J. Biochem. **137**, 205–216 (2005)
DOI: 10.1093/jb/mvi020

Identification and Characterization of the Hesr1/Hey1 as a Candidate *trans*-Acting Factor on Gene Expression through the 3′ Non-Coding Polymorphic Region of the Human Dopamine Transporter (DAT1) Gene

Satoshi Fuke, Noboru Sasagawa and Shoichi Ishiura*

Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902

Received October 25, 2004; accepted December 3, 2004

Expression of the dopamine transporter (DAT) gene affects dopaminergic neurotransmission. A variable number tandem repeat (VNTR) polymorphism in the 3′ non-coding region of the human DAT (DAT1) gene has been reported to affect gene expression as a *cis*-element, and is associated with some neuropsychiatric disorders. In this study, we identified the basic helix-loop-helix (bHLH) transcriptional factor Hesr1 (the Hairy/enhancer of split related transcriptional factor 1 with a YRPW motif, also named Hey1/HERP2/HRT1/CHF2) as a *trans*-acting factor in a yeast one-hybrid system, and showed that Hesr1 down-regulates reporter gene expression with the 3′ non-coding region of *DAT1* gene in mammalian cell lines. The negative regulations depend on bHLH and the Orange domain of the molecule, but not the YRPW motif. The negative regulations affect the VNTR-dependent differences in gene expression. In addition, we identified a splice variant, Hesr1-12nt, with a lower activity. We also show that SNP of *Hesr1*, C386A, causes a lack of activity. These results suggest that Hesr1 and its polymorphism(s) might be involved in dopamine-related polygenic disorders and behavioral traits.

Key words: behavioral traits, polygenic disorder, polymorphism, SNP, VNTR.

Abbreviations: 3-AT, 3-amino-1,2,4-trizole; 5-HTT, serotonin transporter; ADHD, attention deficit hyperactivity disorder; bHLH, basic helix-loop-helix; BMP, bone morphogenetic proteins; CP, core-promoter; CPRG, chlorophenol red-β-D-galactopyranoside; DAT, dopamine transporter; DR, dopamine receptor; HES, hairy/enhancer of split; Hesr1, hairy/enhancer of split related transcriptional factor 1 with YRPW motif; NICD, Notch intracellular cytoplasmic domain; PTSD, posttraumatic stress disorder; SNP, single nucleotide polymorphism; TGF-β1, transforming growth factor-β1; TH, tyrosine hydroxylase; Tukey-Kramer's HSD test, Tukey-Kramer's honestly significant difference test; VEGFR2, vascular endothelial growth factor receptor 2; VNTR, variable number of tandem repeat.

Dopamine is utilized as a neurotransmitter in the central nervous system. Dopaminergic brain systems play important roles in regulating locomotion, cognition, reward, affect, and hormone release (*1*, *2*). Dopamine transporter (DAT) is a member of the sodium- and chloride-dependent family of plasma membrane transport proteins. DAT terminates synaptic transmission by the rapid and specific reuptake of dopamine into presynaptic nerve terminals (*3–6*). DAT is the target of such psychoactive drugs as cocaine, amphetamine, and methylphenidate. These powerful dopaminergic stimulants are known to bind DAT and inhibit dopamine reuptake (*2*, *6–8*).

The levels of the DAT protein are reduced in Parkinson's disease, whereas they are elevated in attention deficit hyperactivity disorder (ADHD), Tourette's syndrome, and major depression (*9–12*). In DAT knockout mice, dopamine clearance is prolonged and the mice show spontaneous hyperactivity (*2*, *8*, *13*). A deficit of DAT results in adaptive changes such as a low content of dopamine in presynaptic terminals, and the misexpression of genes related to dopaminergic brain systems, dopamine receptors (DR), tyrosine hydroxylase (TH) and so on (*13–15*). Furthermore, various phenotypes have been observed in DAT knockout mice (*8*). These suggest that the level of *DAT* gene expression is important for dopaminergic neurotransmission systems *in vivo*.

A 40-bp variable number of tandem repeat (VNTR) polymorphism occurs in exon 15 of the human dopamine transporter gene (*16*), which includes the stop codon, 3′ untranslated region (3′-UTR), and polyadenylation signal (Fig. 1a). Several studies have investigated the association between the VNTR polymorphism and some neuropsychiatric disorders, ADHD, Parkinson's disease, bipolar disorder, schizophrenia, Tourette's syndrome, drug abuse, drug-induced paranoia, alcohol withdrawal, and posttraumatic stress disorder (PTSD), which are thought to be related to dopaminergic systems (*1*, *2*, *17–20*). However, the association between the polymorphism and these disorders remains unclear because diverse results have been reported so far (*1*, *2*, *20*). In addition, the 10-repeat allele has been regarded as a high-risk allele in ADHD (*17*). However, the 10-repeat allele is the allele with the highest frequency among the world population (*21*). The differences in these reports may be

*To whom correspondence should be addressed. Tel/Fax: +81-3-5454-6739, E-mail: cishiura@mail.ecc.u-tokyo.ac.jp

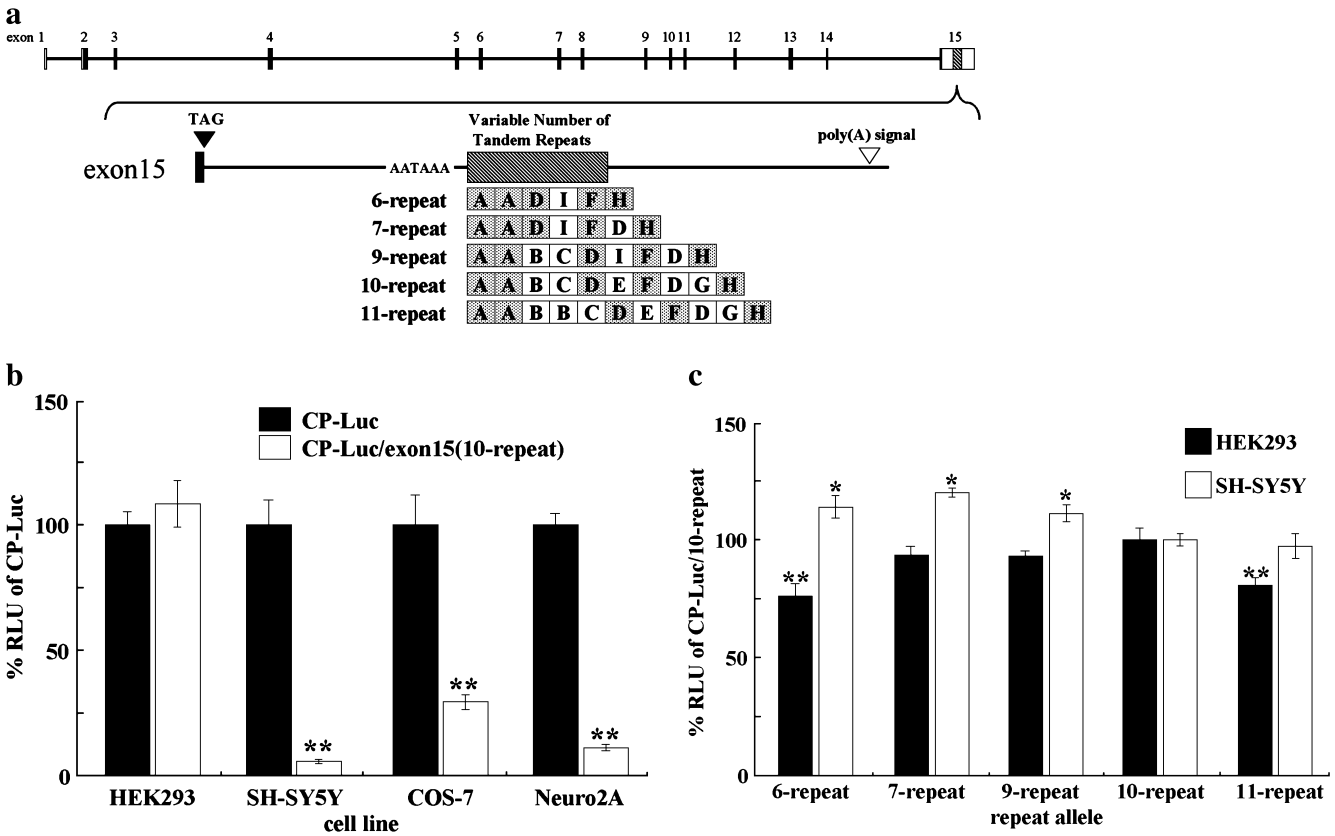

Fig. 1. **The 3′ non-coding region of the *DAT1* gene.** (a) The genomic structure of the *DAT1* gene and allelic variants of VNTR polymorphism in exon15 are shown. The coding region (closed box), non-coding region (open box), VNTR (shaded box), and constant repeat units (gray box) are shown. Exon15 of the *DAT1* gene contains a stop codon (arrowhead) and polyadenylation signal (open arrowhead). Upstream of the VNTR are six nucleotides, AATAAA, that resemble a polyadenylation signal. The allelic variants of VNTR polymorphism indicate repeat units type (A)–(I) in each allele. (b) Negative regulation of gene expression through the 3′ non-coding region of the *DAT1* gene in various cell lines. Relative luciferase activities after transient transfection in SH-SY5Y, HEK293, COS-7, and Neuro2a cell lines are shown. The pGL3-promoter vector containing the SV40 promoter was independently transfected as a control for standardizing each measurement. The relative activities were determined by normalizing the standardized values to the mean CP-luc in each cell line. The CP-Luc/exon15 vector (white columns) shows lower luciferase activity than the CP-Luc vector (black columns) in SH-SY5Y, COS-7, and Neuro2a cells (*$P < 0.001$, $N = 4$). This was not observed in HEK293 cells ($P > 0.16$, $N = 4$). Data represent the means ± SD of four independent transfection experiments. (c) The effect of VNTR polymorphism on gene expression. The luciferase activities of HEK293 (black columns) and SH-SY5Y (white columns) cells transfected with CP-luc/exon 15 containing different alleles with 6, 7, 9, 10, and 11 repeats are shown. The CP-luc vector was independently transfected as a control for standardizing each mesurement. Relative activities were determined by normalizing standardized values to the mean of the CP-luc/10-repeat in each cell line. HEK293 cells transfected with CP-Luc/6- and 11-repeat vectors showed significantly lower luciferase activities (black columns, **$P < 0.01$, $N = 4$). SH-SY5Y cells transfected with the CP-Luc/6-, 7-, and 9-repeat vectors had higher levels of luciferase activity than those transfected with the CP-Luc/10- or 11-repeat vectors (white columns, *$P < 0.05$, $N = 4$).

attributed to the differences in subject groups, previous drug exposure, or influence of other risk factors. This suggests that VNTR polymorphism of the *DAT1* gene may be a modest risk factor in many dopamine-related polygenic disorders and behavioral traits, although polymorphism has biological molecular functions.

Since the VNTR lies in the 3′ non-coding region, the polymorphism should not affect protein structure or function; VNTR polymorphisms might serve as transcriptional or translational regulatory *cis*-acting elements (*22*). For example, the serotonin transporter (5-HTT) gene has a VNTR polymorphism in intron 2 that may function as a transcriptional regulatory region (*23*). Associations between the allelic differences of VNTR polymorphism in intron 2 of the *5-HTT* gene and some neuropsychiatric disorders have been investigated (*20*). Based on the results of a reporter gene expression assay, the polymorphic region is thought to have an enhancer activity that depends on allelic differences (*24*, *25*). Recent reports further suggest that the VNTR polymorphism in the *DAT1* gene has effects on gene expression itself *in vivo* (*26–28*) and on reporter gene expression in mammalian cell lines (*29–31*). Furthermore, a part of the 3' non-coding region containing the VNTR functions as a transcriptional regulatory region, and some proteins bind to the VNTR region in the *DAT1* gene (*32*). These reports suggest that the VNTR polymorphism of the *DAT1* gene has a function as a transcriptional *cis*-acting element.

In this study, to identify other risk factors for many dopamine-related polygenic disorders and behavioral traits, we investigated the role of the *trans*-acting factor in the regulation of gene expression through VNTR polymorphism in the 3′ non-coding region of the *DAT1* gene in mammalian cells.

MATERIALS AND METHODS

Genotyping and Sequencing of VNTR Polymorphisms in DAT1—Genomic DNAs were extracted from whole blood or hair of 181 Japanese individuals. Amplification was performed by the method described previously (*29*). The amplified fragments were visualized by ethidium bromide staining under UV light after electrophoresis in 2% Seaplaque GTG agarose gels (CAMBREX). A maximum of three fragments of each allele were cut out from the gels and purified. The fragments were sequenced directly by the dideoxy chain termination method with CEQ DTCS and CEQ8000 (Beckman Coulter).

Construction of Luciferase Reporter Vectors—Exon15 of *DAT1*, including the stop codon, the VNTR region, and the poly A signal, were amplified from human genomic DNA by PCR using the oligonucleotide primers 5′-CACTGGCTCAAGGTCTAGAGG-3′ and 5′-CCATATGTGGATGTCTTTTATTGTGCTTAG-3′, which were designed to contain restriction enzyme sites (underlined), and annealed at 52°C. The amplified fragments containing 6-, 7-, 9-, 10- or 11-repeat alleles were cloned into pBluescript and sequenced. The fragments were digested with *Xba*I and *Nde*I and cloned into the modified pGL3-Promoter vector (PROMEGA) containing the SV40 promoter, *Xba*I, containing a stop codon and *Nde*I site in the 3′-UTR of the firefly luciferase reporter gene upstream of the SV40 poly(A) signal. From the DAT1-8317 plasmid (*33*), a gift from Dr. Micheal J. Bannon (Wayne State University School of Medicine, Detroit, MI, USA), the *Aat*II–*Hin*dIII fragment containing the human DAT core promoter (*2*, *33*, *34*) segment (bp –240 to +2) was blunted at only the *Aat*II site and cloned into the *Sma*I–*Hin*dIII site of the modified pGL3-Promoter vector to replace the SV40 promoter to construct the CP-Luc reporter vector. Each of the CP-Luc/exon15 vectors was constructed by the same method as the CP-Luc vector. Orientation, junction and cloning fragment sequences were verified by sequencing.

Cloning of the Hesr1 Gene and Construction of GAL4-AD/Hesr1 Expression Vectors for Yeast, and YFP/Hesr1 or Myc/Hesr1 Expression Vectors for Mammalian Cell Lines—The human *Hesr1* and *Hesr1-12nt* genes were amplified from a human fetal brain cDNA library (BD Marathon-Ready cDNA, Clontech) by PCR with oligonucleotide primers 5′-CCCCAGGGAGCCAGCGAATGAAGCGAGCTC-3′ and 5′-CATCAGTTCTTAAAAAGCTCCGATCTCC-3′. The amplified fragment was cloned into pBluescript and sequenced. After cloning, PCRs for each clone were performed using primers containing restriction enzyme sites suitable for the expression of Hesr1, and the products were cloned and sequenced. The fragments were digested and subcloned into each of the expression vectors pACT2 (Clontech), pEYFP-C1 (Clontech), and modified pcDNA3.1 (Invitrogen) containing *c-Myc* epitope tags. Deletion mutants of human Hesr1 (ΔbHLH, ΔOrange, bHLH, ΔC) were generated from Hesr1 clones by PCR. Each expression vector was sequenced, and its expression was confirmed by Western blotting.

Cell Culture and Transient Transfection Assays—The cell lines SH-SY5Y, HEK293, COS-7, and Neuro2a were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2. SH-SY5Y cells were plated at 80% confluency into 24-well plates 24 h prior to transfection. Cells were transfected with 1 μg of each reporter vector using LipofectAmine 2000 reagent (Invitrogen). When the luciferase reporter gene and Hesr1 were co-expressed in cells, 0.5 μg of each vector was co-transfected. In the case of co-transfection with small interfering RNA (siRNA), 0.5 μg of each reporter vector and 40 pmol of each siRNA were co-transfected into SH-SY5Y cells. The plasmid pRL-TK (PROMEGA) containing the HSV-TK promoter and the sea pansy luciferase gene was co-transfected (20 ng) as an internal control for normalization of transfection efficiency in all experiments. After 24 h, luciferase activities were assayed using a Dual-Luciferase Reporter Assay System (PROMEGA). Luciferase activity was measured using a Centro LB 960 (BERTHOLD) for 10 s after a 2 s delay. Experiments were carried out in triplicate, and at least three independent transfections were performed. HEK293, COS-7, and Neuro2a cells were transfected with one-fifth the amount of each vector used for SH-SY5Y cells using FuGENE6 (Roche).

Yeast One-Hybrid Screening—Experiments were performed using a MATCHMAKER One-Hybrid System (Clontech). The human DAT VNTR polymorphism segment containing a 10-repeat allele was amplified from human genomic DNA by PCR using the oligonucleotide primers 5′-TCCCCCGGGCTACTGCCACTCAG-3′, and 5′-GCTCTAGAGGGGTCCCTTCCTG-3′ or 5′-ATAAAACTCGAGTGGGGTCCCTTCCTG-3′, which were designed to contain restriction enzyme sites (underlined). The amplified fragments were cloned into pBluescript II SK and sequenced, digested with *Sma*I–*Xba*I or *Sma*I–*Xho*I, and inserted into pHISi upstream of the HIS3 reporter gene, and also into the pLacZi target-reporter vector upstream of the lacZ reporter gene. The yeast strain YM4271 was transformed with the 10-repeat/lacZ target-reporter vector (1 μg) digested with *Nco*I, and selected on SD/–U plates. Integration of the 10-repeat/lacZ vector into the yeast genome was verified by PCR. The 10-repeat/HIS3 vector (1 μg) was digested with *Xbo*I and integrated into the YM4271 (10-repeat/lacZ) genome. The target-reporter strain was selected on SD/–HU plates and the integration of the vector was verified by PCR.

Yeast cells (5×10^{10}) of the target-reporter strain were transformed with 100 μg of a human fetal brain cDNA GAL4-AD fusion library (BD Matchmaker cDNA Library, Clontech) and plated on SD/–HUL + 1 mM 3-amino-1,2,4-trizole (3-AT), optimal [3-AT] for inhibiting leaky HIS3 expression, and incubated at 30°C for 6 days. Surviving clones were restreaked on SD/–HUL + 1 mM 3-AT plates, and screened using a β-galactosidase colony-lift assay. Clones that remained positive were grown in liquid SD/–L medium, and yeast miniprep DNA containing the cDNAs was prepared by the glass beads method. The human fetal brain cDNA fragments were amplified from yeast miniprep DNA by PCR using 5′ LD Amplimer and 3′ LD Amplimer (LD-Insert Screening Amplimer Sets, Clontech), electrophoresed in 1% agarose gels, cut out, and purified. The cDNA inserts were sequenced directly.

HIS3 and lacZ Reporter Gene Assays in Yeast—Yeast cells (5×10^{9}) of the target-reporter strain were transformed with 10 μg of each GAL-4 AD deletion mutant, and plated on SD/–L plates. The surviving colonies were

picked up and restreaked on SD/–L or SD/–HUL plus 0, 1, 5, 10, 20, 50, 100 and 200 mM 3-AT. The selection plates were incubated at 30°C for 4 days, and the 3-AT concentrations optimal for inhibiting HIS3 reporter gene expression were determined. The colonies of transformed GAL-4 AD deletion mutants were incubated in liquid SD/–L medium at 30°C until the OD_{600} of 1 ml reached 0.5–0.8. Liquid culture β-galactosidase assays using CPRG as a substrate were performed following the Yeast Protocols Handbook (Clontech). After 24 h incubation, measurements were carried out in triplicate; experiments were performed 3 times.

Transfection of siRNA and Semi-Quantitative RT-PCR Analysis—SH-SY5Y cells were plated at 80% confluency into 6 cm dishes 24 h prior to transfection. Cells were transfected with 400 pmol of each siRNA using 20 μl of LipofectAmine 2000 reagent (Invitrogen). The siRNA sequence of the sense strand Hesr1 siRNA was 5′-GCUAGAAAAAGCCGAGAUC-dTdT-3′. The sense strand of the control siRNA was 5′-AGAAGGAGCAGAUACCUCAdTdT-3′. These siRNAs were purified by HPLC (Greiner bio-one). cDNA was synthesized using the ThermoScript RT-PCR System (Invitrogen), starting from 0.5 μg of total RNA after 48 h incubation, according to the manufacturer's protocol. Semi-quantitative RT-PCR analysis was performed. After normalizing for GAPDH and the β-actin gene, endogenous *Hesr1* gene expression was compared by PCR using the oligonucleotides primers 5′-GGAGAGGCGCCGCTGTAGTTA-3′, and 5′-CAAGGGCGTGCGCGTCAAAGTA-3′.

Statistics—The results of quantitative experiments were analyzed by one-way ANOVA after Bartlett's test. Tukey-Kramer's HSD test was used to determine significant differences between each vector. In the case of the comparison in pairs, Student's *t*-test was adopted. Statistical relationships between two independent factors were determined by two-way ANOVA.

RESULTS

Comparison of Allele and Genotype Frequencies—By PCR genotyping of VNTR polymorphism in exon 15 of *DAT1* genes from 181 healthy Japanese individuals, five alleles, 6- (0.003), 7- (0.014), 9- (0.039), 10- (0.906), and 11-repeat (0.039), were recognized and their relative frequencies were calculated. In addition, seven genotypes, 6/10 (0.006), 7/10 (0.028), 9/9 (0.011), 9/10 (0.055), 10/10 (0.834), 10/11 (0.055), 11/11 (0.011), were observed. Most individuals are homozygous or heterozygous for the 10-repeat allele. The allele frequency obtained agrees with those of the previous study (*21*). We clarified differences in the nucleotide sequences between alleles by direct sequencing. The two type (A)s, the first type (D), type (F), and type (H) seem be constant repeat units, because every allele has them (*29*). The other units seem to be variable repeat units (Fig. 1a). A 6-repeat allele was cloned for first time from a Japanese population. In addition, an 11-repeat allele showed a novel sequence.

Down-Regulation of Reporter Gene Expression through the 3′ Non-Coding Region of the DAT1 Gene—To investigate the effect of the 3′ non-coding region of the *DAT1* gene, we compared the expression of reporter genes in the human embryonic kidney cell line HEK293, the human neuroblastoma cell line SH-SY5Y, the African green monkey kidney cell line COS-7, and the mouse neuroblastoma cell line Neuro2a (Fig. 1b). In SH-SY5Y, COS-7, and Neuro2a cells, the CP-Luc/exon15 (10-repeat) vector produced significantly lower luciferase activities than in cells transfected with the CP-Luc vector (Student's *t*-test, $P < 0.001$, $N = 4$). However, this down-regulation was not observed in HEK293 cells (Student's *t*-test, $P > 0.16$, $N = 4$). These results suggest that the 3′ non-coding region of the *DAT1* gene functions as a transcriptional or translational regulatory *cis*-element in gene expression.

The VNTR-Dependent Differences in Gene Expression Are Affected by Down-Regulation through the 3′ Non-Coding Region of the DAT1 Gene—In previous reports, VNTR-dependent differences in reporter gene expression have been examined using virus promoters in various cell lines (*29–31*). These findings prompted us to investigate the interaction between the native *DAT1* promoter and the 3′ non-coding region, and compare it among cell lines. To investigate differences in cell lines, we compared the expression of reporter genes with 6-, 7-, 9-, 10-, or 11-repeat alleles in several cell lines (Fig. 1c).

SH-SY5Y cells transfected with CP-Luc/6-, 7-, or 9-repeat vectors showed significantly higher levels of luciferase activity than cells transfected with CP-Luc/10- or 11-repeat vectors (one-way ANOVA followed by Tukey-Kramer's HSD test, $P < 0.05$, $N = 4$). In HEK293 cells, cells transfected with CP-Luc/6- and 11-repeat reporter vectors showed significantly lower luciferase activity than cells transfected with the CP-Luc/10-repeat vector (one-way ANOVA followed by Tukey-Kramer's HSD test, $P < 0.01$, $N = 4$). There were no significant differences between cells transfected with CP-Luc/7-, 9-, and 10-repeat vectors ($P > 0.1$). In COS-7 and Neuro2a cells, cells transfected with the CP-Luc/6-, 7-, 9- or 10-repeat vectors produced significantly higher activity than cells transfected with the CP-Luc/11-repeat vector (data not shown). In addition, the statistical relationship between alleles and the down-regulation of gene expression with the 3′ non-coding region of the *DAT1* gene was significant (two-way ANOVA, $P < 0.001$). These results indicate that the activity of the 3′ non-coding region of the *DAT1* gene as a *cis*-element is dependent on both the VNTR polymorphic alleles and the cell type.

Yeast One-Hybrid Screening—To identify the *trans*-acting factors involved in the down-regulation mediated by the 3′ non-coding region of *DAT1*, we carried out yeast one-hybrid screening with a target element containing a VNTR region of the 10-repeat allele in part of exon 15. Out of approximately 1×10^7 independent transformants with a GAL4-AD fusion cDNA library of human fetal brain, 141 yeast colonies grew on SD/–HUL plus 1mM 3-AT plates. These colonies were tested for β-galactosidase activity by β-gal colony-lift assay. Of the 17 β-galactosidase–positive clones, which turned blue within 30 min, two independent clones, Nos. 56 and 80, represented the basic helix-loop-helix (bHLH) transcriptional factor *Hesr1* gene (the Hairy/enhancer of split related transcriptional factor 1 with a YRPW motif, also named Hey1/HERP2/HRT1/CHF2) (*35–38*). The clone of No. 56 showed a deletion of 5 amino acids in the N-terminal region. On the other hand, the clone No. 80 had 96 extra

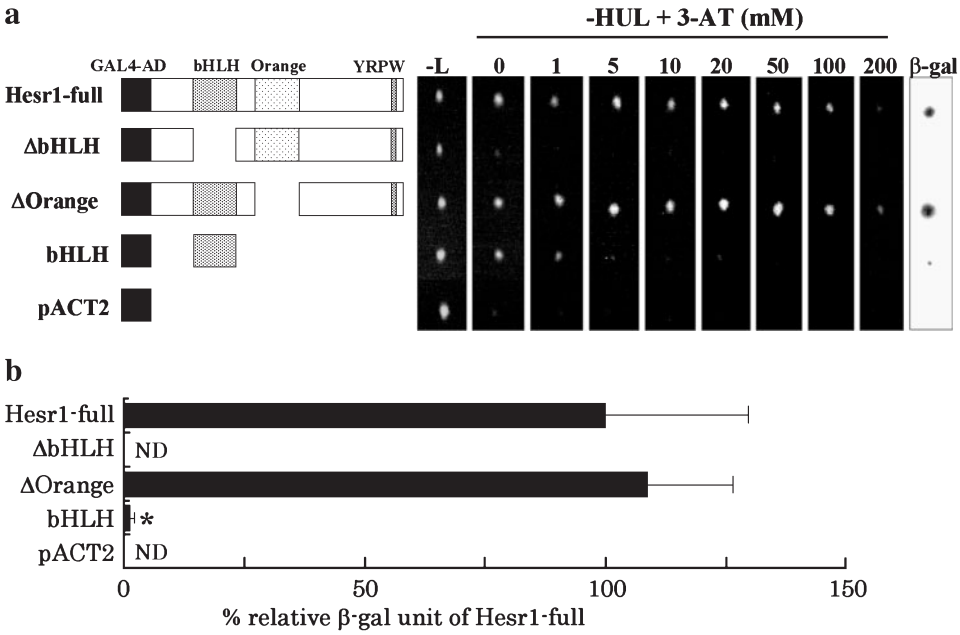

Fig. 2. **Comparison of Hesr1 activity in a yeast one-hybrid system.** A yeast strain containing integrated target-reporter vectors was transformed with expression vectors encoding the GAL4-AD fusion proteins, Hesr1, ΔbHLH, ΔOrange, and bHLH. The pACT2 vector was transformed as a negative control. (a) Comparison of reporter activities using the –H assay. Yeast cells transformed with each expression vector were restreaked on SD/–L or SD/–HUL plus 3-AT. The growth of each transformant after 4 days of incubation is shown in the panel. Cells transformed with Hesr1 and ΔOrange showed high reporter gene expression. No growth was detected with ΔbHLH. All transformants grew on SD/–L transformation selection plates (left panel). In the β-gal colony-lift assay, the ΔbHLH transformant did not turn blue after 30 min incubation (right panel). (b) Quantitative comparison of reporter gene activities in liquid culture β-gal assays with CPRG. Relative β-gal activities were determined by normalizing to the mean unit of the Hesr1 transformant. Cells with Hesr1 and ΔOrange showed significantly higher β-gal activities than other transformants (*$P < 0.001$, $N = 3$). After 24hr incubation, ΔbHLH and pACT2 showed little interaction (less than 0.1 unit of β-gal activity). The data represent the means ± SD of three independent transformation experiments.

nucleotides of the 5′-UTR. Both clones encoded bHLH, Orange domains, and a YPRW motif.

Comparison of the Activities of Hesr1 and Its Deletion Mutants in the Yeast One-Hybrid System—To determine which domain is necessary for the interaction of Hesr1 with the target element, a series of Hesr1 deletion mutants in a GAL4-AD fusion protein were expressed in the target-reporter strain of yeast, and the production of the reporter proteins was analyzed (Fig. 2). We tested two reporter genes, *HIS3* and *LacZ*. The activity of HIS3 was evaluated by –H viability assay with various concentrations of 3-AT, a competitive inhibitor of the HIS3 (Fig. 2a). Both the full-length gene and the mutant with the deletion of the Orange domain, a known repression domain (*39*), showed high reporter gene expression. Deletion of the bHLH domain resulted in a lack of activity. However, the bHLH domain alone showed low activity. We confirmed that these activities are independent of the reporter gene locus using a β-galactosidase colony-lift assay. The expression of these *HIS3* reporter genes was not found in control strains without a target element (data not shown).

Next, we performed a liquid culture β-galactosidase assay using chlorophenol red-β-D-galactopyranoside (CPRG) to compare the activities quantitatively (Fig. 2b). In this experiment, reporter gene expression showed a similar tendency to that seen in the –H assay (Fig. 2a). Yeast cells transformed with full-length or ΔOrange Hesr1 showed significantly higher reporter gene expression than cells transformed with any other vector (one-way ANOVA followed by Tukey-Kramer's HSD test, $P < 0.001$, $N = 3$). Deletion of the bHLH domain resulted in a lack of activity. Therefore, we conclude that Hesr1-dependent reporter gene expressions are dependent on their target element, and the bHLH domain is necessary for the activity.

In SH-SY5Y Cells, the Endogenous Hesr1 Functions as a Negative Regulator of the DAT1 Gene through the 3′ Non-Coding Region.—We tested the effect of endogenous *Hesr1* down-regulation on reporter gene expression with or without the 3′ non-coding region of the *DAT1* gene. The down-regulation of endogenous *Hesr1* was observed after transient transfection with a siRNA specific for *Hesr1*, but not with control siRNA (Fig. 3a). Cells transfected with a siRNA specific for *Hesr1* showed significantly higher luciferase activity than mock-treated cells or cells transfected with control siRNA (one-way ANOVA followed by Tukey-Kramer's HSD test, $P < 0.01$, $N = 3$). There was no significant difference between mock-treated cells and cells transfected with control siRNA ($P > 0.1$). In addition, the diminution of endogenous negative regulation on reporter gene expression was not observed without the 3′ non-coding region of the *DAT1* gene (Fig. 3b). These findings suggest that Hesr1 is involved in the negative regulation of the *DAT1* gene through its 3′ non-coding region in SH-SY5Y cells (Fig. 1b).

Hesr1 Mediates the Down-Regulation of DAT1 Gene Expression in Various Mammalian Cell Lines—We next investigated whether Hesr1-dependent regulation also occurs in other mammalian cell lines (Fig. 4). The CP-Luc/exon15 (10-repeat) reporter vector was transiently co-transfected with expression vectors encoding *Hesr1* into various cultured mammalian cell lines (Fig. 4a). Cells co-transfected with the *Hesr1* expression vector showed significantly lower luciferase activity than cells co-transfected with the expression vector encoding the epitope tag alone (one-way ANOVA followed by Tukey-Kramer's HSD test, $P < 0.001$, $N = 3$).

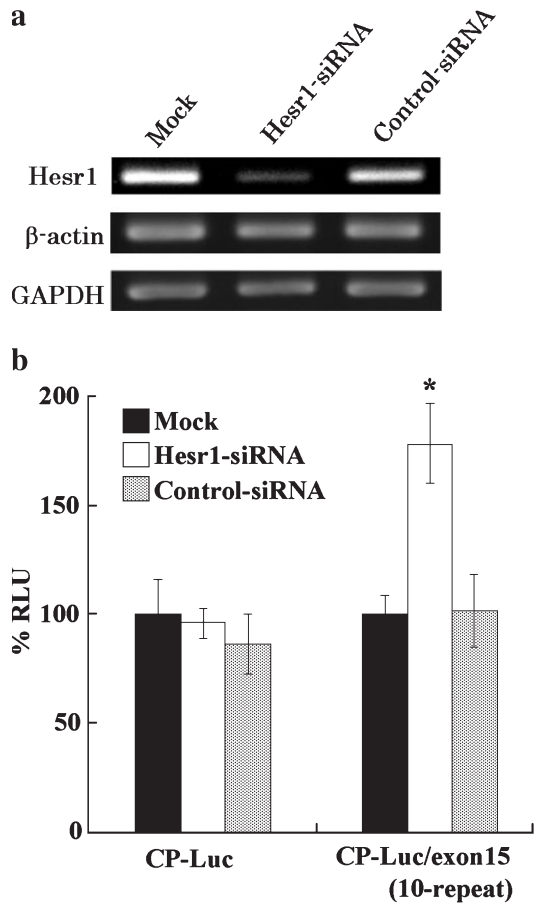

Fig. 3. **Down-regulation of endogenous Hesr1 by siRNA.** (a) The down-regulation of endogenous *Hesr1* was observed after transient transfection of a siRNA specific for *Hesr1* (upper panel). Semi-quantitative RT-PCR analysis was performed using GAPDH (lower panel) and β-actin (middle panel) genes as internal controls for normalizing. (b) Removal of the repressor activity of Hesr1 by siRNA. Cells were co-transfected with siRNAs and relative luciferase activities were determined as described in "MATERIALS AND METHODS." Relative luciferase activities with Hesr1 siRNA (white columns) and control siRNA (gray columns) as well as mock-treated cells (black columns) are shown. These data represent the means ± SD of three independent transfection experiments.

Having confirmed that these activities depend on the function of Hesr1 as a transcription factor, we compared reporter gene activities in cells transiently co-transfected with CP-Luc/exon15 (10-repeat) and expression vectors encoding a series of Hesr1 deletion mutants (Fig. 4b). In HEK293 cells, expression vectors with deletions of bHLH and Orange produced significantly lower activities than the full-length expression vector (one-way ANOVA followed by Tukey-Kramer's HSD test, $P < 0.001$, $N = 3$). However, cells co-transfected with vectors having a C-terminal region deletion had the high activity same as those co-transfected with the full-length vector. In COS-7 cells, the Hesr1 activity was not lost completely when the Orange domain was deleted (one-way ANOVA followed by Tukey-Kramer's HSD test, $P < 0.001$, $N = 3$); the deletion of the Orange domain would cause a lack of activity.

Hesr1 Affects VNTR-Dependent Differences in Reporter Gene Expression in HEK293 Cells—We compared differences in luciferase activities under the expression of Hesr1 to know whether the differences in luciferase activity among alleles are affected by Hesr1-dependent negative regulation. CP-Luc/exon15 reporter vectors containing each of the alleles were transiently co-transfected with expression vectors encoding Hesr1 or epitope tag only into HEK293 cells (Fig. 4c), which show no endogenous down-regulation of the *DAT1* gene through the 3′ non-coding region (Fig. 1b).

In cells co-transfected with the expression vector encoding the epitope tag alone, the difference in the levels of luciferase activity was the same as in cells transfected with the CP-Luc/exon15 reporter vector only (see Figs. 1c and 4c). In cells co-transfected with the expression vector encoding *Hesr1*, no significant difference between luciferase activities in cells transfected with CP-Luc/10-repeat and CP-Luc/6-repeat vectors was detected. Interestingly, the CP-Luc/7-repeat vector produced significantly higher levels of luciferase activity than the CP-Luc/10-repeat vector (one-way ANOVA followed by Tukey-Kramer's HSD, $P < 0.05$, $N = 3$). A significant statistical relationship between repeat alleles and the expression vector was observed (two-way ANOVA, $P < 0.05$). However, in SH-SY5Y, COS-7, and Neuro2a cells, this relationship was not observed (data not shown, two-way ANOVA, $P > 0.1$). The down-regulation of gene expression by the forced expression of Hesr1 changed the VNTR-dependence of reporter gene expression only in HEK293 cells.

Hesr1 Has a Splice Variant, Hesr1-12nt—We found an alternative splice variant of *Hesr1* by PCR cloning of a human fetal brain cDNA library. We named the variant *Hesr1-12nt*. The variant has an insertion of 12 nucleotides (4 amino acids) into the bHLH domain (Fig. 5a). The insertion is found at the 3′ end of exon 2, and has a specific splice donor site (Fig. 5b). The same cDNA clone has been deposited in the NEDO human cDNA-sequencing project (AK092437), implying that our clone is not an artifact.

To investigate the localization of the Hesr1-12nt protein, a YFP/Hesr1-12nt fusion protein was transiently expressed in HEK293 cells and its localization was compared with that of Hesr1. The YFP/Hesr1-12nt fusion protein localizes in nuclei, which is the same as YFP/Hesr1 (Fig. 5c). Next, we compared the activity in the down-regulation of the expression of the reporter gene containing the 3′ non-coding region of the *DAT1* gene (Fig. 5d). Hesr1-12nt showed a significantly lower activity than Hesr1 as a *trans*-acting factor.

C386A Candidate SNP of Human Hesr1—According to the NCBI database, there are many SNPs in the coding and non-coding regions of the *Hesr1* locus. Among the polymorphisms, we are interested in C386A (No. rs11553421) (*40*). This SNP causes the amino acid substitution L94M, and the leucine residue, which is conserved among other members of bHLH families, including the hairy/enhancer of the split family, seems to be important for the structure of the bHLH domain (Fig. 6a).

We investigated whether this substitution affects the activity of Hesr1 or Hesr1-12nt in the expression of the reporter gene with the 3′ non-coding region of the *DAT1* gene. The CP-Luc/exon15 (10-repeat) reporter vector was transiently co-transfected with expression vectors encod-

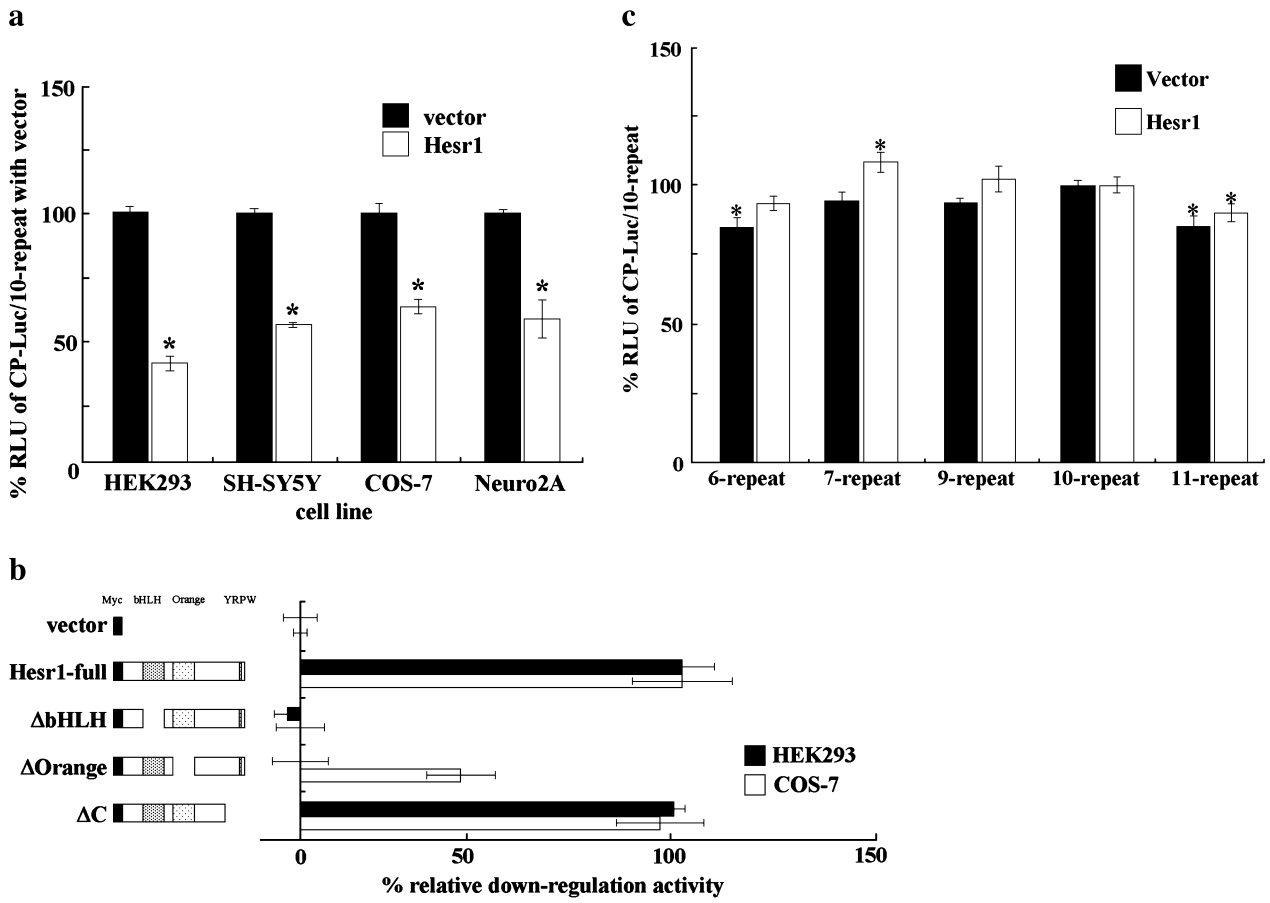

Fig. 4. **Comparison of reporter activity in various mammalian cell lines.** The CP-luc vector was independently transfected as a control for standardizing measurements, and each expression vector was co-transfected. Data represent the means ± SD of three independent transfection experiments. (a) Hesr1 down-regulates reporter gene expression through the 3′ non-coding region of the *DAT1* gene in various cell lines. Relative luciferase activities after transient transfection in cell lines HEK293, SH-SY5Y, COS-7, and Neuro2a are shown. Relative luciferase activities were determined by normalizing the standardized values to the mean of co-transfection with an expression vector encoding only the epitope tag in each cell line (black columns). Relative luciferase activity with Hesr1 is shown as white columns. (b) Cells were co-transfected with expression vectors encoding Hesr1, ΔbHLH, ΔOrange, and ΔC. The relative activities were determined by normalizing the standardized values to the mean of co-transfection with an expression vector encoding Hesr1. In HEK293 cells, the deletions of bHLH and Orange cause a lack of activity (*$P < 0.001$, $N = 3$). The deletion of the C-terminal region containing the YRPW motif had no repressive effect. In other cell lines (COS-7 cells shown as an example), the Hesr1 activity was not lost completely with the deletion of the Orange domain. (c) Hesr1 affects the difference in reporter gene expression between VNTR polymorphism alleles in HEK293 cells. The CP-luc vector was independently transfected as a control for standardizing each measurement and expression vectors were co-transfected. The relative activities were determined by normalizing these standardized values to the mean of the CP-luc/10-repeat co-transfected with each expression vector. The results of co-transfection with the expression vector encoding the epitope tag (black column, $P < 0.05$, $N = 3$) or Hesr1 (white column, $P < 0.05$, $N = 3$) are shown. The statistical significance in the relationships between repeat alleles and the co-transfected expression vector was detected by two-way ANOVA ($P < 0.05$).

ing a series of Hesr1 C386A mutants into HEK293 cells (Fig. 6b). C386A mutants were localized in the nuclei (data not shown). Hesr1 and Hesr1-12nt with C386A showed no activity (one-way ANOVA followed by Tukey-Kramer's HSD test, $P < 0.001$, $N = 3$). Moreover, C386A mutants did not inhibit down-regulation by wild-type Hesr1.

DISCUSSION

Down-Regulation of Gene Expression through the 3′ Non-Coding Region of the DAT1 Gene Is Influenced by trans-Acting Factor(s)—According to our experiments, the down-regulation of gene expression through the 3′ non-coding region of the *DAT1* gene depends on the cell line (Fig. 1b). This suggests that down-regulation is due to the characteristics of the cells, such as cell-specific signals and components. Some unknown *trans*-acting factors possibly work on the regulation of gene expression through the 3′ non-coding region of the *DAT1* gene. In our luciferase assay, it is unclear whether the effect is transcriptional or translational. However, the function of part of exon 15 containing the VNTR as a transcriptional regulatory region has been reported previously (*32*). The *trans*-acting factor(s) may affect the function of VNTR polymorphism of the *DAT1* gene and play an important role in dopamine-related polygenic disorders and behavioral traits.

The VNTR-Dependent Difference in Gene Expression Is Affected by Hesr1-Dependent Regulation—Several studies

suggest that *DAT1* VNTR polymorphism regulates gene expression *in vivo* (*26–28*) and *in vitro* (*29–31*). However, there is no agreement as to the difference in gene expression between alleles. In this study, we confirmed significant differences among alleles in reporter gene analysis of mammalian cell lines (Fig. 1c). Moreover, the down-regulation of gene expression with the 3′ non-coding region of the *DAT1* gene affects VNTR-dependent differences in reporter gene expression. We show that the Hesr1-dependent regulation affects the differences in reporter gene expression between alleles (Fig. 4c). These results suggest that VNTR polymorphism functions as a regulatory region, although the magnitude of the differences is small. Moreover, the accumulation of slight differences that may be secondary to the regulation of *DAT1* gene expression becomes significant *in vivo*. This supports the notion that VNTR polymorphism is a modest risk factor that is influenced by other risk factors in many dopamine-related polygenic disorders and behavioral traits. This provides a good explanation for the various results concerning the association between VNTR polymorphism and some polygenic neuropsychiatric disorders.

The Hesr1 Is a trans-Acting Factor—We identified Hesr1 as a *trans*-acting factor on the element containing the VNTR region using a yeast one-hybrid system. The *Hesr1* gene has been identified and characterized as a member of the subfamily hairy/enhancer of split (*35–38*). And the Hesr family genes, *Hesr1*, *2*, and *3*, encode the bHLH domain and Orange domain. Since the hairy/enhancer of split family genes act as transcriptional repressors and Notch effectors by negative regulation, Hesr may have similar functions (*38*). It has been reported that Hesr is up-regulated by the Notch intracellular cytoplasmic domain (NICD) similar to HES (*41*, *42*). Moreover, Hesr has a repressor activity and promotes cell differentiation (*42–44*).

Recently, an interaction between VNTR in intron 2 of the *5-HTT* gene and the transcription factor Y box binding protein 1 (YB-1) has been reported by using a yeast one-hybrid system (*45*). There is an advantage in screening *trans*-acting factors using a yeast one-hybrid system with two reporter genes with target elements integrated into the same yeast genome at different loci, because chromatin forming or remodeling of target elements play an important role *in vivo*. Our experiments using a yeast one-hybrid system suggest that the bHLH domain, which is indispensable for the DNA-binding activity of Hesr1, is necessary for specific *trans*-acting effects on part of *DAT1* exon 15 (Fig. 2). We believe that the regulation arises from direct binding. In the electrophoretic mobility shift assay (EMSA), recombinant GST-Hesr1 purified from *Escherichia coli* or the whole cell extract of COS-7 cells transfected with the Hesr1 expression vector was incubated with the radiolabeled repeat unit, types (D), (E), and (I). We could not detect specific shifted bands, although it has been suggested that ES cell proteins bind to repeat unit type (D) (*32*). The target element region used in the yeast one-hybrid system has no E-box or N-box known to be a bHLH binding consensus site. It is possible that functions of Hesr1 other than DNA-binding activity affect this down-regulation (*46*).

Hesr1 Functions as a Negative Regulator in Gene Expression through the 3′ Non-Coding Region of the DAT1 Gene in Mammalian Cell Lines—In mammalian cell lines, Hesr1 regulates the expression of reporter genes containing the 3′ non-coding region of the *DAT1* gene (Fig. 4a). Moreover, Hesr1 shows its highest activity in HEK293 cells, which show no endogenous down-regulation of the *DAT1* gene through the 3′ non-coding region (Fig. 1b). Moreover, that the negative regulation is dependent on bHLH and the Orange domain suggests that regulation through the 3′ non-coding region of the *DAT1* gene is caused by Hesr1 functioning as a transcriptional repressor (Fig. 4b). In addition, the knockdown of endogenous Hesr1 gene expression by RNAi caused the diminution of down-regulation on reporter gene expression depending on the presence of the 3′ non-coding region in SH-SY5Y cells (Fig. 3), but not on the expression of the endogenous *DAT1* gene (data not shown). These results suggest that regulation through the 3′ non-coding region of the *DAT1* gene does not depend simply on the expression of Hesr1, but depends on interactions with other factor(s) in mammalian cells. For example, Hesr family members are also known to undergo heterodimerization with other transcriptional factors, HES1, dHAND, eHAND, and Arnt (*43*, *47*, *48*). In addition, another bHLH protein, dHAND, regulates dopamine-β-hydroxylase in a manner dependent on the presence of another transcriptional factor Arix/Phox2a (*49*, *50*). The regulation of the expression of the *DAT1* gene with the 3′ non-coding region may be controlled by a complicated mechanism.

Interestingly, the deletion of the YRPW motif of Hesr1 does not affect the luciferase reporter activity (Fig. 4b). The YRPW tetrapeptide motif seems to be a variant of the WRPW motif in HES that is known to be active in the recruitment of TLE/Groucho and repression (*51*). However, the YRPW motif is not necessary for the activities of Hesr1 and Hesr1-12nt in the expression of the *DAT1* gene. It is possible that the motif has other functions. It was reported previously that the YRPW motif does not contribute to the repressor activity of Hesr2 (*43*).

Dopaminergic Neurons and Hesr1—Although it is debatable whether our *in vitro* assay reflects the *in vivo* situation due to several limiting conditions, the results are sufficient to raise a new question about the interaction between Hesr1 and the dopaminergic nervous system. Hesr1 is known to be a Notch effecter, and no evidence for a direct relationship with the dopamine system has been reported. However, in the relationship between the dopaminergic nervous system and angiogenesis, a dopamine stimulant to the presynaptic dopamine autoreceptor D2 receptor induces endocytosis of vascular endothelial growth factor receptor 2 (VEGFR2) (*52*), which is down-regulated by Hesr1 (*42*). In addition, transforming growth factor-β1 (TGF-β1), the level of which is elevated in the striatum of patients with Parkinson's disease and has been shown to cause a decrease in the number of dopaminergic neurons in the mouse ventral mesencephalon (*53*), controls the activation of Hesr1 in epithelial cells (*54*). A relationship between TGF-β signaling and the dopamine system in development has been reported (*55*).

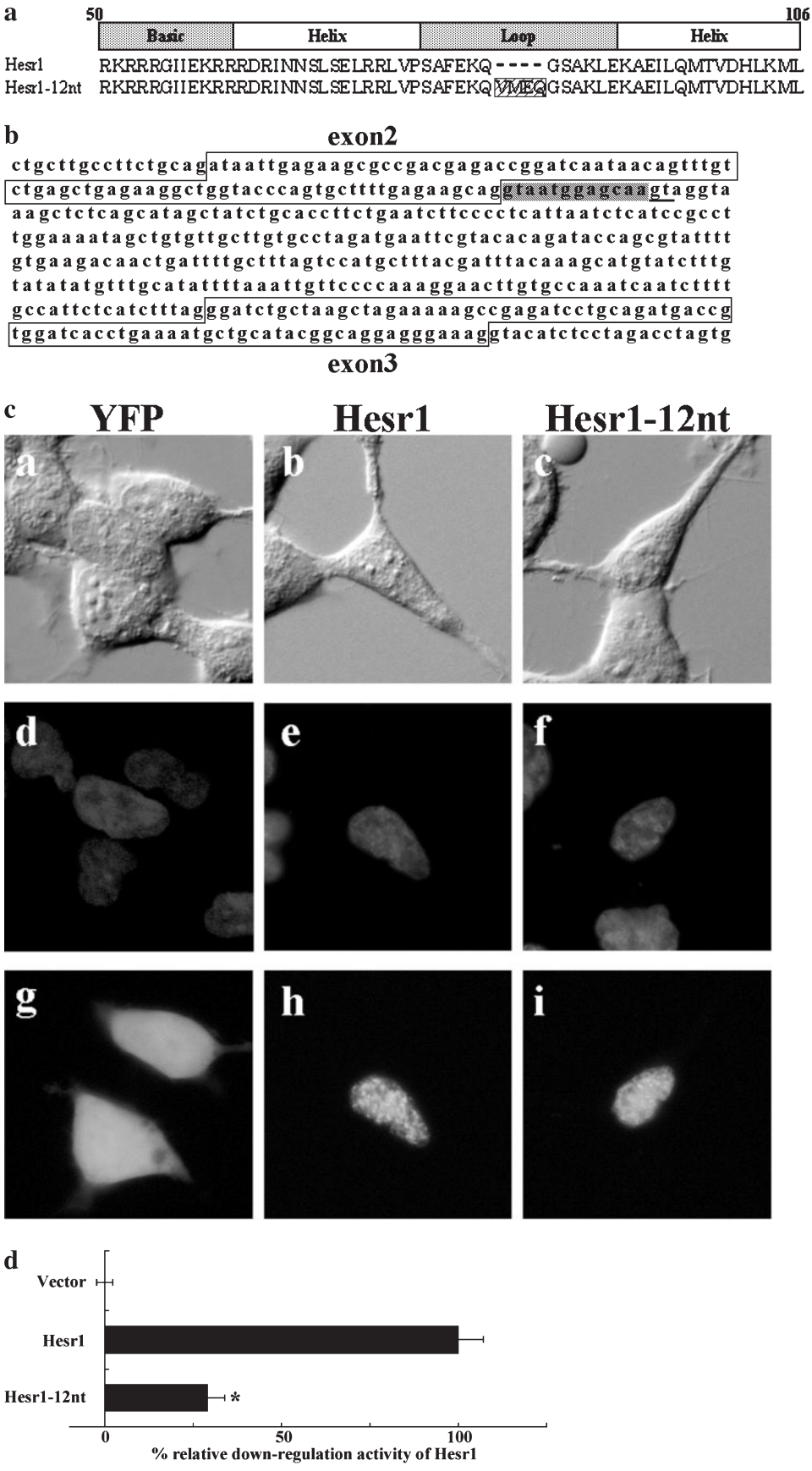

Fig. 5. **Comparison of Hesr1 and Hesr1-12nt.** (a) The bHLH domains of the Hesr1 and Hesr1-12nt proteins are aligned. The variant has an insertion of 4 amino acids in the "Loop" region. Amino acids Val-Met-Glu-Gln (shaded box) are inserted between 83Q and 84G in the center of the "Loop" region. (b) The partial genomic sequence of the human *Hesr1* gene is shown. Two open squares indicate exon2 and exon3. *Hesr1-12nt* has a 12-nucleotide insertion (gray box). There is a different splice site (underline). (c) Localization of the Hesr1 and Hesr1-12nt proteins in HEK293 cells. The pEYFP-C1 vector was transfected as a negative control (a, d, g). EYFP/Hesr1 localized in nuclei (b, e, h), and the localization of EYFP/Hesr1-12nt was the same (c, f, i). Nomarski interference microscopic images are shown (a-c). Hoechst33342 staining shows nuclei (d–f). Transiently transfected cells expressing EYFP were identified by fluorescence microscopy (g–i). (d) Comparison of repressor activities between Hesr1 and Hesr1-12nt. The relative activities calculated by normalizing the standardized values to the mean of Hesr1 are shown ($P < 0.01$, $N = 3$).

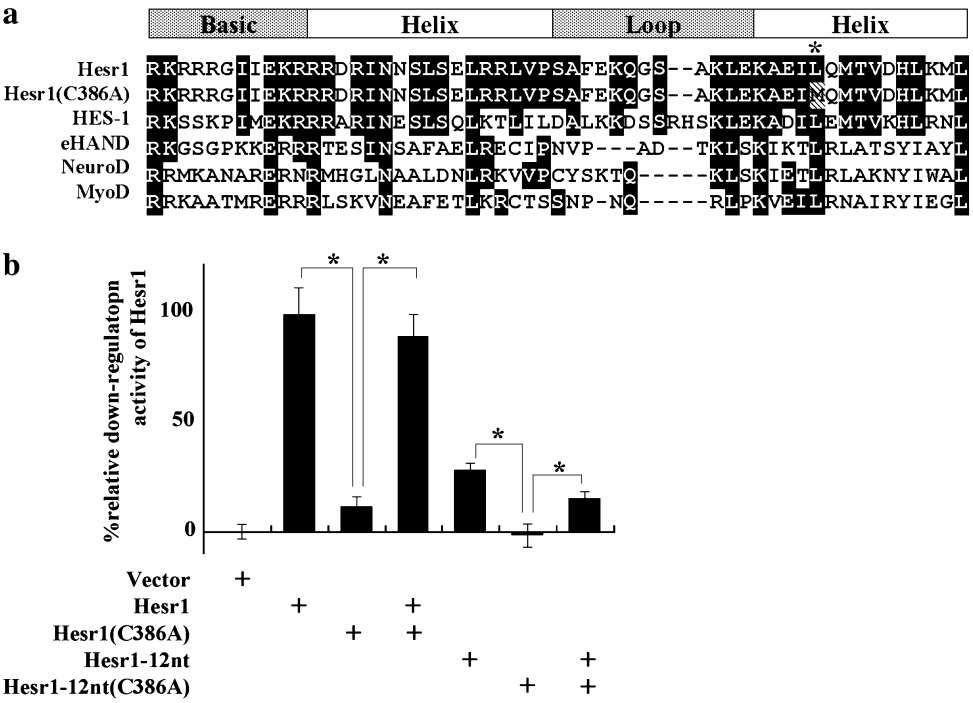

Fig. 6. **The candidate SNP of human *Hesr1*, C386A, causes a loss of down-regulatory activity.** (a) The bHLH domains of Hesr1, Hesr1 (C386A), and other members of the bHLH families are aligned. Identical amino acid sequences are shown (black boxes). C386A causes a single amino acid substitution (gray box), highlighted with an asterisk. (b) The activity of Hesr1 (C386A) on reporter gene expression through the 3′ non-coding region of the *DAT1* gene in HEK293 cells. The relative activities were determined by normalizing the standardized values to the mean of co-transfection with an expression vector encoding Hesr1. SNP causes a lack of activity (*P < 0.001, N = 3). Hesr1 (C386A) did not inhibit down-regulation by Hesr1. These data represent the means ± SD of three independent transfection experiments.

In rat embryos, dopaminergic neurons have been detected in the midbrain, adjacent to the floor plate where Hesr1 has been shown to be expressed (*36*). In addition, Hesr1 is expressed in the dorsal aorta (*36*), in which the walls contain localized bone morphogenetic proteins (BMP) that have been shown to be essential for the induction of TH in neural crest–derived cells (*55*). Members of the dpp subgroup, including BMP-2, the stimulation of which induces the expression of Hesr1 and cooperates in Notch signaling, and BMP-4, which promotes the expression of Hesr1 in neural stem cells isolated from the lateral ventricle of adult mouse brain, are capable of inducing TH expression in cultured neural cells from the mouse embryonic striatum (*56–58*). Although these references seem to provide circumstantial evidence only, it is enough to make the argument that there is an interaction between Hesr1 and the dopaminergic nervous system. It is possible that Hesr1 is involved in the differentiation, survival, or maintenance of dopaminergic neurons. According to a previous report, a deficit of both Hesr1 and Hesr2 results in embryonic death after day E9.5, but Hesr1 knockout mice show no observed major developmental or functional impairments (*59*). However, our findings suggest that the neuropsychiatric behavior of Hesr1 KO mice should be examined.

Polymorphism of the Hesr1 Gene—We should not overlook the fact that there are many SNPs in the coding and non-coding regions of the *Hesr1* locus. Some SNPs may affect the structure or function of the protein, specific gene expression, alternative splicing, and translation. It is possible that these SNPs are more important risk factors in dopamine-related polygenic disorders and behavioral traits.

In this study, we show that the C386A candidate SNP of the *Hesr1* gene causes a lack of activity itself on the expression of the reporter gene with the 3′ non-coding region of the *DAT1* gene (Fig. 6b). Therefore, we identified one of the SNPs with molecular functions that affect the activity of Hesr1. The data suggest that C386A polymorphism of the *Hesr1* gene might result in a knockdown of the function of Hesr1 *in vivo*. It is possible that C386A polymorphism of the *Hesr1* gene is an important risk factor in dopamine-related polygenic disorders and behavioral traits. Moreover, Hesr1 regulates the expression of many genes other than *DAT1*. Therefore, the polymorphism might be positively involved in many other polygenic disorders and behavioral traits.

We thank Dr. M.J. Bannon of Wayne State University School of Medicine at Detroit for kindly providing us with the DAT1-8317 plasmid. We also thank Dr. H. Kokubo, Dr. S. Suo, and Mr. A. Yoshikawa for valuable discussions and encouragement. S.F. is supported by a JSPS Research Fellowship for Young Scientists. This work was supported in part by grants from the Ministry of Health, Labor and Welfare and the Ministry of Education, Science, Culture, Sports and Technology, Japan.

REFERENCES

1. Bannon, M.J., Michelhaugh, S.K., Wang, J., and Sacchetti, P. (2001) The human dopamine transporter gene: gene organization, transcriptional regulation, and potential involvement in neuropsychiatric disorder. *Eur. Neuropsychopharmacol.* **11**, 449–455
2. Uhl, G.R. (2003) Dopamine transporter: Basic science and human variation of a key molecule for dopaminergic function, locomotion, and Parkinsonism. *Mov. Disord.* **18**, 71–80
3. Giros, B., el Mestikawy, S., Bertrand, L., and Caron, M.G. (1991) Cloning and functional characterization of a cocaine-sensitive dopamine transporter. *FEBS Lett.* **295**, 149–154
4. Kilty, J.E., Lorang, D., and Amara, S.G. (1991) Cloning and expression of a cocaine-sensitive rat dopamine transporter. *Science* **254**, 578–579
5. Shimada, S., Kitayama, S., Lin, C.L., Patel, A., Nanthakumar, E., Gregor, P., Kuhar, M., and Uhl, G.R. (1991) Cloning and

expression of a cocaine-sensitive dopamine transporter complementary DNA. *Science* **254**, 576–578
6. Giros, B., el Mestikawy, S., Godinot, N., Zheng, K., Han, H., Yang-Feng, T, and Caron, M.G. (1992) Cloning, pharmacological characterization, and chromosome assignment of the human dopamine transporter. *Mol. Pharmacol.* **42**, 383–90
7. Giros, B. and Caron, M.G. (1993) Molecular characterization of the dopamine transporter. *Trends Pharmacol. Sci.* **14**, 43–49
8. Gainetdinov, R.R., Sotnikova, T.D., and Caron, M.G. (2002) Monoamine transporter pharmacology and mutant mice. *Trends Pharmacol. Sci.* **23**, 367–373
9. Madras, B.K., Gracz, L.M., Fahey, M.A., Elmaleh, D., Meltzer, P.C., Liang, A.Y., Stopa, E.G., Babich, J., and Fischman, A.J. (1998) Altropane, a SPECT or PET imaging probe for dopamine neurons: III. Human dopamine transporter in postmortem normal and Parkinson's diseased brain. *Synapse* **29**, 116–127
10. Muller-Vahl, K.R., Berding, G., Brucke, T., Kolbe, H., Meyer, G.J., Hundeshagen, H., Dengler, R., Knapp, W.H., and Emrich, H.M. (2000) Dopamine transporter binding in Gilles de la Tourette syndrome. *J. Neurol.* **247**, 514–520
11. Krause, K.K., Dresel, S.H., Krause, J., Fougere, C., and Ackenheil, M. (2003) The dopamine transporter and neuroimaging in attention deficit hyperactivity disorder. *Neurosci. Biobehav. Rev.* **27**, 605–613
12. Brunswick, D.J., Amsterdam, J.D., Mozley, P.D., and Newberg, A. (2003) Greater availability of brain dopamine transporters in major depression shown by [99m Tc] TRODAT-1 SPECT imaging. *Amer. J. Psychiatry*. **160**, 1836–1841
13. Giros, B., Jaber, M., Jones, S.R., Wightman, R.M., and Caron, M.G. (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* **379**, 606–612
14. Jaber, M., Dumartin, B., Sagne, C., Haycock, J.W., Roubert, C., Giros, B., Bloch, B., and Caron, M.G. (1999) Differential regulation of tyrosine hydroxylase in the basal ganglia of mice lacking the dopamine transporter. *Eur. J. Neurosci.* **11**, 3499–3511
15. Fauchey, V., Jaber, M., Caron, M.G., Bloch, B., and Moine, C.L. (2000) Differential regulation of the dopamine D1, D2 and D3 receptor gene expression and changes in the phenotype of the striatal neurons in mice lacking the dopamine transporter. *Eur. J. Neurosci.* **12**, 19–26
16. Vandenbergh, D.J., Persico, A.M., Hawkins, A.L., Griffin, C.A., Li, X., Jabs, E.W., and Uhl, G.R. (1992) Human dopamine transporter gene (DAT1) maps to chromosome 5p15.3 and displays a VNTR. *Genomics* **14**, 1104–1106
17. Cook, Jr. E.H., Stein, M.A., Krasowski, M.D., Cox, N.J., Olkon, D.M., Kieffer, J.E., and Leventhal, B.L. (1995) Association of attention-deficit disorder and dopamine transporter gene. *Amer. J. Hum. Genet.* **56**, 993–998
18. Vandenbergh, D.J., Thompson, M.D., Cook, E.H., Bendahhou, E., Nguyen, T., Krasowski, M.D., Zarrabian, D., Comings, D., Sellers, E.M., Tyndale, R.F., George, S.R., O'Dowd, B.F., and Uhl, G.R. (2000) Human dopamine transporter gene: coding region conservation among normal, Tourette's disorder, alcohol dependence and attention-deficit hyperactivity disorder populations. *Mol. Psychiatry* **5**, 283–292
19. Segman, R.H., Cooper-Kazaz, R., Macciardi, F., Goltser, T., Halfon, Y., Dobroborski, T., and Shalev, A.Y. (2002) Association between the dopamine transporter gene and posttraumatic stress disorder. *Mol. Psychiatry* **7**, 903–907
20. Ueno, S. (2003) Genetic polymorphisms of serotonin and dopamine transporters in mental disorders. *J. Med. Invest.* **50**, 25–31
21. Mitchell, R.J., Howlett, S., Earl, L., White, N.G., McComb, J., Schanfield, M.S., Briceno, I., Papiha, S.S., Osipova, L., Livshits, G., Leonard, W.R., and Crawford, M.H. (2000) Distribution of the 3′ VNTR polymorphism in the human dopamine transporter gene in world populations. *Hum. Biol.* **72**, 295–304
22. Nakamura, Y., Koyama, K., and Matsushima, M. (1998) VNTR (variable number of tandem repeat) sequences as transcriptional, translational, or functional regulators. *J. Hum. Genet.* **43**, 149–152
23. Battersby, S., Ogilvie, A.D., Smith, C.A.D., Blackwood, D.H.R., Muir, W.J., Quinn, J.P., Fink, G., and Harmar, A.J. (1996) Structure of a variable number tandem repeat of the serotonin transporter gene and association with affective disorder. *Psychiatr. Genet.* **6**, 177–181
24. Fiskerstrand, C.E., Lovejoy, E.A., and Quinn, J.P. (1999) An intronic polymorphic domain often associated with susceptibility to affective disorders has allele dependent differential enhancer activity in embryonic stem cells. *FEBS Lett.* **458**, 171–174
25. Mackenzie, A. and Quinn J. (1999) A serotonin transporter gene intron 2 polymorphic region, correlated with affective disorders, has allele-dependent differential enhancer-like properties in the mouse embryo. *Proc. Natl. Acad. Sci. USA* **96**, 15251–15255
26. Heinz, A., Goldman, D., Jones, D.W., Palmour, R., Hommer, D., Gorey, J.G., Lee, K.S., Linnoila, M., and Weinberger, D.R. (2000) Genotype influences in vivo dopamine transporter availability in human striatum. *Neuropsychopharmacology* **22**, 133–139
27. Jacobsen, L.K., Staley, J.K., Zoghbi, S.S., Seibyl, J.P., Kosten, T.R., Innis, R.B., and Gelernter, J. (2000) Prediction of dopamine transporter binding availability by genotype: a preliminary report. *Amer. J. Psychiatry*. **157**, 1700–1703
28. Mill, J., Asherson, P., Browes, C., D'Souza, U., and Craig, I. (2002) Expression of the dopamine transporter gene is regulated by the 3′UTR VNTR: Evidence from brain and lymphocytes using quantitative RT-PCR. *Amer. J. Med. Genet.* **114**, 975–979
29. Fuke, S., Suo, S., Takahashi, N., Koike, H., Sasagawa, N., and Ishiura, S. (2001) The VNTR polymorphism of the human dopamine transporter (DAT1) gene affects gene expression. *Pharmacogenomics J.* **1**, 152–156
30. Miller, G.M. and Madras, B.K. (2002) Polymorphisms in the 3′-untranslated region of human and monkey dopamine transporter genes affect reporter gene expression. *Mol. Psychiatry* **7**, 44–55
31. Inoue-Murayama, M., Adachi, S., Mishima, N., Mitani, H., Takenaka, O., Teraco, K., Hayasaka, I., Ito, S., and Murayama, Y. (2002) Variation of variable number of tandem repeat sequences in the 3′-untranslated region of primate dopamine transporter genes that affects reporter gene expression. *Neurosci. Lett.* **334**, 206–210
32. Michelhaugh, S.K., Fiskerstrand, C., Lovejoy, E., Bannon, M.J., and Quinn, J.P. (2001) The dopamine transporter gene (SLC6A3) variable number of tandem repeats domain enhances transcription in dopamine neurons. *J. Neurochem.* **79**, 1033–1038
33. Sacchetti, P., Brownschidle, L.A., Granneman, G.J., and Bannon, M.J. (1999) Characterization of the 5'-flanking region of the human dopamine transporter gene. *Brain. Res. Mol. Brain. Res.* **74**, 167–174
34. Kouzmenko, A.P., Pereira, A.M., and Singh, B.S. (1997) Intronic sequences are involved in neural targeting of human dopamine transporter gene expression. *Biochem. Biophys. Res. Commun.* **240**, 807–811
35. Kokubo, H., Lun, Y., and Johnson, R.L. (1999) Identification and expression of novel family of bHLH cDNAs related to Drosophila hairy and enhancer of split. *Biochem. Biophys. Res. Commun.* **260**, 459–465
36. Leimeister, C., Externbrink, A., Klamt, B., and Gessler, M. (1999) Hey genes: a novel subfamily of hairy- and Enhancer of split related genes specifically expressed during mouse embryogenesis. *Mech. Dev.* **85**, 173–177
37. Nakagawa, O., Nakagawa, M., Richardson, J.A., Olson, E.N., and Srivastava, D. (1999) HRT1, HRT2, and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somatic, and pharyngeal arch segments. *Dev. Biol.* **216**, 72–84

38. Iso, T., Kedes, L., and Hamamori, Y. (2003) HES and HERP families: multiple effectors of the Notch signaling pathway. *J. Cell Physiol.* **194**, 237–255
39. Dawson, S.R., Turner, D.L., Weintraub, H., and Parkhurst, S.M. (1995) Specificity for the hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression. *Mol. Cell. Biol.* **15**, 6923–6931
40. Buetow, K.H., Edmonson, M.N., and Cassidy, A.B. (1999) Reliable identification of large numbers of candidate SNPs from EST data. *Nat Genet.* **21**, 323–325
41. Wang, W., Campos, A.H., Prince, C.Z., Mou, Y., and Pollman, M.J. (2002) Coordinate notch3-hairy-related transcription factor pathway regulation in response to arterial injury. *J. Biol. Chem.* **277**, 23165–23171
42. Henderson, A.M., Wang, S.J., Taylor, A.C., Aitkenhead, M., and Hughes, C.C. (2001) The basic helix-loop-helix transcription factor HESR1 regulates endothyelial cell tube formation. *J. Biol. Chem.* **276**, 6169–6176
43. Iso, T., Sartorelli, V., Poizat, C., Iezzi, S., Wu, H.Y., Chung, G., Kedes, L., and Hamamori, Y. (2001) HERP, a novel heterodimer partner of HES/E(spl) in notch signaling. *Mol. Cell. Biol.* **21**, 6080–6089
44. Sakamoto, M., Hirata, H., Ohtsuka, T., Bessho, Y., and Kageyama, R. (2003) The basic helix-loop-helix genes hesr1/hey1 and hesr2/hey2 regulate maintenance of neural precursor cells in the brain. *J. Biol. Chem.* **278**, 44808–44815
45. Klenova, E., Scott, A.C., Rpberts, J., Shamsuddin, S., Lovejoy, E.A., Bergmann, S., Bubb, V.J., Royer, H.D., and Quinn, J.P. (2004) YB-1 and CTCF differentially regulate the 5-HTT polymorphic intron 2 enhancer which predisposes to a variety of neurological disorders. *J. Neurosci.* **24**, 5966–5973
46. Kamakura, S., Oishi, K., Yoshimatsu, T., Nakafuku, M., Masuyama, N., and Gotoh, Y. (2004) Hes binding to STAT3 mediates crosstalk between Notch and JAK–STAT signaling. *Nat. Cell Biol.* **6**, 547–554
47. Chin, M.T., Maemura, K., Fukumoto, S., Jain, M.K., Layne, M.D., Watanabe, M., Hsieh, C.M., and Lee, M.E. (2000) Cardiovascular basic helix loop helix factor 1, a novel transcriptional repressor expressed preferentially in the developing and adult cardiovascular system. *J. Biol. Chem.* **275**, 6381–6387
48. Firulli, B.A., Hadzic, D.B., McDaid, J.R., and Firulli, A.B. (2000) The basic helix-loop-helix transcription factors dHAND and eHand exhibit dimerization characteristics that suggest complex regulation of function. *J. Biol. Chem.* **275**, 33567–33573
49. Xu, H., Firulli, A.B, Zhang, X., and Howard, M.J. (2003) HAND2 synegistically enhances transcription of dopamine-β-hydroxylase in the presence of Phox2a. *Dev. Biol.* **262**, 183–193
50. Rychlik, J.L., Gerbasi, V., and Lewis, E.J. (2003) The interaction between dHAND and Arix at the dopamine-β-hydroxylase promoter region is independent of direct dHAND binding to DNA. *J. Biol. Chem.* **278**, 49652–49660
51. Fisher, A.L. and Caudy, M. (1998) Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. *Genes Dev.* **12**, 1931–1940
52. Basu, S., Nagy, J.A., Pal, S., Vasile, E., Eckelhoefer, I.A., Bliss, V.S., Manseau, E.J., Dasgupta, P.S., Dvorak, H.F., and Mukhopadhyay, D. (2001) The neurotransmitter dopamine inhibits angiogenesis induced by vascular permeability factor/vascular endothelial growth factor. *Nat. Med.* **7**, 569–574
53. Sanchez-Capelo, A., Colin, P., Guibert, B., Biguet, N.F., and Mallet, J. (2003) Transforming growth factor beta1 overexpression in the nigrostriatal system increases the dopaminergic deficit of MPTP mice. *Mol. Cell. Neurosci.* **23**, 614–625
54. Zavadil, J., Cermak, L., Soto-Nieves, N., and Bottinger, E.P. (2004) Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. *EMBO J.* **23**, 1155–1165
55. Farkas, L.M., Dunker, N., Roussa, E., Unsicker, K., and Krieglstein, K. (2003) Transforming growth factor-beta(s) are essential for the development of midbrain dopaminergic neurons in vitro and in vivo. *J. Neurosci.* **23**, 5178–5186
56. Stull, N.D., Jung, J.W., and Iacovitti, L. (2001) Induction of a dopaminergic phenotype in cultured striatal neurons by bone morphogenetic proteins. *Brain. Res. Dev. Brain. Res.* **130**, 91–98
57. Dahlqvist, C., Blokzijl, A., Chapman, G., Falk, A., Dannaeus, K., Ibanez, C.F., and Lendahl, U. (2003) Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. *Development* **130**, 6089–6099
58. Takizawa, T., Ochiai, W., Nakashima, K., and Taga, T. (2003) Enhanced gene activation by Notch and BMP signaling crosstalk. *Nucleic Acids Res.* **31**, 5723–5731
59. Fischer, A., Schumacher, N., Maier, M., Sendtner, M., and Gessler, M. (2004) The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev.* **18**, 901–911