disease (52,58,59). Here we found that *DBH* C-970T predicted two such heritable traits in predominantly normotensive twin pairs: catecholamine secretion and environmental stress BP change (22).

Third, we conducted *in cella* functional studies to confirm differential effects of the C-970T alleles on *DBH* expression. Expression of *DBH* promoter variants on a variety of haplotypic backgrounds, both natural and artificial, indicated that C-970T exerted functional changes upon reporter activity. Follow-up experiments indicated that this variant harbored partial consensus matches for two transcriptional control motifs: MYC and MEF-2. Context/haplotype background-dependence was observed for *trans*-activation by either n-MYC or MEF-2 at C-970T. Finally, ChIP demonstrated interactions of *endogenous* MYC and MEF-2 with the C-970T region in nucleosomes. Taken together, these complementary results indicate that particular transcriptional motifs function differentially at C-970T.

Mechanisms *in cella* versus those *in vivo*—We were initially surprised to note that the *DBH* haplotypes and individual variants tested had directionally *opposite* effects on transcription *in cella* (reporter gene activity in transfected cells) and plasma DBH activity *in vivo*. However, norepinephrine has very different actions on BP *centrally* and *peripherally*. Indeed, such actions are *reciprocal*: while peripherally (e.g., intravenously) administered norepinephrine elevates BP, centrally administered norepinephrine acts in the vasomotor center of the brainstem to decrease sympathetic outflow and hence BP (Figure 4).

Alternatively, negative feedback mechanisms can be proposed to explain the contrasting results *in vivo* and *in cella*. After exocytosis, norepinephrine acts via presynaptic α_2 -receptors on sympathetic termini, both decreasing catecholamine biosynthesis and inhibiting vesicle exocytosis. Blood pressure elevation by catecholamines also activates the baroreceptor mechanism, thereby diminishing efferent sympathetic activity and consequently decreasing vesicle exocytosis. We have previously reported a similar effect for the catecholamine release-inhibitory catestatin region of *CHGA*, for which we proposed reciprocal central and peripheral actions on BP (30).

Conclusions and perspectives

Our data suggest that common genetic variation in the promoter region of *DBH*, a necessary enzyme in catecholamine biosynthesis, confers a series of changes in autonomic traits (Figure 4): *DBH* expression and catecholamine secretion, heritable environmental stress-induced BP increments, and ultimately risk for hypertension. Studies with the isolated *DBH* promoter established the role of particular *cis/trans*-interactions in the nucleus, especially at promoter position C-970T. Since this promoter variant is so common (indeed, trait-elevating C allele is the major/most frequent allele in each population studied), our observations are consistent with the “common disease/common allele” hypothesis for frequent traits in the population (60), and suggest new molecular strategies for probing the pathophysiology, risk, and rational treatment of hypertension.

Since catecholamine biosynthesis proceeds by a sequence of reactions in series (61,62). Further analysis of genetic interactions between variants of *DBH* and other genes in the biosynthetic pathway may assist in defining contributions of such genes to susceptibility for hypertension.

Condensed abstract

We systematically probed dopamine *beta*-hydroxylase polymorphisms associated with enzymatic activity as well as autonomic and BP/disease phenotypes *in vivo*. *DBH*

secretion was predicted by genetic variants in the *DBH* promoter, rather than the coding region. The C allele of variant C-970T increased plasma DBH activity, epinephrine excretion, the heritable change in BP during environmental stress in twin pairs, and also predicted higher basal BP in three independent populations. Mutagenesis and expression studies with isolated/transfected *DBH* promoter/luciferase reporters in chromaffin cells indicated that variant C-970T was functional. C-970T partially disrupted consensus transcriptional motifs for *n-MYC* and *MEF-2*, and this variant affected not only basal expression, but also the response to exogenous/co-transfected *n-MYC* or *MEF-2*; during ChIP, these two endogenous factors interacted with the motif. These results suggest that *DBH* variant C-970T may play a role in the pathogenesis of human essential hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations (alphabetical)

A-A	African American
BP	Blood Pressure
ChIP	CHromatin ImmunoPrecipitation
CPT	Cold Pressor Test
DBH	Dopamine Beta-Hydroxylase
DZ	Dizygotic twin
E-A	European American
GEE	Generalized Estimating Equations (in SAS)
LD	Linkage Disequilibrium
MZ	Monozygotic twin
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
SNPSpD	SNP SPectral Decomposition
QTL	Quantitative Trait Locus

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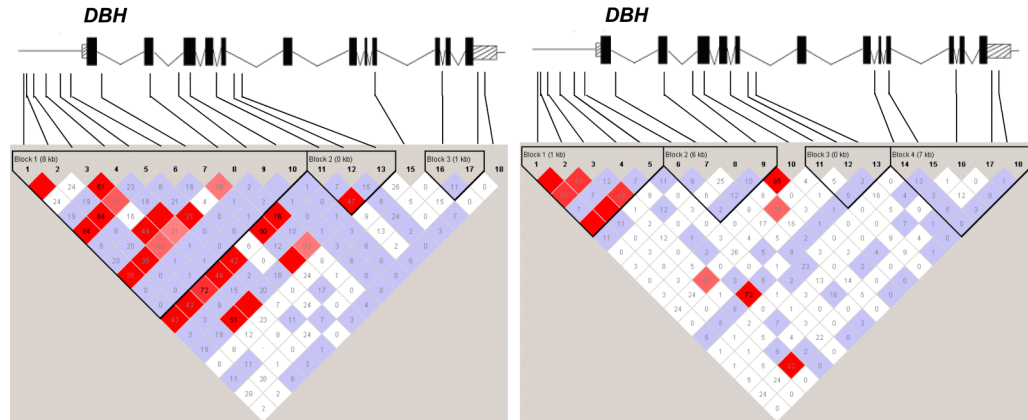
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DBH polymorphism discovery

Haplotype blocks at the *DBH* locus for American populations of European and African ancestry



White: 2n=46 chromosomes

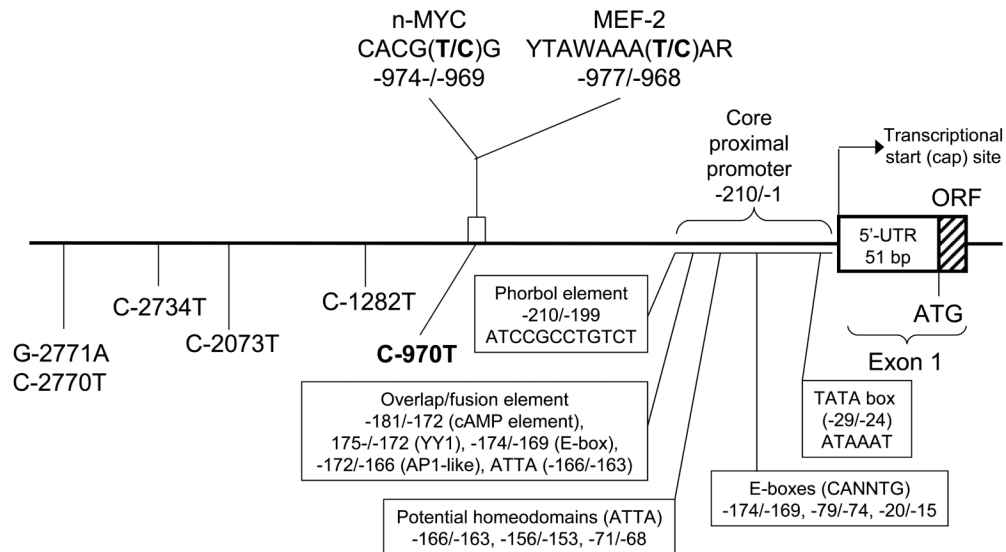
Black: 2n=50 chromosomes

Red: $D' > 1$, $LOD \geq 2$. Blue: $D' > 1$, $LOD < 2$. Pink: $D' < 1$, $LOD \geq 2$. White: $D' < 1$, $LOD < 2$.
Numbers in diagonals: $r^2 * 100$. LD blocks by solid spline method in Haploview.

A

DBH polymorphism discovery

Common genetic variation in the human *DBH* promoter



(Bold): Polymorphic base disrupting a motif

B

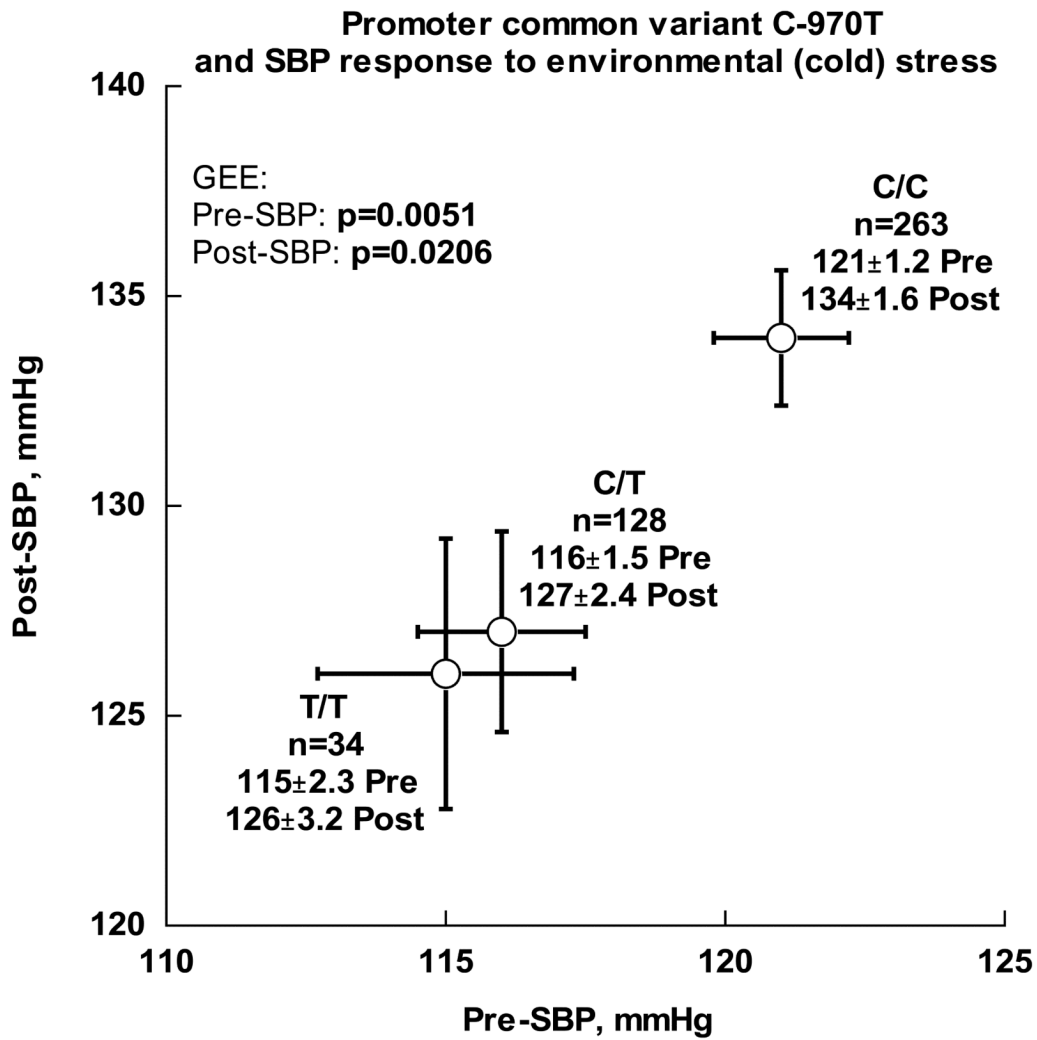
Figure 1. DBH polymorphism discovery

1A. Haplotype blocks at the *DBH* locus for American populations of European and African ancestry. Haplotype blocks in the *DBH* region for European American population (left) and African population (right). LD map and haplotype blocks were constructed by Haploview, using our resequencing data. The “solid spine” method was used to position haplotype block boundaries. The four-color scheme (from white, blue, pink, red) represents

the increased value of LD with maximum D' as one in bright red. Block-1 is longer in Whites than in Blacks, extending further into the coding region.

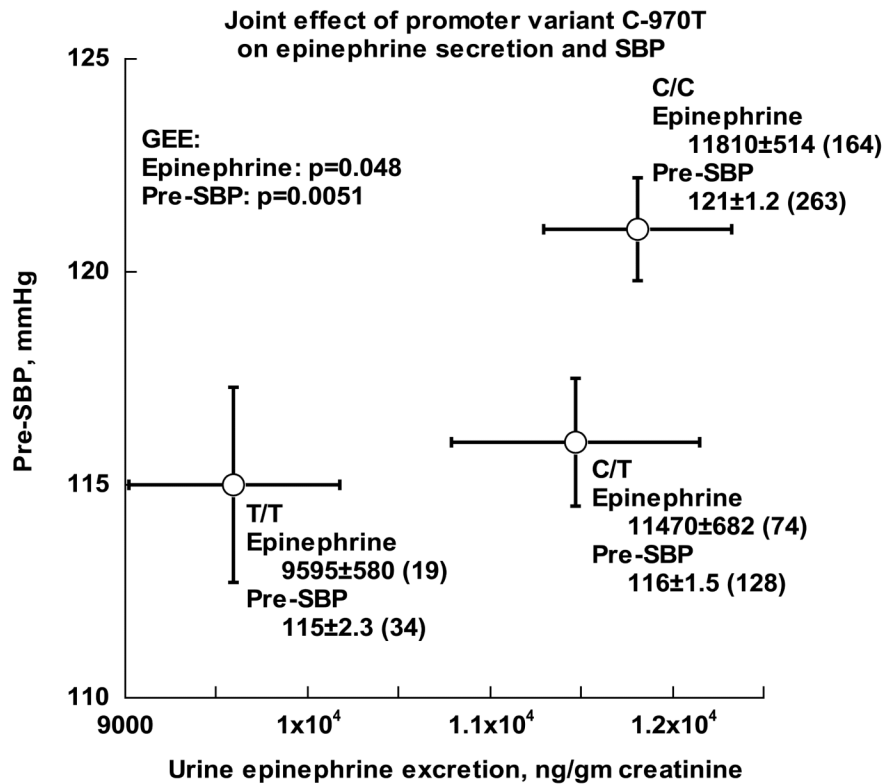
1B. Human *DBH* proximal promoter elements, common polymorphisms, and motifs altered by common genetic variants. Motifs altered by common variant C-970T were predicted by phylogenetic footprinting, as detailed in Methods. Elements within the core proximal promoter are assembled from several previous reports in the literature, rather than being identified in this report; of note, this very proximal region (-210/-1 bp) was invariant during our resequencing of $n=88$ subjects ($2n=176$ chromosomes). The short homeodomain 4-bp motif (ATTA) indicates *potential* homeodomain sites. Regions are not drawn to scale.

DBH promoter polymorphism: Effects on autonomic traits in San Diego twin pairs.



A

**DBH promoter polymorphism:
Effects on autonomic traits in San Diego twin pairs.**



B

Figure 2. DBH promoter variants: Effects on BP and catecholamines in San Diego twin pairs
Mean values \pm SEM are shown, calculated from all individuals as indicated in each bar (both members of each twin pairs, MZ and DZ) in that group, by GEE. Analyses were adjusted by age and sex. BP results were also adjusted for the effects of antihypertensive medications in the 8.8% treated for hypertension.

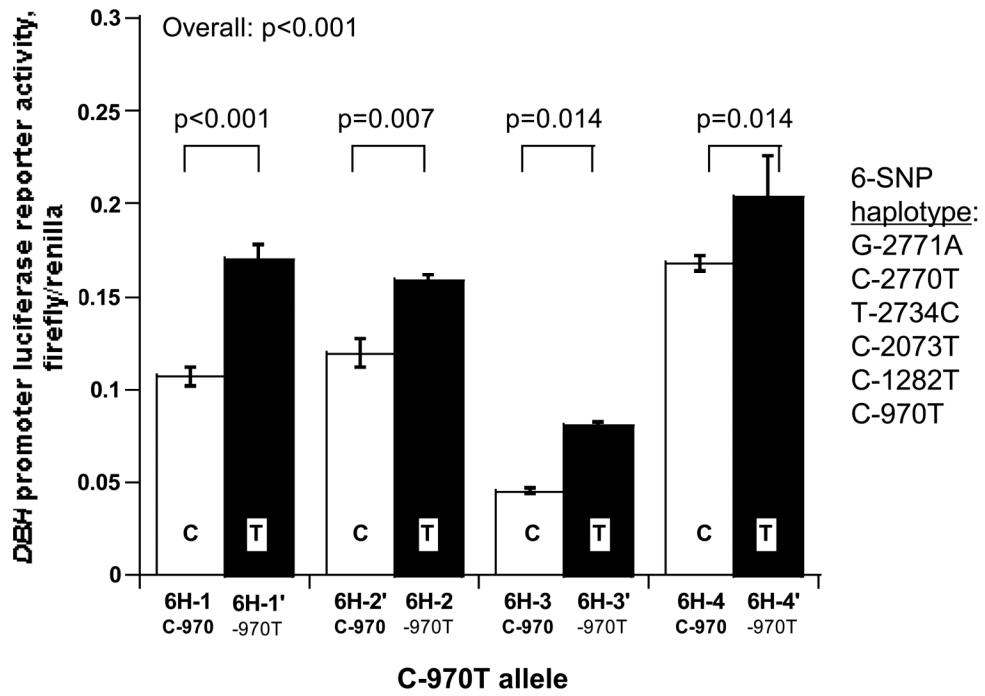
2A: Haplotype effects on basal/resting BP. 7-SNP haplotype-1 (suppl. Table 5B) is significantly associated with basal SBP.

2B: Stress-induced BP changes. Effects of C-970T genotype on both pre- and post-cold stress SBP in twins.

2C: Catecholamines. Joint effects of C-970T genotype on both urine epinephrine excretion and blood pressure.

DBH promoter haplotype/luciferase reporter activity *in cella*.

Promoter variant C-970T: Site-directed mutagenesis.

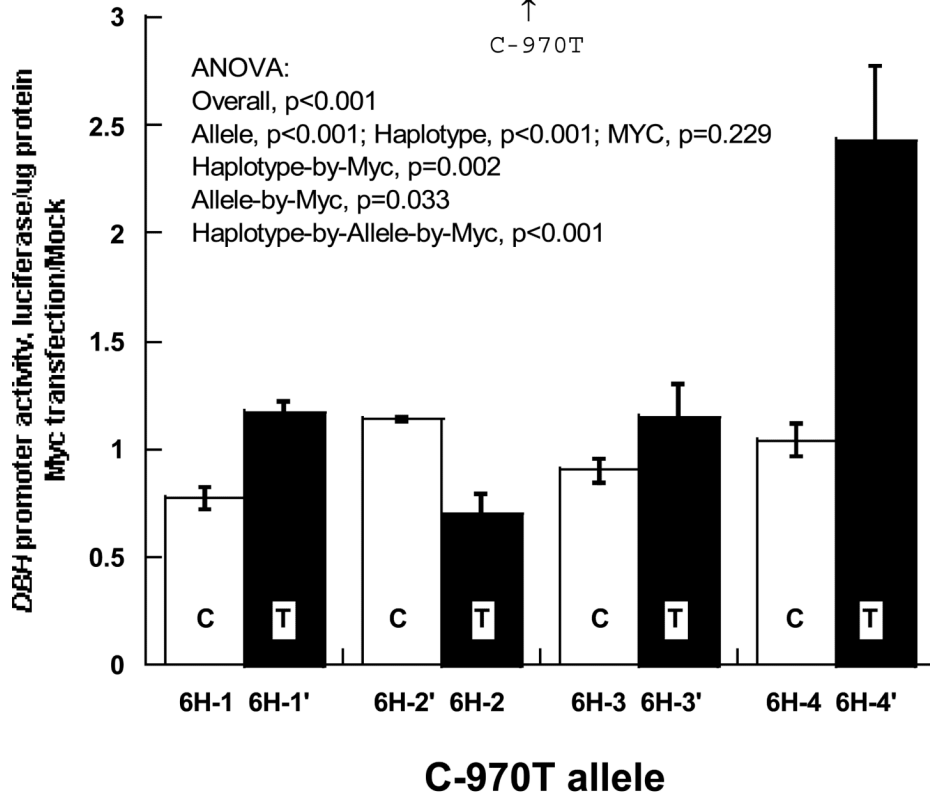


A

Trans-activation of *DBH* promoter variant C-970T *in cella*

Haplotype-dependent effect of n-MYC trans-activation.

<i>n-Myc</i> motif:	C	A	C	G	T	G	Match
-970T allele:	C	T	T	G	T	G	4/6
C-970 allele:	C	T	T	G	C	G	3/6
Chimp:	C	T	T	G	C	G	3/6
Rat:	C	T	T	G	C	G	3/6



6-SNP
haplotype:
 G-2771A
 C-2770T
 T-2734C
 C-2073T
 C-1282T
 C-970T

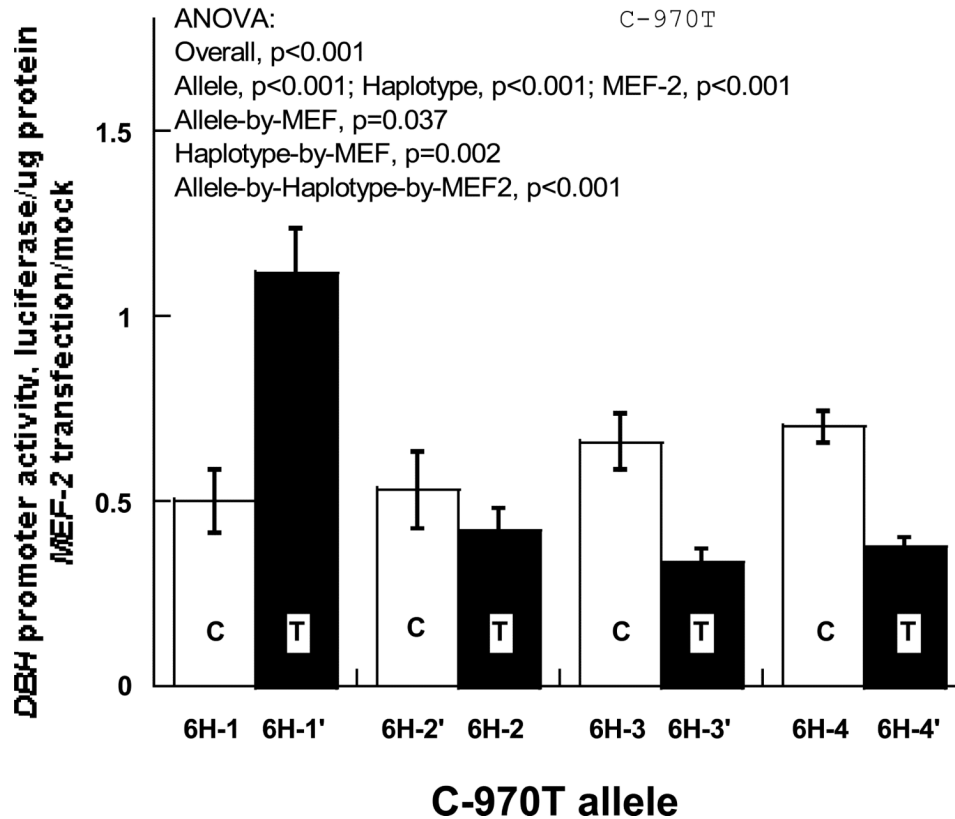
B

Trans-activation of *DBH* promoter variant C-970T *in cella*

Haplotype-dependent effect of *MEF-2* trans-activation.

<i>MEF-2</i> motif:	Y T A W A A A T A R	Match
-970T allele:	C T A C T T G T G G	5/10
C-970 allele:	C T A C T T G C G G	4/10
Chimp:	C T A C T T G C G G	4/10
Rat:	C T A C T T G C G G	4/10

↑
C-970T

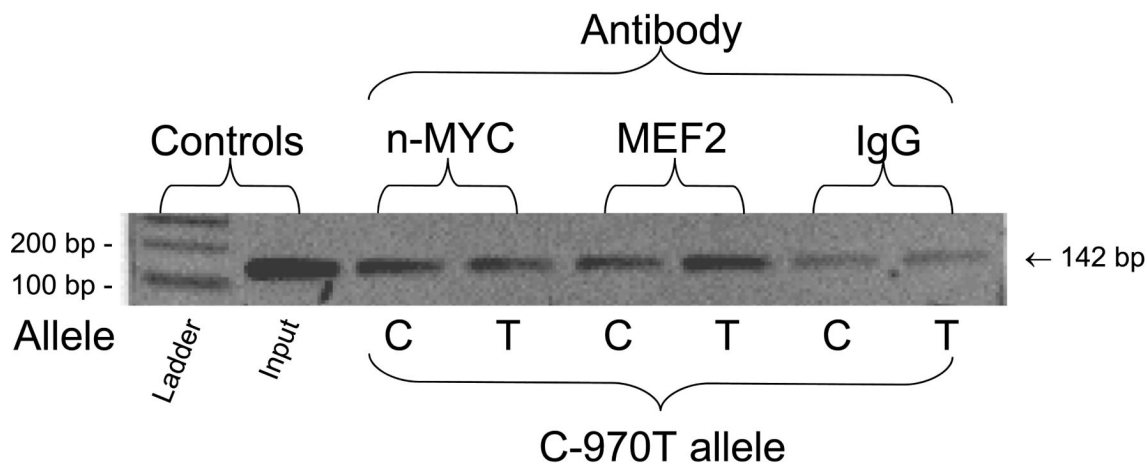


6-SNP
haplotype:
G-2771A
C-2770T
T-2734C
C-2073T
C-1282T
C-970T

C

Trans-activation of *DBH* promoter variant C-970T *in cella*

ChIP (Chromatin Immunoprecipitation) at C-970T.



D

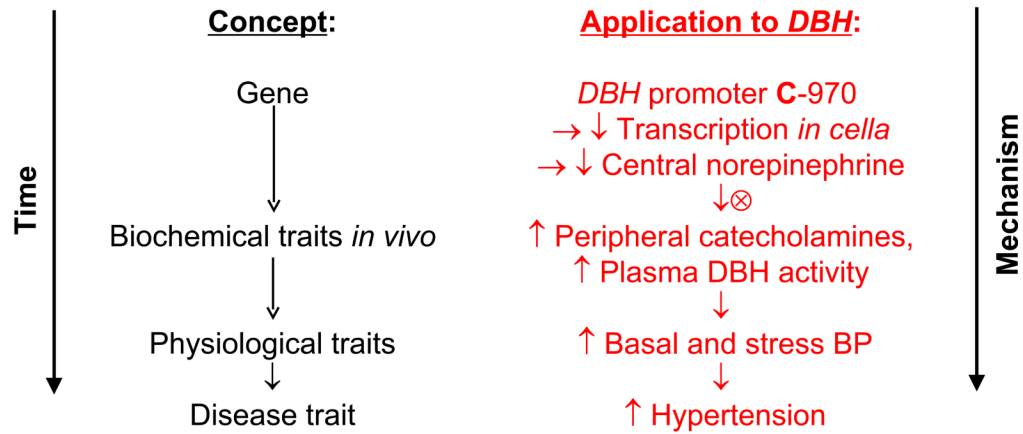
Figure 3. *DBH* promoter activity: Role of common variant C-970T

3A. Site-directed mutagenesis at C-970T. On 4 common natural haplotype backgrounds, C-970 or -970T were point-mutated to -970T or C-970, creating 4 non-natural/artificial haplotypes. Results are compared by ANOVA followed by post-hoc T-tests. These basal (unstimulated) activity results are presented as firefly luciferase activity normalized to Renilla activity in the same cell lysate. As a negative control, the ratio of firefly/renilla luciferase activities for the promoterless luciferase reporter vector (empty vector pGL3-Basic) co-transfected with transfection efficiency control plasmid Renilla plasmid (pTK-RL) was 0.008 ± 0.001 . The absolute firefly luminescence for haplotype-1 \rightarrow firefly luciferase transfection was 1334.7 ± 198.2 RLU/sec; 50 μ l from each 500 μ l cell lysate were used for luciferase assay. Each experiment was performed in triplicate, and such experiments were repeated at least three times.

3B. Trans-activation of C-970T by n-MYC. The *n-MYC* motif in the sequence is shown. Results are presented as fold-stimulation by exogenous/co-transfected n-MYC for each haplotype. Mock: Co-transfected n-MYC empty expression vector. The RLU/protein for haplotype-1 \rightarrow firefly luciferase transfection (without co-transfected n-MYC) was 9.98 ± 0.31 RLU/ μ g, with an absolute firefly RLU/sec of 346 ± 19.2 . 50 μ l of each 500 μ l cell lysate was used for luciferase assay.

3C, Trans-activation of C-970T by MEF-2. The *MEF-2* motif in the sequence is shown. Results are presented as fold over basal activity for each haplotype. Mock: Co-transfected MEF-2 empty expression vector. Basal RLU values were as reported in Figure 3A. **3D. ChIP (Chromatin ImmunoPrecipitation) at C-970T.** Results of nucleosomal immunoprecipitations from PC12 cells. “Input” DNA derives from the nucleosomal preparation prior to immunoprecipitation. Antibodies directed against MYC or MEF-2 (or pre-immune serum) were used to precipitate nucleosomes derived from PC12 cells transfected with either the C-970 or -970T alleles, on promoter/reporter plasmids. The nucleosomal immunoprecipitates were interrogated with PCR primers spanning a 142 bp amplicon centered upon C-970T.

Hypertension: “Intermediate” phenotypes and candidate genes. Application to catecholamine biosynthesis.



⊗: Inhibition or negative feedback
(or reciprocal central/peripheral effects of catecholamines on BP)

Figure 4. Human *DBH* genetic variation: Schema for effects on autonomic and disease traits Intermediate phenotype hypothesis for *DBH*

Framework for integration of the experimental results on *DBH* genetic variation, early/proximate autonomic traits, later disease traits (such as hypertension), and changes in *DBH* secretion.