High regulatability favors genetic selection in *SLC18A2*, a vesicular monoamine transporter essential for life

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ABSTRACT **_SLC18A2_ encodes the vesicular monoamine transporter 2 protein that regulates neurotransmission and reduces cytosolic toxicity of monoamines. Deletion of this gene causes lethality in mice, and DNA sequence variation in this gene is associated with alcoholism and Parkinson's disease, among other disorders. The Caucasian _SLC18A2_ promoter has at least 20 haplotypes (*A–T*), with *A* representing two-thirds of 1460 chromosomes. It is not known why *A* is selected in the human lineage. To understand the selection, here we took a functional approach by investigating the regulations of 4 representative haplotypes (*A*, *C*, *G*, and *T*) by 17 agents. We show that 76.5% of the agents were able to regulate *A* but only 11.8–23.5% of them regulated the 3 other infrequent ones, observing a positive correlation between haplotype frequency and regulatability. Pathway and molecular analyses revealed five signaling hubs that regulate the four haplotypes differentially, probably through targeting the polymorphic core promoter region. These findings suggest that greater diversity of transcriptional regulations is the driving force for the haplotype selection in _SLC18A2_.—Lin, Z., Zhao, Y., Chung, C. Y., Zhou, Y., Xiong, N., Glatt, C. E., Isacson, O. High regulatability favors genetic selection in _SLC18A2_, a vesicular monoamine transporter essential for life. _FASEB J._ 24, 2191–2200 (2010). www.fasebj.org**

Key Words: human · promoter haplotypes · evolution · signaling cascades · transcription

SLC18A2, THE HUMAN VESICULAR monoamine transporter 2 gene, is associated with a number of brain disorders, including alcoholism, Parkinson's disease (PD), and schizophrenia (1–6). Molecular studies have revealed that the low activity-associated haplotypes are risk factors for these diseases. Consistently, one copy deletion of the gene increases both alcohol consumption and dopaminergic vulnerability in +/− mice (7–9), demonstrating the contribution of reduced *SLC18A2* expression to alcoholism and PD. Interestingly, the *SLC18A2* promoter has at least 20 haplotypes (*A–T*, where *T* is newly identified in this study), and one of them, designated *A*, had a frequency of 66.7% in 730 unrelated Caucasians from 2 independent collections. *In vitro* analyses showed that a medium level of promoter activity was displayed by *A*, whereas higher activity by *C* and lower activity by *G* were observed consistently in different cellular systems. Although highly selected, *A* was among the disease-associated haplotypes (3, 4).

The vesicular monoamine transporter 2 (VMAT2) is an important molecule for the function of monoaminergic neurons that are key participants in locomotion, reward, working memory, and mnemonic brain systems. Acting to remove cytosolic monoamines [dopamine (DA), serotonin, norepinephrine, and histamine] by uptake into intracellular vesicles and to discharge the monoamines into extracellular space, VMAT2 prevents neurotoxicity of these monoamines in the cytosol and regulates neurotransmission (10). VMAT2 is expressed in central, peripheral, and enteric neurons as well as in platelets (11, 12). The expressional difference between different human brain regions can be >16-fold (13). Data from knockout mice indicate that expression of VMAT2 is essential for survival and that different expression levels have altered behavioral consequences. Homozygous (−/−) knockout mice survive only about 1 postnatal week because of developmental defects, which is in contrast to other monoamine transporters, for which homozygous deletions (−/−) cause no lethality (14). Nobably, environmental factors can regulate expression of the VMAT2 gene, including stress, clozapine, and environmental contaminants (15–17). Despite the small number of regulation studies available, these data all suggest that the VMAT2 gene is under sensitive physiological regulation.

By investigating haplotype-dependent regulation of *SLC18A2*, in this study, we took a functional analysis approach to understanding why *SLC18A2* promoter haplotype *A* is highly selected in the human lineage, whereas others are deselected. Elucidation of the biological drive for the selections may help our under-

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standing of the contributions of *SLC18A2* to human evolution and diseases.

MATERIALS AND METHODS

Haplotype-reporter hybrid constructions

SLC18A2 promoter region-luc (fruit fly luciferase gene) hybrids were constructed by shuttling 8.5-kb *Nhe*I/*Bam*HI fragments of plasmids pGL3e-hVMAT2–6.1A and pGL3e-hVMAT2–6.1C (3) into pGL4.14[*luc2*/Hygro] (Promega, Madison, WI, USA), resulting in 12.1-kb plasmids pSLC18A2–6.3A-luc and pSLC18A2–6.3C-luc. A 5.5-kb haplotype *G* or *T* fragment was cloned by PCR from a *G* or *T* carrier and after confirmation of PCR fidelity by DNA sequencing (Supplemental Fig. S1*B*; Supplemental Table S1*A, B*) was shuttled into pSLC18A2–6.3A-luc, resulting in pSLC18A2–6.3G-luc or pSLC18A2–6.3T-luc. The control vector was generated by replacing the 2.0-kb *Nhe*I/*Hind*III fragment in pGL4.14[*luc2*/Hygro] with the 2.2-kb *Nhe*I/*Hind*III fragment of pGL3-Enhancer. To linearize the plasmids, *Nhe*I located immediately upstream of *SLC18A2* DNA was used for restriction digestion (Supplemental Fig. S1*A*), followed by purification with a QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA, USA). Haplotype confirmation of SY-SY5Y genomic DNA for *SLC18A2* used PCR and DNA resequencing primers listed in Supplemental Table S1*E*.

Transfection, agent treatments, and luciferase activity measurements

The transfection procedure has been described before (3). Eighteen-hour treatments of transfected cells with agents, as listed in **Table 1**, started 27 h after transfection. For luciferase (Luc) activity measurements, a Luciferase Assay System Kit (Promega) in Bio-Tek Synergy HT/KC4 and a 96-well format were used, according to the manufacturer's instructions. Cell numbers in each well were estimated by protein amount based on Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). An arbitrary unit of *SLC18A2* promoter activity is calculated as Luc activity: $(\text{readout/protein})_{(SLC18A2-\text{MOCK})}/$

TABLE 1. *Agents used in regulation studies*

Symbol	Name	Major activity	Solvent	Stock conc.	Working conc.	Source
AMPH	*d*-Amphetamine	Treatment of ADHD; psychostimulant	H_2O	50 mM	10 μM	Sigma-Aldrich Corp., St. Louis, MO, USA
DA	Dopamine	Neurotransmitter	50 μM ascorbic acid	50 mM[a]	10 μM	Sigma-Aldrich Corp.
Dyn[b]	Dynorphin A	Endogenous κ-agonist	H_2O	0.5 mM	0.25 μM	Phoenix Pharmaceuticals, Belmont, CA, USA
Forskolin		Adenylyl cyclase activator	DMSO	50 mM	10 μM	EMD Biosciences, San Diego, CA, USA
IGF-1	Insulin-like growth factor	AKT signaling activator	H_2O/0.1% BSA	0.1 mg/ml	50 ng/ml	Sigma-Aldrich Corp.
KN-62		CaM kinase inhibitor	DMSO	15 mM	3 μM	Tocris Cookson, Inc., Ellisville, MO, USA
LY294002		PI3 kinase inhibitor	DMSO	15 mM	3 μM	Tocris Cookson, Inc.
MK801	(+)-MK-801 or dizocilpine	NMDA receptor inhibitor	H_2O	5 mM	1 μM	Sigma-Aldrich Corp.
MPP+	Methylpiperidinopyrazole	Neurotoxin	H_2O	0.5 mM[a]	5 μM	Sigma-Aldrich Corp.
OA	Okadaic acid	Protein phosphatase inhibitor	DMSO	0.1 mM	20 nM	Tocris Cookson, Inc.
6-OHDA	6-Hydroxydopamine	Neurotoxin	50 μM ascorbic acid	50 mM	10 μM	Sigma-Aldrich Corp.
PACAP38	Pituitary adenylyl cyclase activating polypeptide 38 amino acids	Adenylate cyclase stimulator	H_2O	50 μM	10 nM	EMD Biosciences. . . .
4α-PDD	4α-Phorbol 12,13-didecanoate	Negative control of PMA, PDD	DMSO	5 mM	1 μM	EMD Biosciences
PDD	Phorbol 12,13-didecanoate	PKC activator	DMSO	5 mM	1 μM	EMD Biosciences
PMA	Phorbol 12-myristate 13-acetate	PKC activator	DMSO	5 mM	1 μM	EMD Biosciences
SB202190		p38 kinase inhibitor	DMSO	50 mM	10 μM	Tocris Cookson, Inc.
TNF-α	Tumor necrosis factor-α	Apoptosis inducer	H_2O	0.1 mg/ml	100 ng/ml	Sigma-Aldrich Corp.
U0126		MEK1/2 inhibitor	DMSO	50 mM	10 μM	Tocris Cookson, Inc.

ADHD, attention deficit/hyperactivity disorder; CaM kinases, Ca^{2+}/calmodulin-dependent protein kinase II; NMDA, *N*-methyl-D-aspartate. [a]Freshly prepared. [b]Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln.

$(\text{readout/protein})_{(\text{Vector}-\text{MOCK})}$, where MOCK means transfection without DNA, a background control.

Stable cell line establishment and immunocytochemistry

Establishment of stable cell lines followed a previous protocol (18), using *Bst*EII-linearized pSLC18A2–6.3A-luc (Supplemental Fig. S1*A*) and cloning medium containing 100 μg/ml hygromycin B (Invitrogen, Carlsbad, CA, USA) until colonies appeared, followed by Luc activity and immunocytochemical analyses (19). Luc activity uses $(\text{readout/protein})_{(SLC18A2-\text{MOCK})}$.

Electrophoretic mobility shift assay (EMSA)

For ^{35}S-labeling of oligonucleotides (oligos) (listed in Supplemental Table S1*C*), double-stranded (ds) oligos were prepared in annealing buffer (10 mM Tris-HCl, pH 8.0, and 100 mM $MgCl_2$), and 20 μM ds oligo was labeled in a 30-μl solution containing 0.5 μl of 20 μM annealed ds oligos, 3 μl of 10× Reaction Buffer 2 (New England Biolabs, Ipswich, MA, USA), 6 μl of [^{35}S]dATP (60 pmol; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA), and 1 μl of Large Fragment DNA Polymerase I (Invitrogen), incubated at room temperature for 28 min followed by purification in a Chroma Spin-10 column (Clontech, Mountain View, CA, USA). DNA binding reactions were conducted with a Novagen EMSA Accessory Kit (EMD Biosciences, Gibbstown, NJ, USA), and electrophoresis was performed with 0.6% nondenaturing DNA retardation gels according to the EMSA kit instructions. Gels were dried on filter papers and exposed to X-ray film for 3–5 d for autoradiography.

Quantitation of mRNA levels by quantitative real-time PCR (qRT-PCR)

Reverse transcription was performed with Superscript II reverse transcriptase (Qiagen) oligo(dT) as the primer. Quantitative PCR reactions was conducted with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and primers listed in Supplemental Table S1*D*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-*actin* were used as internal controls. Relative expression levels were calculated by using the $2^{-\Delta\Delta CT}$ method (20). *SLC18A2* primers showed amplification efficiency approximately equal (within 5% difference) to that of the internal controls.

Southern blotting

Biotin labeling of a probe (1.1-kb *Ava*I/*Hind*III fragment within the *luc* gene) was performed with the BrightStar Psoralen-Biotin Nonisotopic Labeling Kit and associated protocols (Ambion; Applied Biosystems Inc.). Genomic DNA, isolated from cultured cells by using the Blood & Cell Culture DNA Midi Kit (Qiagen), was digested with *Kpn*I/*Bam*HI, followed by 0.7% agarose electrophoresis. After capillary transfer, the DNA on the positively charged nylon membrane (Ambion) was hybridized with the biotin-labeled probe in ULTRAhyb hybridization buffer (Ambion) at 42°C for 24 h. After several washes with SSC/0.1% SDS and 1× wash buffer, membranes were treated with strep-alkaline phosphatase in blocking buffer for 15 min, washed with 1× wash buffer, and treated with detection solution CDP-Star (BrightStar Bio-Detect Kit; Ambion), followed by exposure to a film.

RESULTS

Expression systems for regulation study

To study regulation of the *SLC18A2* promoter, human cell lines that express endogenous *SLC18A2* were searched among 5 human cell lines, including 4 DA cell lines [SH-SY5Y, IMR-32, SK-N-AS, and BE(2)-M17] and a non-neuronal cell line (HEK293T) by using qRT-PCR. As a result, SH-SY5Y expressed the highest levels of endogenous *SLC18A2* mRNA and IMR-32 the second highest (**Fig. 1*A***). The data on BE(2)-M17 were

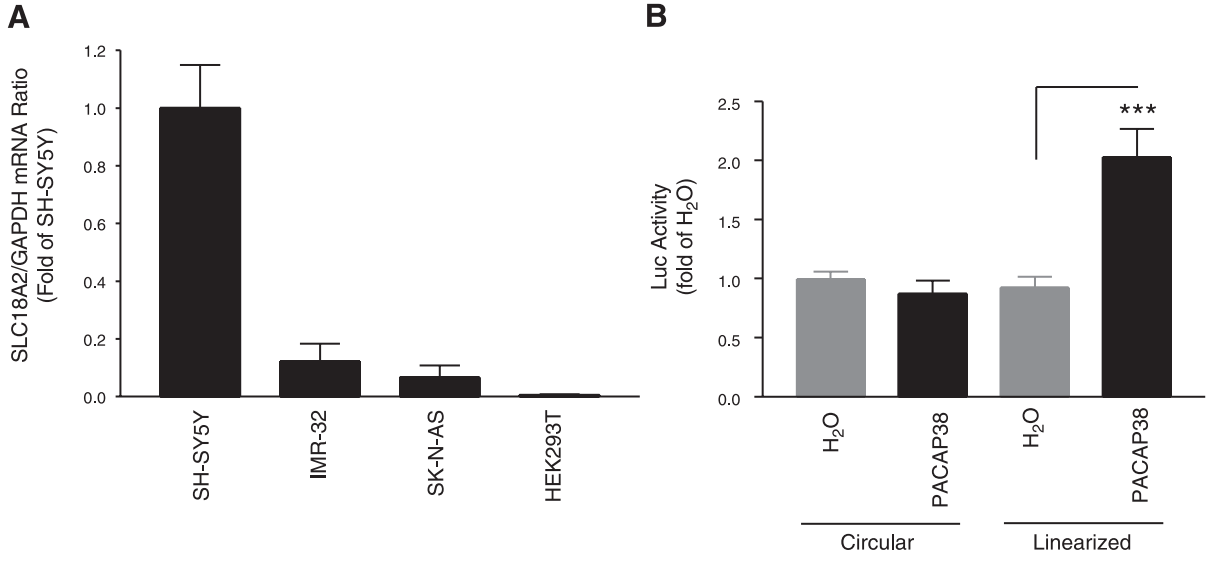

Figure 1. Search for expression systems. *A*) Endogenous *SLC18A2* expression in human cell lines. The DA cell line SH-SY5Y expressed endogenous *SLC18A2* the most, compared with two other DA cell lines, IMR-32 and SK-N-AS, based on qRT-PCR. Non-neuronal cell line HEK293T did not express detectable mRNA levels (n=3 each in duplicate). $P = 0.0006$ overall, $P < 0.001$ for SH-SY5Y *vs.* others; ANOVA. *B*) PACAP38 regulation of haplotype *A* in circular *vs.* linearized pSLC18A2–6.3A-luc in SH-SY5Y (n=6–12). *$P < 0.05$; Student's t test.

unreliable and are not shown here. We therefore used the human DA cell lines SH-SY5Y and IMR-32 as two systems for *SLC18A2* regulation analysis that mimic different brain regions to some extent.

In this study we also chose linearized plasmid DNAs for expressional analyses. In an animal study, Miller and colleagues (21) had reported that PACAP38 up-regulated VMAT2 levels in mouse striatum. However, PACAP38 failed to up-regulate *SLC18A2* promoter activity when pSLC18A2–6.3A-luc (6.3-kb haplotype *A* in a Luc reporter vector; see Materials and Methods) was transiently expressed in SH-SY5Y. With the plasmid DNA linearized, PACAP38 was able to up-regulate the promoter activity (Fig. 1*B*). These data suggested that the supercoiled structure of pSLC18A2–6.3A-luc affected regulation of the promoter activity. Therefore, regulation data from transient expression systems in this study were obtained with *Nhe*I-linearized DNAs.

Regulation of transient expression in SH-SY5Y and IMR-32

Seventeen agents (substances) were used in this regulation study, including 5 endogenous ones (listed in Table 1). All of the resultant regulation data are summarized in **Table 2** (see Supplemental Material for details), and only those showing statistical significance based on *t* tests (in pairwise treatments) or by ANOVA posttest (Tukey's test) are described here.

Endogenous molecules

DA is an endogenous substrate of the VMAT2 protein and binds to DA receptors, inducing intracellular signaling that could regulate *SLC18A2*. In SH-SY5Y, treatment with 10 μM DA for 18 h tended to up-regulate promoter activity of haplotype *A* by 1.55-fold ($P=0.058$ by *t* tests), compared with the negative control (H_2O Vc). DA had no effect on three other haplotypes in SH-SY5Y or IMR-32 (data not shown). Another four endogenous agents studied were PACAP38, Dyn, IGF-1, and TNF-α, among which PACAP38 was the only one that up-regulated the promoter activity of *A* (2.03-fold) and *C* (2.02-fold) in SH-SY5Y and of *A* (1.46-fold), *C* (1.75-fold), and *T* (2.34-fold) in IMR-32, compared with the negative control.

Exogenous molecules, including AMPH, MK801, and forskolin

In SH-SY5Y/*A* (pSLC18A2–6.3A-luc) cells, MK801 up-regulated *A* (2.06-fold), *C* (2.37-fold), and *G* (2.48-fold) but not *T*. Forskolin up-regulated the promoter activity of *A* by 2.02-fold but not the promoter activity of any other haplotypes in SH-SY5Y.

In IMR-32, haplotype dependence was less evident. Forskolin up-regulated promoter activity by the largest amount (2.80-fold) on *G*, second (1.84-fold) on *A* or

TABLE 2. *Summary of haplotype-dependent regulations*

	Haplotype								
	SH-SY5Y				IMR-32				
Agent	*A*	*C*	*G*	*T*	*A*	*C*	*G*	*T*	Reference figure
Endogenous									
DA	↑ [a,**]								2*A*, 3*B*
PACAP38	↑ [##]	↑ [#]			↑ [#]	↑ [#]		↑ [#]	2*B*, S2
Dyn	↑ [b,###]								3*B*, S2
IGF-1									3*B*, S2
TNF-α	↑ [a,#]								2*B*, S2
Exogenous									
AMPH									S3
MK801	↑ [###]	↑ [##]	↑ [#]						S3
Forskolin	↑ **				↑ **	↑ **	↑ ***	↑ *	S3
PMA/ PDD	↓ [###,###]	↓ [##,#]	↓ [#,#]	↓ **, NA					S4
OA	↑ [#]								S4
LY294002[c]	↓ [a,###]								2*D*
U0126[c]	↓ [a,#]								2*D*
SB202190[c]	↓ [a,###;b,#]								2*D*, 3*B*
KN-62									S4
MPP+	↑ [a,*,***]		↑ **	NA	↓ **			NA	2*E*, S5
6-OHDA	↑ [a,*,**]	↑ **		↑ *	↑ *				2*E*, S5
Up	9	3	2	1	3	2	1	2	
Down	4	1	1	1	1	0	0	0	
Total	13	4	3	2	4	2	1	2	

All data from the transient expression experiments SH-SY5Y and IMR-32 as indicated, unless noted. No arrow indicates no significant effect based on either ANOVA posttests or *t* tests. NA, not analyzed. [a]Data from endogenous expression of SH-SY5Y. [b]Data from stable expression of A7. [c]Unreliable data in transient expression systems. $*P<0.05$, $**P<0.01$, $***P<0.001$; Student's *t* tests of pairwise treatments. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$; ANOVA posttest (Tukey's test).

(1.74-fold) on *C* and least (1.66-fold) on *T*. None of the other treatments including those with MK801 had any effect on the promoter activities. That is, AMPH did not affect any of these haplotypes in either of the two cell lines (Supplemental Fig. S3).

PKC, CaMKII, and phosphatase effectors: two PKC activators, PMA and PDD, the CaMK inhibitor KN-62, and the protein phosphatase inhibitor OA

In SH-SY5Y, PMA and PDD down-regulated the promoter activities of all 4 haplotypes, by ~0.6-fold for *C*, 0.5-fold for *A*, 0.4-fold for *G*, and 0.14-fold for *T*. OA up-regulated the *A* promoter activity (by 2.02-fold) but had no significant effect on 3 others; KN-62 had no effect on any of the haplotypes. In IMR-32, no significant effects were observed for any of the haplotypes (Supplemental Fig. S4).

Neurotoxins: MPP^+ (a substrate of VMAT2) and 6-OHDA, the two most common toxins used in animal models for PD

In SH-SY5Y/*A*, MPP^+ and 6-OHDA up-regulated the promoter activity by 1.62- and 2.24-fold. In SH-SY5Y/*C*, 6-OHDA displayed an up-regulation of 1.56-fold, and MPP^+ up-regulation was not statistically significant. In SH-SY5Y/*G*, only MPP^+ displayed a significant up-regulation (1.95-fold). In SH-SY5Y/*T*, 6-OHDA displayed a significant up-regulation (2.92-fold). In IMR-32/*A*, MPP^+ down-regulated but 6-OHDA up-regulated the promoter activity by 0.75- and 1.39-fold. Neither of the toxins regulated *C* or *G*, and 6-OHDA also did not affect *T* in IMR-32 (Supplemental Fig. S5).

Regulation of endogenous expression in SH-SY5Y

Selected agents were examined for their effects on endogenous mRNA levels of *SLC18A2* in SH-SY5Y by using qRT-PCR. DA was able to up-regulate *SLC18A2* by 1.57-fold (**Fig. 2*A***), a finding consistent with what was observed in the transient expression. TNF-α up-regulated the endogenous activity by 1.71-fold (Fig. 2*B*). PMA had no effect (Fig. 2*C*). The MAP kinase inhibitors LY294002 (PI3 kinase), U0126 (MEK1/2), and SB202190 (p38 kinase) down-regulated the endogenous activity by 0.61-, 0.80-, and 0.64-fold (Fig. 2*D*). In the transient expression systems, the regulation data were generally unreliable because of vector effects, but haplotype dependence was observed for PI3 kinase inhibitor-mediated down-regulation in *A* (0.65-fold) *vs. G* (1.10-fold) in SH-SY5Y. No haplotype dependence was observed in IMR-32. Consistent with the transient expression, both MPP^+ and 6-OHDA up-regulated the endogenous activity by 2.02- and 1.71-fold, compared with the negative controls (Fig. 2*E*). These regulation data, except for PACAP38 and PMA, were all consistent with the findings from transient expression of haplotype *A* in SH-SY5Y. More consistently, *SLC18A2* haplotyping of the SH-SY5Y genomic DNA confirmed that this cell line carried homozygous *A*.

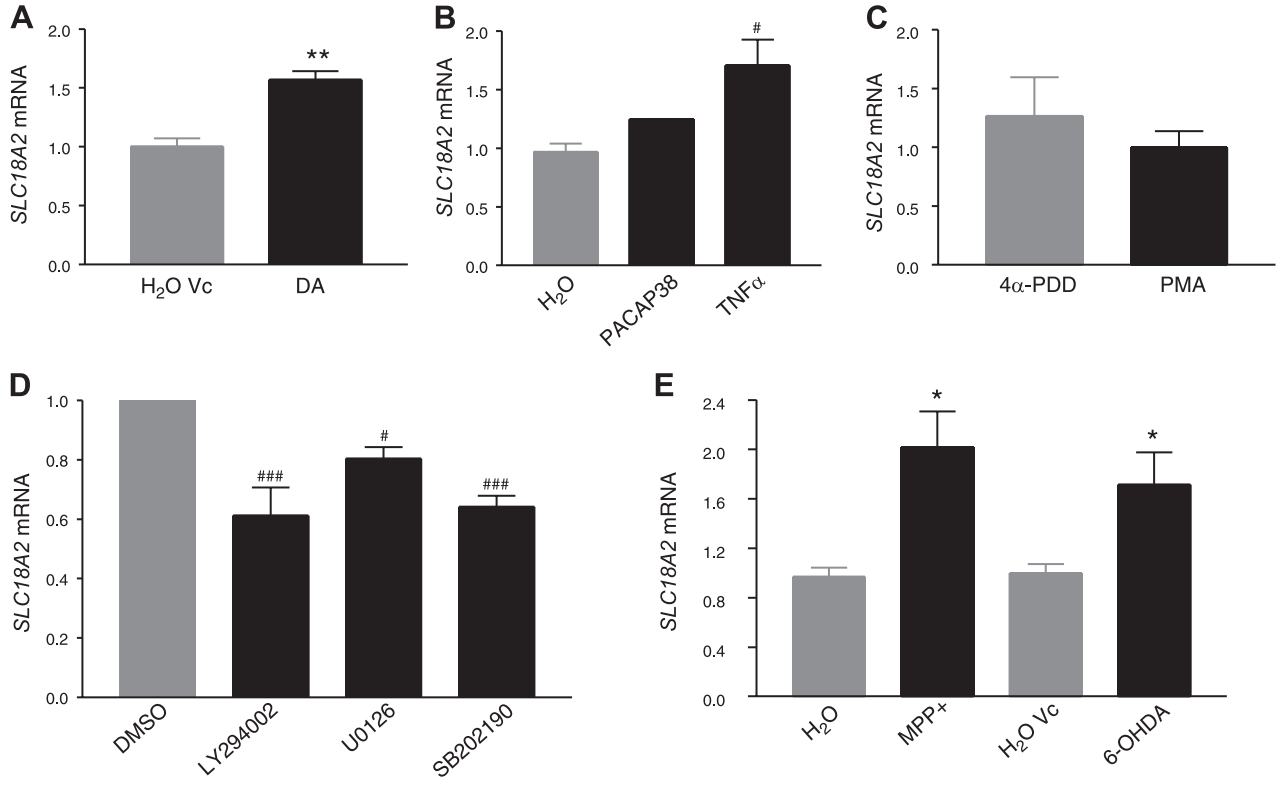

Figure 2. Regulation of endogenous *SLC18A2* activity in SH-SY5Y by various agents, based on qRT-PCR analysis of mRNA levels. *A*) DA; $n = 4$. *B*) Other endogenous agents; $n = 3$. $P = 0.021$ overall, $^{\#}P < 0.05$ vs. H_2O; ANOVA. *C*) PKC activator PMA; $n = 5$. *D*) MAP kinase inhibitors; $n = 3$–4. $P < 0.0001$ overall; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *vs.* DMSO; ANOVA. *E*) Neurotoxins; $n = 3$–5. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. corresponding controls; Student's *t* test. Shaded bars indicate solvent controls.

Regulation of stable expression

Chromatin structure may influence promoter regulation (22), suggesting that expression from plasmid DNAs may or may not be able to reflect the chromosomal expression. To understand the potential discrepancy in the regulation of *SLC18A2* between plasmids and chromosomes, stable cell lines were generated for haplotype *A*-luc hybrid to integrate into host chromosomes and to see whether the same regulations could be observed in the stable cell lines. Among 12 colonies obtained, one stable cell line, termed A7, expressed normal Luc activity (within 1- to 2-fold of the activity observed in transient expression) and was a pure cell line, based on immunocytochemical staining of Luc (**Fig. 3*A***). Regulation analyses showed that Dyn upregulated the promoter activity expressed in the host chromosome, whereas SB202190 again down-regulated the activity in this stable cell line (Fig. 3*B*). Generally speaking, A7 displayed a regulation pattern similar to those observed in the transient or endogenous expression system. Southern blotting analyses of the genomic DNA digested with *Kpn*I/*Bam*HI showed that A7 carried a single copy of the hybrid and that this hybrid had

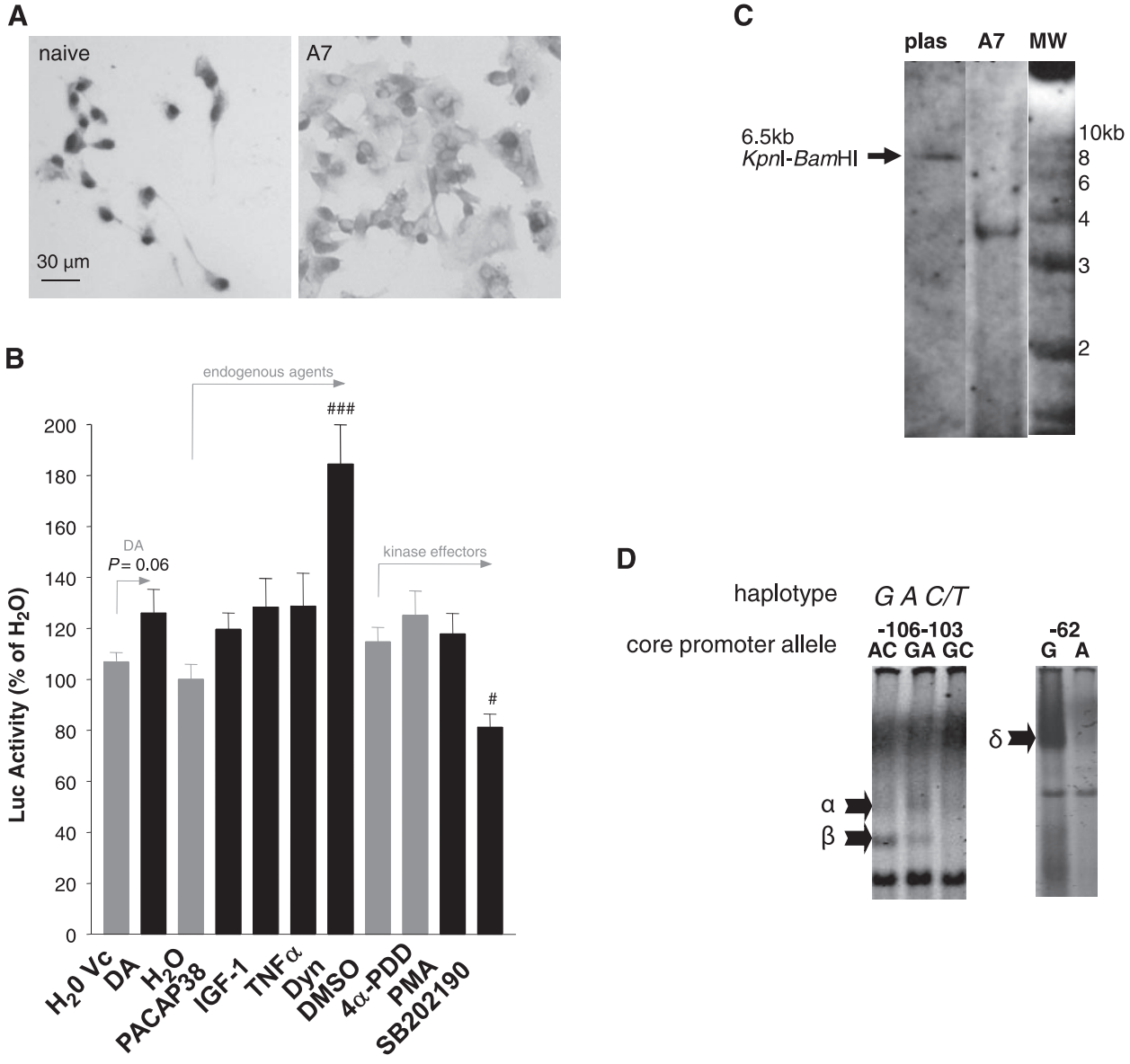

Figure 3. Characterization of stable SH-SY5Y/pSLC18A2–6.3A-luc cell line A7. *A*) Luc immunoreactivity in naive and A7 cells. *B*) Regulation of *A* by DA, other endogenous agents, and kinase effectors. Shaded bars indicate solvent controls; bent arrow indicates a negative control and its comparison. ANOVA: $P < 0.0001$ overall, $^{###}P < 0.001$ *vs.* H_2O for endogenous agents; $P = 0.0005$ overall, $^{\#}P < 0.05$ *vs.* DMSO for kinase effectors; *P* value is indicated by *t* tests, compared with corresponding control H_2O Vc (n=12–14). *C*) Southern blotting analysis of A7. plas, plasmid DNA as a positive control for the 6.5-kb target. *D*) EMSA analysis of SNPs −106A/G, −103C/A, and −62G/A (Supplemental Figure S1*C*). SNPs at −106 and −103 were linked and analyzed together as AC in *G*, GA in *A*, and GC in *C* or *T*. Three nuclear proteins with differential binding activities are termed α, β, and δ here. Oligo specificity of these binding activities has been confirmed by competition experiments; the persistent band below the arrows was poly(A/T)-related activity.

been truncated by ~3 kb, probably at the 5′ end (Fig. 3*C*). The exact location of truncation has not been mapped yet, but A7 appeared to carry a 1- to 1.5-kb core promoter region upstream of the *luc* gene, and the observed regulations were executed by this core promoter.

Transcription factor (TF) binding to polymorphic sites in the core promoter

Because EMSA may faithfully reveal TF-promoter interactions that occur *in vivo* (23–25), this method was used to investigate whether polymorphic sites in the core promoter region were bound by nuclear proteins. This core promoter region harbored 3 single-nucleotide polymorphisms (SNPs) at −106, −103, and −62 (3). To understand whether TFs could recognize these polymorphic sites, EMSA analyses were performed by using ^{35}S-labeled ds oligos (Supplemental Table S1*C*) and nuclear proteins isolated from SH-SY5Y. As a result, there appeared to be 3 nuclear proteins that bound to these sites: α bound to −106 to −103 G–A in haplotype *A* but not to other haplotypes; β bound most to haplotype *G*, less to *A*, and not at all to *C/T*; and δ bound to the −62G allele that was carried by *A*, *G*, or *T* (Fig. 3*D*; Supplemental Fig. S1*C*). These differential binding activities were consistent with the haplotype-dependent regulations observed in our transient expression systems.

DISCUSSION

Functional basis for haplotype selection

The critical finding in our analyses is that haplotype *A*, the highly selected one, is more regulatable than the infrequent haplotypes *C*, *G*, and *T*. *A* was regulated by 13 (76.5%) of the 17 agents tested, displaying the most responses relative to *C* (4 agents or 23.5%), *G* (3 agents or 17.6%), and *T* (2 agents or 11.8%) in SH-SY5Y. In particular, *A* was regulated by 4 of the 5 endogenous agents, compared with only 1 agent for *C* and none for *G* or *T*. These agents were relatively less active in IMR-32, probably because *SLC18A2* is less active in this cell line, as IMR-32 expresses only 12% of the mRNA levels in SH-SY5Y (Fig. 1*A*). The lesser activity suggests that fewer signaling pathways activate *SLC18A2* in IMR-32. *A* was regulated by 4 (23.5%) agents, compared with *C* or *T* by 2 (11.8%) agents and *G* by 1 (5.9%) agent in IMR-32, a high A-regulatability pattern similar to that in SH-SY5Y (Table 2). These data on more regulations of *A* are consistent with the fact that *A* is recognized by more nuclear proteins than the other haplotypes (Fig. 3*D*).

Interestingly, haplotype frequency is positively and almost linearly correlated with the number of significant regulations (**Fig. 4**). Cladistic analysis of the four haplotypes shows a large distance between the two most frequent haplotypes, *A* and *C*. Evidently, *A* has been heavily favored during human biological history; *T* is relatively close to *A* but is deselected (Fig. 4*A*). These data imply that high *SLC18A2* regulatability is critical for survival during human evolution, which is consistent with variations in VMAT2 expression influencing brain integrity, function, and behaviors in knockout mice. In particular, the high regulatability may underlie forms of synaptic plasticity in which regulation of neurotransmission relies on variation in VMAT2-facilitated quantal size of transmitter release (26). An intriguing question is the following: because low-activity haplotypes are associated with brain diseases, why did high-activity haplotypes such as *C* not have a greater survival advantage and were not highly selected (the frequency of *C* was 14.4% in the Caucasian populations)? Among other speculations (14, 27), we may

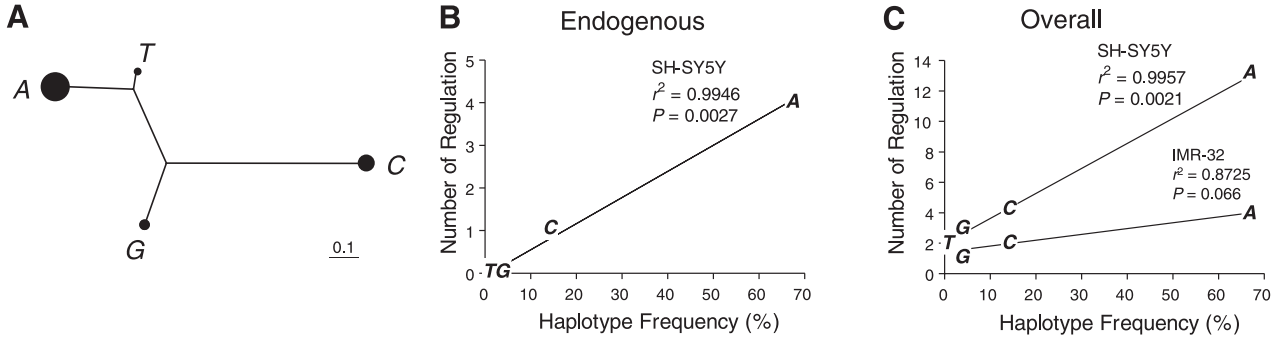

Figure 4. *A*) Cladogram of *SLC18A2* promoter haplotypes *A*, *C*, *G*, and *T* (see ref. 3 for method). Scale bar = 0.1 substitution/site. *B*, *C*) Positive correlation between haplotype frequency and regulatability for 5 endogenous agents (*B*) or all studied agents (*C*) in 2 DA cell lines, SH-SY5Y and IMR-32. IMR-32 had too few endogenous agent-based regulations to show a correlation in *B*. Haplotype frequency: 66.7% for *A*, 14.4% for *C*, 3.68% for *G*, and ~1% for *T*, based on 1460 Caucasian chromosomes combined from 2 independent studies (3, 4). Haplotype *T* appeared to be a variant of haplotype *H* (3), due to a different simple sequence length polymorphism allele at −5199 and 2 different intron 1 alleles at +321 and +517 (Supplemental Figure S1*C*; Table 3 in ref. 3), possibly representing a subgroup of the haplotype 1 that had a 2% frequency in >300 Caucasians (4). Because haplotypes *G*, *H*, *K*, and *M* together counted for 6% of the total haplotypes in 333 Caucasians (3), we estimate that this new haplotype, termed haplotype *T*, has a frequency of ~1%. The r^2 value indicates degree of linear fitness in cell type as noted; *P* indicates significance for the nonzero slope of linearity; letters indicate haplotypes.

postulate that *C* is much less regulatable, reducing the capacity for synaptic plasticity. It would be interesting to see whether *SLC18A2* is associated with other monoamine-related features of human beings.

Dependence in *SLC18A2* activity

The most consistent findings from this study are the haplotype dependence and the cell type-dependence in regulation of *SLC18A2* promoter activity. Such haplotype dependence could range from 0.91-fold for *G* to 2.24-fold for *A* (a 2.5-fold difference) with 6-OHDA or from 0.14-fold for *T* to 0.48-fold for *A* (a 3.4-fold difference) with PMA in SH-SY5Y. Among the 17 agents investigated, 76.5% (13 agents) of them showed haplotype dependence in either of the DA cell lines (Table 2). Some agents regulated one haplotype but not another, including DA, Dyn, TNF-α, forskolin, OA, and 6-OHDA. Others affected different haplotypes to various extents, including PMA and 6-OHDA in SH-SY5Y, forskolin in IMR-32, and PACAP38 in both cell lines. These haplotype-dependent regulations suggest that the 6.3-kb haplotypes are regulated by multiple signaling cascades, among which some may recognize TFs that bind to conserved elements or polymorphic sites in the core promoter.

The *SLC18A2* regulatome: *A*-dominant

Several signaling cascades appear to contribute to the selection of *A*, based on the regulation data. These cascades are conjoined by five hubs including cAMP response element-binding protein (CREB) 1, β-arrestin 1, G-protein α_s, c-Raf-1, and ERK-1 (**Fig. 5**) and may make few points. Foremost, upstream of CREB1 is CaMKII that had been expected to activate CREB1, an activator of the VMAT2 gene (28). However, this activation was not present in our expression systems because the CaMKII inhibitor KN-62 did not affect the promoter activity. Second, Ca^{2+} appeared to play an

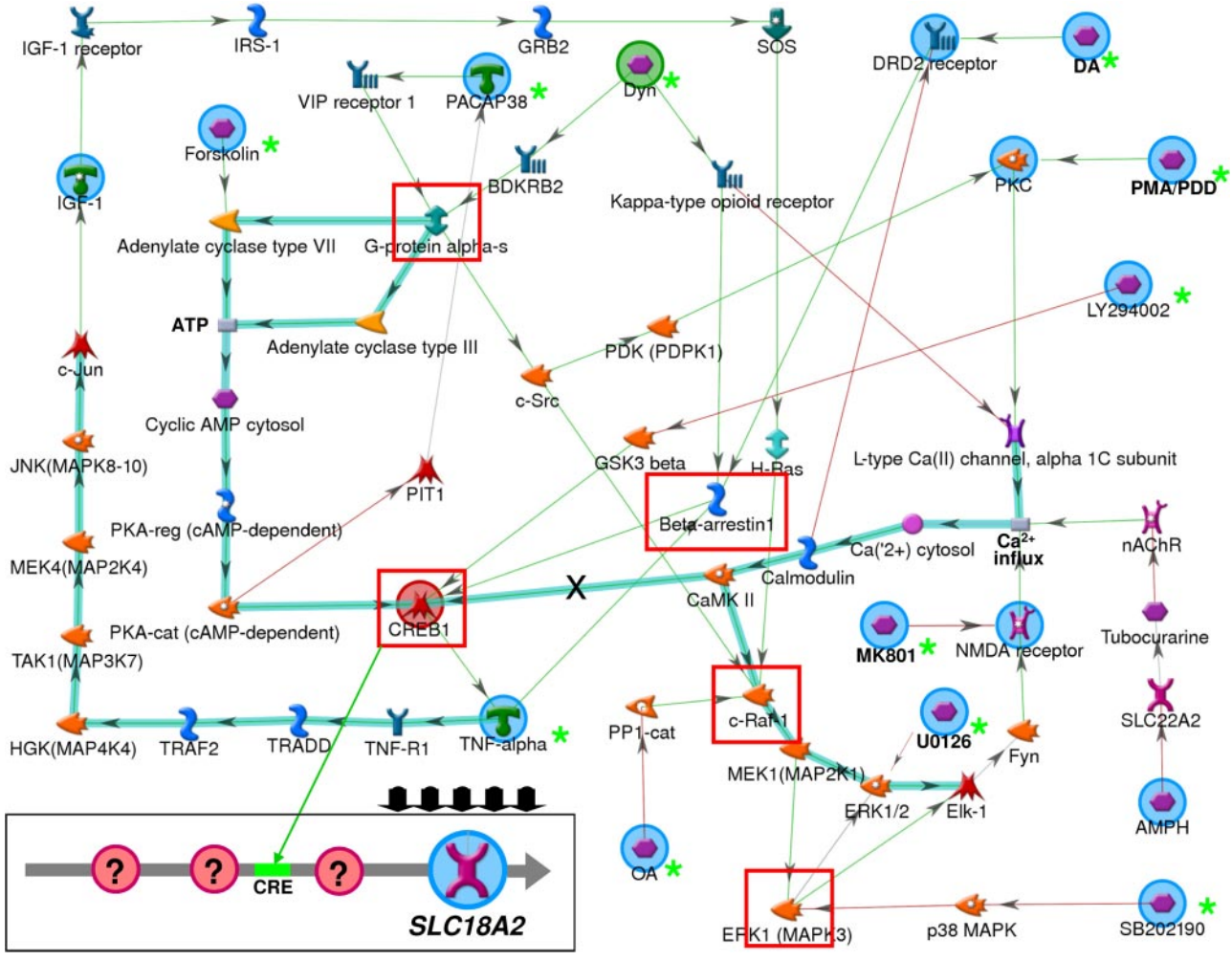

Figure 5. Signaling cascades that regulate *A*. Green asterisks, agents that regulated *A* (see Table 2); red boxes, major hubs each with ≥4 interactions; thick aquamarine lines, canonical pathways; thin green arrows, activation; thin red arrows, inhibition; thin gray arrows, unspecified; X, pathway that was not supported by the data from this study. Oxidative stress-related (MPP^+ and 6-OHDA) pathways are not included. Bottom rectangle: schematic diagram of *SLC18A2* regulated by TFs, including CREB1 and others (indicated by question mark) such as those indicated in Fig. 3*D*; CRE (green), cAMP-responsive element; black arrows, unknown links between TFs and pathways. Pathways analysis was performed with MetaCore (36).

inhibitory role here because its reduced concentration seems to correlate with increased promoter activity, as suggested by data with PMA, PDD, MK801, and SB202190. This Ca^{2+}-reverse correlation is further supported by the nicotine/nicotinic acetylcholine receptor (nAChR)-mediated down-regulation of *A* by 22% (P=0.010, n=4; compared with the negative control), consistent with the finding that expression of a hypersensitive nAChR caused neurotoxicity and DA neuron death in mice (29). Third, a single agent such as Dyn may regulate *A via* multiple pathways (bradykinin receptor B2, G-protein α_s, κ-type opioid receptor, the L-type Ca^{2+} channel α1C subunit, and β-arrestin 1). Fourth, expression of an endogenous agent such as TNF-α can regulate as well as be regulated by related pathways (β-arrestin 1, c-Jun pathway, and CREB1), implying the regulation of *A* by balancing of different related pathways. Finally, infrequent haplotypes are regulated by a limited number of pathways that involve CREB1 and Ca^{2+}. Because agents that regulated the rare haplotypes all regulated the dominant *A* (Table 2), these rare haplotypes may miss regulatory elements due to DNA sequence variation, which merits further investigation.

Technical caveats associated with reporter systems

Transient expression of the Luc-based reporter is used for transcriptional analyses by many biological fields (30–34), and data obtained by using such a reporter system could be spurious according to our observations. Foremost, regulation of the promoter on linearized plasmid DNA expressed transiently could result in data more similar to those obtained with endogenous, stable, or even *in vivo* expression than data obtained with circular plasmid DNA, based on the PACAP38 and DA data (35) (Figs. 1*B*; 2*A, B*; and 3*B*), suggesting that the regulations required an open DNA conformation (21). The second observation was that kinase effectors could regulate mysterious promoters of the vector and such regulations of vector depended on the expression systems. These technical caveats associated with transient expression of reporter assays mandate data confirmation by a means different from transient expression.

CONCLUSIONS

Haplotype *A* is regulated by more pathways than the infrequent haplotypes *C, G,* and *T*, suggesting a positive correlation between haplotype frequency and number of regulators. TF-based further dissection of the haplotype-specific regulatory cascades can reveal specific mechanisms for haplotype selections. Haplotype-dependence can also be cell type-dependent and transgenic technology will help to clarify specific contributions of distinct *SLC18A2* haplotypes to human evolution and diseases. FJ

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