mini-gene assays using fibroblasts from DM patients [58]. The expression of insulin receptors was altered to predominantly the non-muscle type in DM patients, and a contribution by CUGBP1 was indicated in this insulin receptor abnormality [59]. The function of the chloride channel was reduced in mice over-expressing the CTG repeat, and this was attributed to the abnormal splicing of the chloride channel premature mRNA confirmed in these mice and DM1 patients [60]. The skeletal muscle ryanodine receptor and sarcoplasmic/ endoplasmic reticulum calcium-ATPase are also candidate genes for altered splicing in mice over-expressing the CTG repeat and in DM1 patients [61]. CUGBP1 has been reported to bind at the intron of the chloride channel gene [62]. Myotubularin-related 1 was found to change its splicing patterns in DM1 muscle cells [63]. The expression pattern of tau protein was altered in transgenic mice carrying the DMPK region of the human genome containing a long CTG repeat [37], and abnormal tau RNA maturation was observed in DM1 patients [64]. Splicing abnormalities were observed for NMDA and amyloid beta precursor protein in DM1 patients, and decreased expression of MBNL1 was also detected in the same samples [65]. These data lead us to conclude that the splicing patterns of many genes change in the presence the expressed long CTG triplet repeat and that DM could be categorized as a "splicing syndrome."

Concluding remarks

Recent evidence has suggested an unexpected mechanism of DM pathogenesis (Fig. 3). The expanded CTG triplet repeat expressed in DM patients can itself have a physiological function that leads to abnormal gene expression through its binding to RNA-binding proteins, resulting in the depletion of these proteins. This fact strongly indicates that the purpose of RNA molecules is not only to mediate the transfer of DNA information into protein but also to serve as physiologically functioning molecules in the cell. Recently, the functions of many small RNAs have been reported, revealing that such RNAs work ubiquitously in cellular gene systems. Investigations on DM pathogenesis have also been able to uncover new possibilities for RNA functions.

There are still many questions to be answered. The normal functions of RNA-binding proteins such as CUGBP1 (or CELF family proteins) and MBNL1 are unclear, as are their target RNAs. CUGBP1 does not seem to be a key factor in binding long CUG hairpin RNA, although some evidence implicates CUGBP1 in DM pathogenesis. MBNL1, however, does appear to directly bind the CUG repeat hairpin RNA, but the pathway involving MBNL1 has not been elucidated. It is possible that CUGBP1 (and CELF family proteins) and MBNL1 work together, and the possibility remains that another

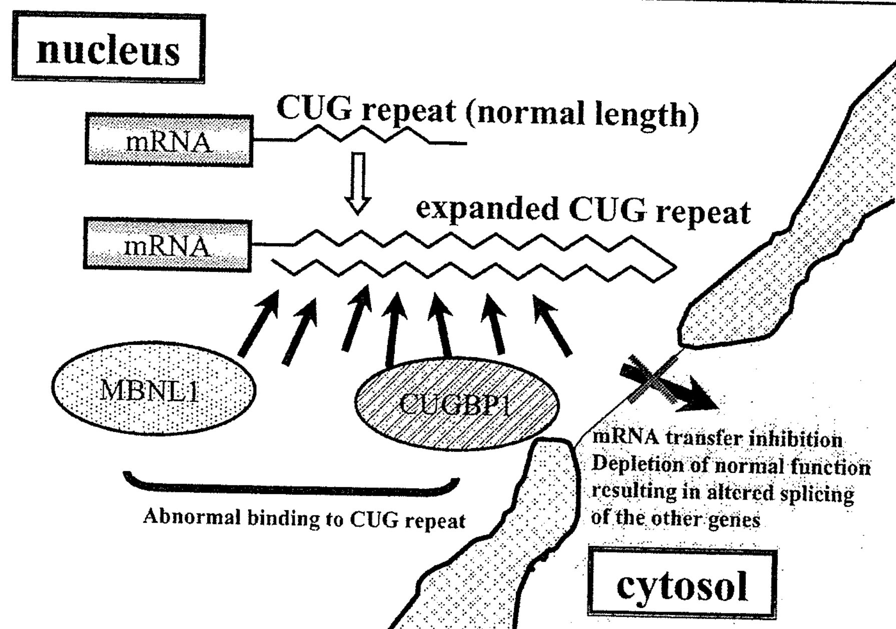

Figure 3. Scheme for the molecular pathogenesis of myotonic dystrophy.

new candidate RNA-binding protein is involved in DM pathogenesis. These investigations and future studies on the molecular basis of myotonic dystrophy will help to establish new methods for DM therapy.

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Notes & Tips

A simple, one-step cloning method to obtain long artificial triplet repeats

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Tandem trinucleotide repeats are common in the genomes of many organisms. In humans, triplet repeat expansion causes various hereditary diseases such as Huntington's disease [1] and myotonic dystrophy [2–4]. Because these repeats must involve abnormal physiological functions at the transcribed mRNA or translated protein level, it is critical to establish an easy method for making constructs with artificially expanded triplet repeats.

Previously, we reported a method of obtaining long CTG/CAG triplet repeats using a polymerase chain reaction (PCR)[1] with $(CTG)_7$ and $(CAG)_7$ primers without a template (i.e., nontemplate PCR method) [5]. In this method, we needed to ligate the expanded triplet repeat into blunt-ended plasmid vectors. Therefore, we sometimes had difficulty in adapting the codon frame, and if we wanted to use restriction endonucleases with sticky ends, we needed to clone the repeat into a specific cloning vector, such as pUC118, in the first step and then use multiple subcloning steps to obtain the final construction.

Here we report an improved technique for subcloning expanded triplet repeats into vectors with any restriction sites directly (Fig. 1). The primers (10 μM) *Eco*RI–CTG7 (5′-GGAATTCTAACTGCTGCTGCTGCTGCTGCTG-3′) and CAG10 (5′-CAGCAGCAGCAGCAGCAGCAGCA GCAGCAG-3′) were mixed and used for PCR without any template (tube A). We used LA–*Taq* DNA polymerase with GC buffer (TaKaRa, Tokyo, Japan) and a standard PCR protocol consisting of 25 cycles at 95 °C for 15 s, 57 °C for 1 min, and 72 °C for 1.5 min in an Eppendorf Mastercycler personal thermal cycler (Eppendorf Scientific, Hamburg, Germany). The reaction volume was 20 μl. Similarly, 10 μM each of primers *Xba*I–CAG7 (5′-GCTCTAGACAGCAG CAGCAGCAGCAGCAG-3′) and CTG10 (5′-CTGCTG CTGCTGCTGCTGCTGCTGCTG-3′) were mixed, and we conducted another PCR (tube B) in exactly the same way as with tube A. Then we mixed 10 μl each of the solutions from tubes A and B (tube C, final 20 μl) and continued the PCR. The PCR reagents, reaction conditions, and cycle program for tube C were exactly the same as for tubes A and B. The final PCR product was electrophoresed in 1% agarose gels, and the DNA extracted from the agarose was cut using an *Eco*RI/*Xba*I double digestion.

A derivative of the vector pUC118 was used in the experiment and was cut using *Eco*RI and *Nhe*I (note that the *Nhe*I and *Xba*I sites have compatible cohesive ends). The vector and triplet repeat were ligated and then transformed into *Escherichia coli* XL-1 blue. The positive colonies were selected, and the sequence was confirmed using a Beckman CEQ8000 DNA sequencer (Beckman Coulter, Fullerton, CA, USA).

The PCR products from tubes A and B produced a smear (Fig. 2A), indicating that various lengths of tandem repeats with a restriction site at one end were made successfully in tubes A and B. The PCR product from tube C also produced a smear, and we expected it to consist of tandem repeats with restriction sites at both ends (5′ and 3′). We collected the tandem repeats from tube C, cut them using *Eco*RI/*Xba*I, and ligated them into a vector. Fig. 2B shows the result of a tandem repeat successfully ligated into a vector at the predicted restriction site. Sequence analysis revealed that the vector contained a maximum of more than 100 tandem repeats (Fig. 2B, lane 3). This method is suitable for any plasmid vector. We also tested the vector pGEX and had success (data not shown). The upper limit of the number of repeats is dependent on the insert retention capacity of the vector; we always observed instability with more than 100 CTG triplet repeats in a plasmid. It is necessary to determine a suitable vector, including an artificial chromosome, to clone more than 1000 tandem triplet repeats. Nevertheless, the protocol presented here is still effective for cloning long triplet repeats.

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[1] *Abbreviation used:* PCR, polymerase chain reaction.

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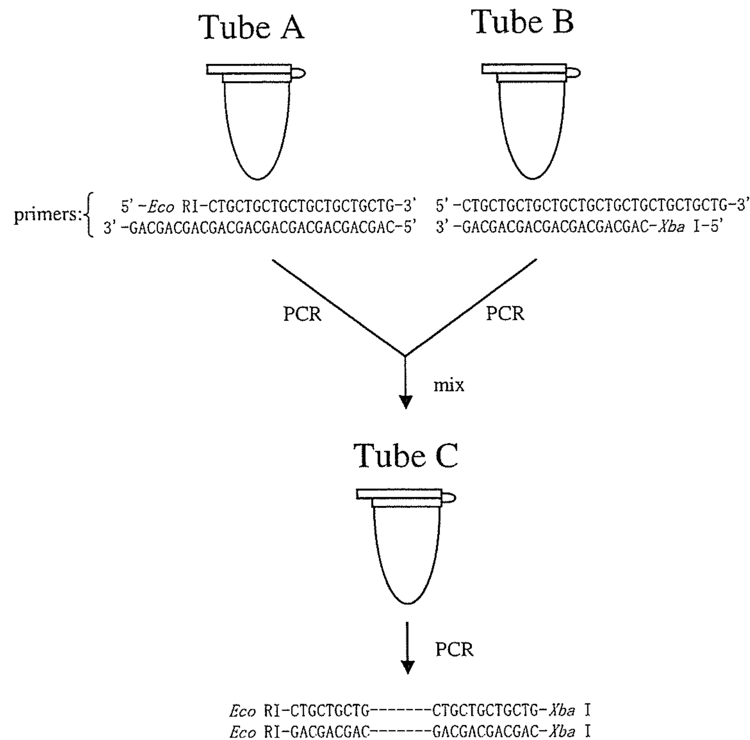

Fig. 1. Scheme used to obtain long triplet repeats with one-step cloning. The final PCR product in tube C consists of various lengths of CTG/CAG repeats with *Eco*RI/*Xba*I sites at each end.

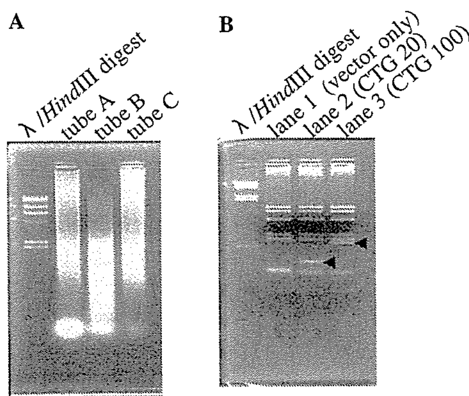

Fig. 2. (A) PCR products from tubes A, B, and C were electrophoresed in 1% agarose gels. All of the lanes show smears, indicating wide variation in the numbers of tandem triplet repeats. (B) The subcloned constructs with the CTG/CAG triplet repeat were cut using *Dra*I and electrophoresed in 2% agarose gels. The arrows show fragments containing the CTG/CAG repeat region. The rate of movement of each band revealed that the clone in lane 3 contains approximately 100 CTG/CAG repeats.

We consider that the key point in this protocol is the use of a proofreading PCR polymerase. Tandem repeats anneal with each other at slipped CTG–CAG repeat positions, and the exonuclease activity of a proofreading polymerase digests the mismatched 3′ end. Then the proofreading polymerase starts a 5′–3′ extension reaction. The repeated reaction of slipped annealing and proofreading extension results in the production of a long tandem repeat. Although we have not tested it, we should be able to skip the tube B step by adding primer *Xba*I–CAG7 to the PCR product in tube A and performing the PCR step in tube C to amplify the desired PCR product.

Consequently, we can make tandem repeat constructs at any desired restriction site. The merit of this method is that we can adopt many other tandem repeats for construction involving one-step ligation.

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Hesr1 Knockout Mice Exhibit Behavioral Alterations Through the Dopaminergic Nervous System

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The basic helix-loop-helix (bHLH) transcriptional factor *Hesr1* gene (hairy and enhancer of split-related 1, also called *Hey1/HRT1/CHF2/HERP2*) has been identified and characterized as a member of the subfamily of *hairy/Enhancer of split*, and shown to be involved in cardiovascular and neural development. We report that HESR1 binds directly to a part of the 3′ non-coding region of the human dopamine transporter (*DAT1*) gene and represses the endogenous *DAT1* gene in HEK293 cells. To investigate functions of the *HESR1* gene in the dopaminergic nervous system in vivo, we analyzed the expressions of dopamine-related genes in the postnatal day 0 whole brains of *Hesr1* knockout mice by real-time RT-PCR analysis. Several dopamine-related genes, such as *DAT*, dopamine receptors *D1*, *D2*, *D4*, and *D5*, were significantly upregulated. Moreover, young adults of *Hesr1* knockout mice showed a decrease in spontaneous locomotor activity and a reduction in exploratory behavior or behavioral responses to novelty in the open-field, and elevated plus-maze tests. These results indicate that the *HESR1* gene is related to neuropsychiatric disorders and behavioral traits through the dopaminergic nervous system. © 2006 Wiley-Liss, Inc.

Key words: attention deficit hyperactivity disorder (ADHD); behavior; dopamine transporter; major depression; schizophrenia; hesr1

The dopaminergic nervous system plays important roles in regulating locomotion, cognition, reward, affect, and hormone release (Missale et al., 1998; Bannon et al., 2001; Uhl, 2003). Dopamine and its related genes are thought to be involved in neuropsychiatric disorders and behavioral traits. The dopamine transporter (*DAT1*) gene is particularly important among the many dopamine-related genes. The levels of DAT are reduced in Parkinson's disease (PD) and elevated in attention deficit hyperactivity disorder (ADHD), Tourette's syndrome, and major depression (Madras et al., 1998; Muller-Vahl et al., 2000; Krause et al., 2003; Brunswick et al., 2003). In addition, several psychoactive drugs, including cocaine, amphetamine, and methylphenidate, are known to inhibit dopamine reuptake by the DAT protein (Giros et al., 1991, 1992, 1993; Kilty et al., 1991; Shimada et al., 1991). Mice lacking the *DAT* gene decreased intraneural storage of dopamine, and showed spontaneous hyperlocomotion and the downregulation of several dopamine-related genes, such as dopamine receptor *D1*, *D2*, and tyrosine hydroxylase (*TH*) (Giros et al., 1996; Jaber et al.,

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Abbreviations used: 3-AT, 3-amino-1, 2, 4-trizole; ADHD, attention deficit hyperactivity disorder; bHLH, basic helix-loop-helix; BMP, bone morphogenetic proteins; DAT, dopamine transporter; DR, dopamine receptor; HEK293, human embryonic kidney 293; HES, hairy/enhancer of split; hesr1, hairy/enhancer of split related transcriptional factor 1 with YRPW motif; SNP, single nucleotide polymorphism; TGF-β1, transforming growth factor-β 1; TH, tyrosine hydroxylase; Tukey-Kramer HSD test, Tukey-Kramer honestly significant difference test; VNTR, variable number of tandem repeat.

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1999; Fauchey et al., 2000; Gainetdinov et al., 2002). There is a transcriptional regulatory *cis*- element in a part of the 3′ non-coding region included in exon 15 of the *DAT1* gene (Michelhaugh et al., 2001). The *cis*- element contains a 40-bp variable number tandem repeat (VNTR) polymorphism and its surroundings (Vandenbergh et al., 1992; Michelhaugh et al., 2001). VNTR polymorphism is known to be associated with many neuropsychiatric disorders, such as ADHD, PD, and drug abuse (Cook et al., 1995; Vandenbergh et al., 2000; Ueno S., 2003), and modified gene expression depending on the genotype in vivo (Heinz et al., 2000; Jacobsen et al., 2000; Mill et al., 2002) and in mammalian cell lines (Fuke et al., 2001, 2005; Miller and Madras, 2002; Inoue-Murayama et al., 2002). In our previous report, we identified and characterized the *HESR1* gene as a *trans*-acting repressor of gene expression through the *cis*- element in the 3′ non-coding region of the *DAT1* gene (Fuke et al., 2005).

The *Hesr* family genes, *Hesr1*, *2*, and *3*, have been identified as a *hairy/enhancer of split*-type bHLH gene, and characterized as a direct transcriptional target of the Notch signaling pathway and its effector (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999, 2000; Iso et al., 2001, 2003; Henderson et al., 2001; Wang et al., 2002; Sakamoto et al., 2003). The *Hesr* family genes encode the bHLH domain and Orange domain, and HESR proteins bound to E-box or N-box, known to be bHLH binding consensus sites, and repress the expression of target genes (Iso et al., 2001, 2003; Nakagawa et al., 2000). Each *Hesr* gene is known to play an important role in development or cell differentiation (Henderson et al., 2001; Satow et al., 2001; Wang et al., 2002; Sakamoto et al., 2003; Dahlqvist et al., 2003; Zavadil et al., 2004). The *Hesr1/2* double mutation is known to be embryonic lethal due to cardiac and vascular dysplasia, and they have been shown to be involved in neural development (Sakamoto et al., 2003; Fischer et al., 2004; Kokubo et al., 2005). In addition, *Hesr2* knockout mice show heart dysfunction (Kokubo et al., 2004). However, *Hesr1* single knockout mice show no obvious morphological or anatomical phenotype (Fischer et al., 2004; Kokubo et al., 2005). On the other hand, interactions between the *Hesr1* gene and transforming growth factor-β (TGF-β) signaling or bone morphogenetic proteins (BMP) signaling (Dahlqvist et al., 2003; Takizawa et al., 2003; Zavadil et al., 2004), which function in the differentiation or the maintenance of dopaminergic nervous system, have been reported (Stull et al., 2001; Sanchez-Capelo et al., 2003; Farkas et al., 2003). It is possible that the *HESR1* gene plays important roles in the dopaminergic nervous system with the regulation of dopamine-related genes, such as the *DAT* gene. Therefore, the deficit of the *Hesr1* gene might show a phenotype in dopamine-related neuropsychiatric behaviors.

In this study, we show that HESR1 binds directly to the *cis*-element in 3′ non-coding region of the *DAT1* gene through dimerization with the bHLH domain of the molecule, and represses the expression of the endogenous *DAT1* gene in a mammalian cell line. Moreover, we report a novel phenotype of mice lacking the *Hesr1* gene. Enhancements in the expression of the *DAT* gene and some dopamine-related genes were observed in the brains of *Hesr1* knockout mice. As a result, *Hesr1* knockout mice show a decrease in spontaneous locomotor activity and a reduction in exploration to novelty. These findings suggest that the human *HESR1* gene and its polymorphisms could be related to many psychiatric disorders and behavioral traits.

MATERIALS AND METHODS

Cell Culture, Transfection Assay, and Semi-Quantitative RT-PCR Analysis

The human embryonic kidney cell line, HEK293, was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) at 37°C in an atmosphere of 5% CO_2. The cells were plated at 60% confluency into 6 cm dishes 24 hr before transfection. Twenty-four hours after transfection with 2 µg of each expression vector encoding Myc/HESR1 (Fuke et al., 2005) into HEK293 cells using the Fugene 6 Transfection Regent (Roche, Basel, Switzerland), the cells were exposed to 1 mg/ml of the antibiotic G-418 (Sigma). After 7 days incubation with the medium exchanged every 2 days, total RNA was extracted from the cells using an RNeasy mini kit (Qiagen, Valencia, CA). First-strand complementary DNA (cDNA) was synthesized using an oligo(dT) primer in the ThermoScript RT-PCR System (Invitrogen), starting from 0.5 µg of total RNA. cDNA templates were diluted 10-fold before use in PCR. A semi-quantitative RT-PCR analysis was carried out after normalizing for the *GAPDH* gene. The endogenous *DAT1* gene was amplified by PCR using the oligonucleotides primers listed in Supplementary Table I. The amplified fragments were visualized by ethidium bromide staining under UV light after electrophoresis in a 2% Seaplaque GTG agarose gel (BioWhittaker Molecular Applications, East Rutherford, NJ). PCR amplifications were carried out in triplicate. Transfections were carried out three times independently.

Electrophoretic Mobility Shift Assay (EMSA)

The in vitro-translated proteins were prepared from each expression vector using the TNT T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI). EMSA was carried out with the SmaI-XbaI fragment from the 10-repeat/LacZ target reporter vector (Fuke et al., 2005), which was purified after electrophoresis in a 1% Seaplaque GTG agarose gel (Bio-Whittaker Molecular Applications) using a Min Elute Gel Extraction kit (Qiagen). The DNA fragments were labeled at one end by filling in with the Klenow enzyme (Takara, Otsu, Japan) in the presence of 6,000 Ci/mmol [α-^{32}P]dCTP (Amersham Biosciences, Piscataway, NJ). DNA-Protein complexes were formed by incubating of the proteins with 10,000 cpm of the radiolabeled DNA fragments for 20 min at room temperature in 20 µl of binding buffer (20 mM HEPES-KOH [pH 7.5], 80 mM KCl, 12% glycerol, 0.1% NP-40, 10 µM $ZnSO_4$, 1 mM DTT, 100 ng/µl poly(dI-dC), containing 5 µl protein solution). DNA-protein complexes were resolved by electrophoresis in a 5% acrylamide gel containing 2.5% glycerol in TBE buffer (25 mM Tris, 0.5 mM EDTA, 24 mM

boric acid) at 4°C. The dried gel was exposed to an Imaging Plate (Fujifilm, Minamiashigara, Japan) as well as being subjected to an image analyzer BAS-2000 (Fujifilm). Super-shift assays were carried out with 10 ng/ul Anti-hHESR1-C antibody (HRT-1(C-20): sc-16424, Santa Cruz Biotechnology, Santa Cruz, CA). Inhibition assays were carried out with an Anti-*myc* antibody (Invitrogen), which recognizes the Myc epitope tag in the N-terminal region of each fusion protein. The in vitro-translation proteins and the radiolabeled DNA fragments were prepared at least three times independently. The experiment was carried out three times.

Animals

The Animal Experiment Committee of the University of Tokyo approved all animal experiment protocols. The *Hesr1* knockout strains and the PCR method for genotyping have been described (Kokubo et al., 2005). Mouse tail tips were used for genotyping by PCR. In the case of the mouse at postnatal day 0, mice were genotyped as to the XX or XY karyotype by PCR for the Y chromosome gene *Zfy* and X chromosome gene *Zfx* (Nagamine at al., 1999). C57BL/129Svj wild-type mice and their littermates heterozygous and homozygous were used between the ages of 2–3 months for the behavioral analysis. All animals were housed in standard mouse cages at 22–24°C on a 12 hr light/dark cycle with access to food and water freely.

Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted from the whole brains of four male mice of each genotype at postnatal day 0 using an RNeasy midi kit (Qiagen) after homogenization. First-strand cDNA was synthesized by priming 1 μg of total RNA with oligo(dT) using the ThermoScript RT-PCR System according to the manufacturer's instructions (Invitrogen). cDNA templates were diluted 10-fold before use in real-time RT-PCR. Real-time quantitative PCR was carried out using the Mx3000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA). Each PCR product was synthesized using the SYBR Premix Ex Taq reagent (Takara) with the oligonucleotide primers (Supplementary Table I). The amount of double-stranded PCR product was measured by fluorescence at the end of the annealing step of each cycle to monitor amplification. In each reaction, the expression level of each gene for each template was extrapolated from a standard curve generated from Ct values calculated by simultaneous amplifications of quantified PCR fragments as templates. The expression levels in each genotype and each animal were calculated in simultaneous reactions and confirmed on two separate reaction plates. Average putative expression values from replicate PCR reactions were normalized to the average expression values for the *GAPDH* gene as an internal control from each template. The ratios of gene expression levels of heterozygous or homozygous mice to wild-type were then calculated.

Behavioral Analysis

In the open-field test, the open-field was a square arena (44 × 44 × 39.5 [H] cm) with acrylic clear walls and black floor. Each mouse was placed in the center of the open-field and allowed to explore freely for a period of 20 min. The computer defined grid lines that divided each open field into nine separate regions: one square region in center, four conner regions, four wall or "side" regions, with each of four lines being 11 cm from each wall. Behavior was recorded by a centrally placed video camera using an automated video tracking software (LimeLight, Actimetrix, IL) to assess the distance traveled per second, the number of entries into the center, and the total time spent in each separate region. Moreover, non-locomotor time was quantified by accumulation of no traveled seconds. Spontaneous alternation was assessed using a Y-maze test following a procedure modified from Fraser et al. (1997).

The elevated plus-maze procedure was a modified version of that of Holmes et al., 2002. The apparatus was constructed of clear acrylic walls and floor covered with a brown rubber sheet (a thickness of 0.3 cm). The maze comprised two open arms (27 × 6 cm) and two closed arms (27 × 7 × 4.5 [H] cm) extending from a common central platform (6 × 6 cm), and elevated to a height of 43 cm above floor level. A small raised lip (0.5 cm) around the perimeter of the open arms prevented the mouse from falling. Each mouse was placed on the center square, facing an open arm, and allowed to explore freely for a period of 5 min. Behavior was recorded by a centrally placed video camera using automated video tracking software (LimeLight, Actimetrix) to measure the distance traveled and time spent in the platform, outer and inner halves of each type of arm. Motor coordination and balance were assayed with an accelerating rotorod (Ugo Basile, Italy). Mice were placed on a slowly rotating drum, which accelerated gradually from 5.5 to 26 rpm over a 3 min period. The time to fall off the drum was recorded.

Each mouse was placed in the open field (45 × 45 × 40 [H] cm) set in the behavioral analyzing system (SCANET, Melquest, Japan), and was allowed to explore freely for a period of 30 min. After the habituation period, mice were treated with cocaine HCl at a dose of 10 mg/kg, i.p. (base equivalent dose, dissolved in saline) and their locomotor activity were observed for 60 min.

Statistics

The results of quantitative experiments were analyzed by one-way ANOVA or Welch's ANOVA after Bartlett's test. Student's *t*-test or Tukey-Kramer HSD test was used to determine significant differences in multiple comparisons. These statistical significances were determined by JMP (SAS Institute, Cary, NC).

RESULTS

HESR1 Represses the *DAT1* Gene by Binding to a Part of the 3′ Non-Coding Region of the Gene

We compared expression levels of the *DAT1* gene in HEK293 cells transfected with each *HESR1* expression vector by semi-quantitative RT-PCR analysis to investigate the function of HESR1 in the expression of the endogenous *DAT1* gene. The endogenous *DAT1* gene was potentially repressed in cells transfected with the expression vector encoding the human *HESR1* gene

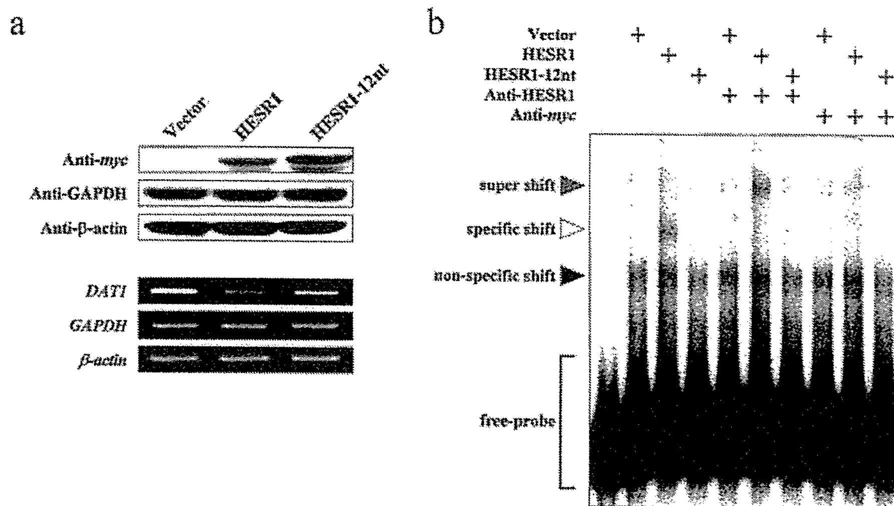

Fig. 1. **A:** The endogenous *DAT1* gene was repressed in cells transfected with the expression vector encoding the human *Hesr1* gene. Comparison of expression levels of the *DAT1* gene in HEK293 cells by semi-quantitative RT-PCR after normalizing for the *GAPDH* gene (lower three panels). Cells expressing *HESR1*, *HESR1-12nt* showed a lower repression activity than those expressing *Hesr1*. Western blot analyses were carried out simultaneously (upper three panels). **B:** Investigation of the binding of HESR1 to a part of the 3′ non-coding region of the *DAT1* gene using EMSA. DNA-Protein complexes were formed by incubating of the in vitro-translation proteins with radiolabeled DNA fragments. The specific mobility shift of the radiolabeled DNA fragments was observed after incubation with the HESR1 proteins (open arrowhead), but not the HESR1-12nt or Myc epitope tag proteins. Super shift was observed after incubation with an anti-HESR1(C-terminus) antibody (shaded arrowhead). Incubation with an anti-*myc*(N-terminus) antibody caused the disappearance of the specific mobility shift. The signals of the free-probe and the non-specific mobility shift (arrowhead) are shown.

in contrast to cells transfected with the vector encoding the epitope tag alone (Fig. 1a). The expression of the β-*actin* gene was not affected. The expression of *HESR1-12nt*, which is an alternative splice variant of human *HESR1* (Fuke et al., 2005), showed a lower repression activity than that of the *HESR1* gene. According to Western blot analysis, the difference in repression activity did not depend on the expression levels of the exogenous gene (Fig. 1a).

We next investigated whether HESR1 directly represses the *DAT1* gene by the EMSA method (Fig. 1b). The mobility shift of the radiolabeled DNA fragments was observed after incubation with the in vitro-translated HESR1 proteins. Then, the super shift was observed after incubation with an anti-HESR1 antibody, which recognizes the C-terminal region of the HESR1 protein. Incubation with an anti-*myc* antibody, which recognizes the Myc epitope tag in the N-terminal region of fusion proteins containing the bHLH DNA-binding and dimerization domain, inhibits the formation of DNA-protein complexes. These findings suggest that HESR1 binds directly to a part of the 3′ non-coding region of the *DAT1* gene.

Deficit of the *Hesr1* Gene Affects the Expressions of Several Dopamine-Related Genes In Vivo

To know the relationship between *Hesr1* and the dopaminergic nervous system, we investigated the effect of the *Hesr1* gene on the expressions of not only the *DAT* gene, but also other dopamine-related genes (Table I). In a real-time quantitative RT-PCR analysis, we used cDNA synthesized from the total RNA from whole brain at postnatal day 0. Although the expression level of the *Hesr1* gene in heterozygous mice was lower than that in wild-type mice (Student's *t*-test, $P < 0.05$), the expression in homozygous mice was undetectable. Homozygous mice showed higher expression of the *DAT* gene than wild-type mice (one-way ANOVA followed Tukey-Kramer HSD test, $F(2,9) = 9.79$, $P < 0.01$). The expression levels of the *D1* and *D5* dopamine receptor genes were significantly high (one-way ANOVA followed Tukey-Kramer HSD test, $F_{D1}(2,9) = 6.95$, $F_{D5}(2,9) = 5.69$, $P < 0.05$). Although the expressions of the *DAT*, *D1*, and *D5* genes in heterozygous mice were also significantly higher than in wild-type mice (Student's *t*-test, $P < 0.05$). Both heterozygous and homozygous mice showed higher expression levels of the *D2* gene than wild-type mice (one-way ANOVA followed Tukey-Kramer HSD test, $F(2,9) = 7.40$, $P < 0.05$). Moreover, the expression level of the *D4* gene in homozygous mice was higher than in either heterozygous or wild-type mice (one-way ANOVA followed Tukey-Kramer HSD test, $F(2,9) = 6.47$, $P < 0.05$); there was no significant difference between heterozygous mice and wild-type mice (Student's *t*-test, $P > 0.1$). On the other hand, the β-*actin* gene and other dopamine-related genes, *D3*, *TH*, *nurr1*, and α-*synuclein*, showed no differences in expression in any genotype (one-way ANOVA, $F_{\beta\text{-}actin}(2,9) = 0.232$, $F_{D3}(2,9) = 2.40$, $F_{TH}(2,9) = 0.287$, $F_{nurr1}(2,9) = 0.144$, $F_{\alpha\text{-}synuclein}(2,9) = 0.787$, $P > 0.1$).

TABLE I. Comparison of the Expressions of Several Dopamine-Related Genes by Real-Time Quantitative RT-PCR Analysis

Gene	+/+	+/−	−/−
Hesr1	1.00 ± 0.15	0.69 ± 0.03**	Undetectable
Dopamine transporter (DAT)	1.00 ± 0.09	1.32 ± 0.22§	1.59 ± 0.23**
Dopamine receptor			
D1	1.00 ± 0.22	1.29 ± 0.14§	1.45 ± 0.13*
D2	1.00 ± 0.12	1.28 ± 0.07*	1.26 ± 0.14*
D3	1.00 ± 0.17	0.96 ± 0.11	0.79 ± 0.14
D4	1.00 ± 0.13	1.04 ± 0.10	1.27 ± 0.12*
D5	1.00 ± 0.07	1.16 ± 0.06§	1.22 ± 0.14*
Tyrosine hydroxylase (TH)	1.00 ± 0.11	1.03 ± 0.13	0.96 ± 0.15
Nurr1	1.00 ± 0.12	1.02 ± 0.17	1.06 ± 0.22
α-*synculein*	1.00 ± 0.09	1.12 ± 0.17	1.13 ± 0.22
β-*actin*	1.00 ± 0.23	1.09 ± 0.10	1.02 ± 0.20

Average putative expression values of genes are shown after normalization to the average expression values of the *GAPDH* gene from each template. Data represent the means ± SD of four male mice of each genotype after calculation of ratios to expression levels in wild-type mice. Although the expression of the *hesr1* gene in heterozygous mice was lower than that in wild-type mice, expression in homozygous mice was undetectable. The expressions of several genes, *DAT, D1, D2, D4,* and *D5* in the brains of homozygous mice were higher than those in wild-type mice. The β-*actin* gene and other dopamine-related genes, *D3, TH, nurr1,* and α-*synuclein*, showed no significant differences in expression.

*$P < 0.05$.

**$P < 0.005$, one-way ANOVA followed Tukey-Kramer HSD test.

§$P < 0.05$, Student's *t*-test.

Hesr1 Knockout Mice Exhibit Behavioral Alterations

In this study, we carried out behavioral analyses to determine the functions of the *Hesr1* gene and the different expressions of dopamine-related genes in vivo. In the open-field test, *Hesr1* knockout mice ambulated for shorter distances, accompanied by longer non-locomotor times, than wild-type mice (Fig. 2a,b; one-way ANOVA, $F_{distance}(1,10) = 18.57$, $F_{non\text{-}locomotor}(1,10) = 14.65$, $P < 0.005$). Calculation of the actual moving speed, with an exclusion of non-locomotor time, *Hesr1* knockout mice ambulated more slowly than that of wild-type mice (Fig. 2c; one-way ANOVA, $F(1,10) = 13.86$, $P < 0.005$). Moreover, *Hesr1* knockout mice spent significantly more time in the corner than wild-type mice (Fig. 2d; one-way ANOVA, $F(1,10) = 5.14$, $P < 0.05$). Although *Hesr1* knockout mice spent less time in the center and made fewer entries into the center than wild-type mice, significant differences were not detected (data not shown; one-way ANOVA, $F_{time}(1,10) = 4.29$, $P = 0.065$, $F_{entry}(1,10) = 3.89$, $P = 0.077$). No significant difference in time in side region was observed between genotype (data not shown; one-way ANOVA, $F(1,10) = 3.70$, $P = 0.083$).

In the elevated plus-maze test, *Hesr1* knockout mice made fewer entries into all arm than the wild-type mice (Fig. 3a; one-way ANOVA, $F(1,10) = 5.65$, $P < 0.05$).

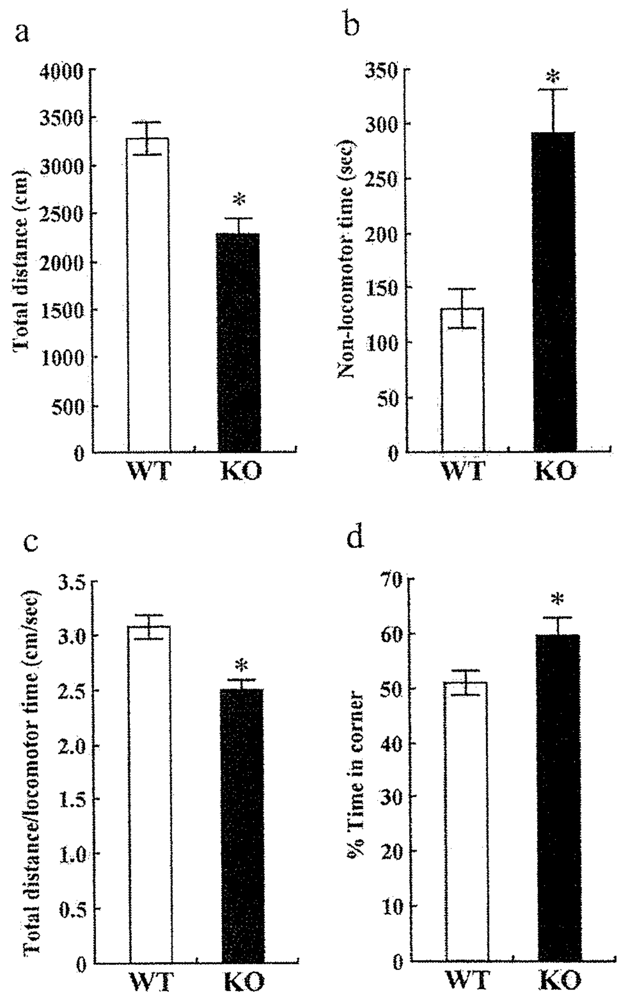

Fig. 2. Open-field test. The total distance traveled (**A**), non-locomotor time (**B**), moving speed calculated by dividing total distance by the locomotor time (**C**), the total time spent in the corners (**D**) are shown for the wild-type (white column, n = 6) and the *Hesr1* knockout mice (black column, n = 6) in the open-field test during 20-min exposures. Data represent the means of actual values ± SE for six male mice of both genotypes. *$P < 0.05$ vs. the wild-type mice with ANOVA.

Although we did not find significantly differences in the ratio of entry into open arms (data not shown; one-way ANOVA, $F(1,10) = 0.0047$, $P = 0.95$), *Hesr1* knockout mice spent significantly less time in open arms compared to wild-type mice (Fig. 3b; one-way ANOVA, $F(1,10) = 5.45$, $P < 0.05$).

We also compared these indices between heterozygote and wild-type mice, and found that heterozygous mice show significant behavioral alterations similar to homozygous mice compared to wild-type mice in spontaneous locomotor activities (one-way ANOVA, < 0.05), but not in terms of time in the corners in the open-field test, and time in open arms in the elevated plus-maze test

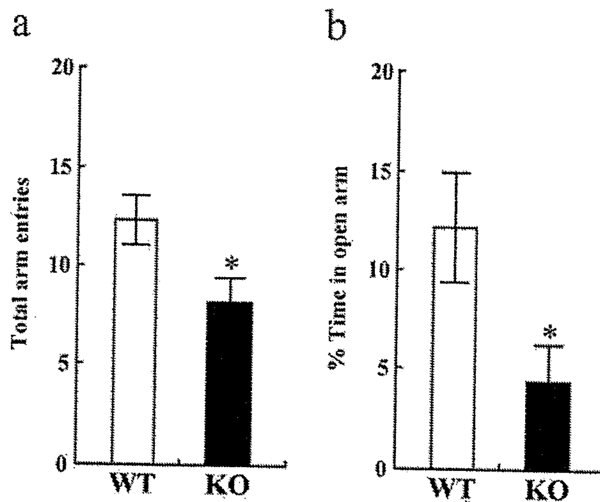

Fig. 3. Elevated plus-maze test. The total number of entries into the arms (**A**), the percentage of the total time in two open arms (**B**) are shown for the wild-type (white column, n = 6) and the *Hesr1* knockout mice (black column, n = 6) in the elevated plus-maze test during 5-min exposures. Data represent the means of actual values ± SE for six male mice of both genotypes. $*P < 0.05$ vs. the wild-type mice with ANOVA.

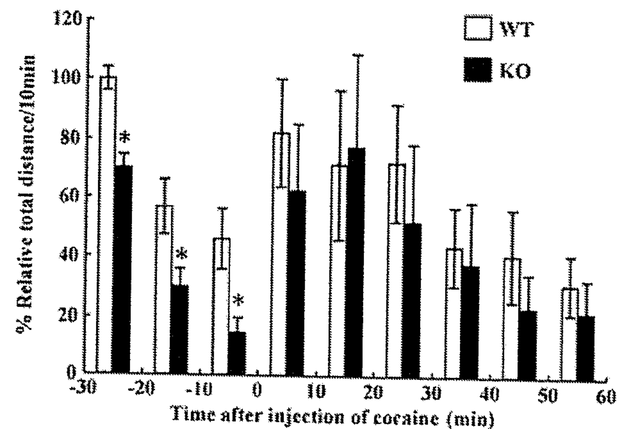

Fig. 4. The distance traveled is plotted for 10-min periods for the wild-type (white column, n = 6) and the *Hesr1* knockout mice (black column, n = 6) in the open-field test during 90-min exposures. After 30-min habituation period, mice were injected with 10 mg/kg cocaine, and the total distance was recorded for the next 60-min. Data represent the means of actual values ± SE for six male mice of both genotypes. $*P < 0.05$ vs. the wild-type mice with ANOVA.

(data not shown; one-way ANOVA, $P > 0.5$). In the Y-maze spontaneous alternation test, although the difference in spontaneous alternation for each genotype was not significant, the total number of arms entered was less for *Hesr1* knockout mice than for wild-type mice (Supplementary data; one-way ANOVA, $F_{\text{spontaneous alternation}}(1,10) = 1.02$, $P = 0.34$, F_{entry} $(1,10) = 11.13$, $P < 0.01$).

Furthermore, we confirmed that there were no difference in body weight and rotorod performance between each genotype (data not shown; one-way ANOVA, $F_{\text{body weight}}$ $(1,10) = 0.18$, $F_{\text{rotorod}}(1,10) = 0.028$, $P > 0.5$).

To assess the involvement of DAT and dopaminergic nervous system in behavioral alterations of the *Hesr1* knockout mice, we investigated the behavioral effects of treatment with cocaine (Fig. 4). Although *Hesr1* knockout mice showed significantly lower locomotor activity than wild-type during the habituation period (one-way ANOVA, $F_{-30\sim-20}(1,10) = 25.82$, $P < 0.0005$, $F_{-20\sim-10}(1,10) = 5.76$, $P < 0.05$, $F_{-10\sim0}(1,10) = 7.76$, $P < 0.05$), there were no difference in the locomotor activity after cocaine challenge (one-way ANOVA, $F(1,10) < 1.0$, $P > 0.5$).

DISCUSSION

bHLH Domain of HESR1 Plays an Important Role in Repressing of the *DAT1* Gene and Binding to the Target Element in the 3′ Non-Coding Region of the *DAT1* Gene

In our previous report, we showed that the expression of the *HESR1* gene suppresses the expression of the luciferase reporter gene with the 3′ non-coding region of *DAT1* (Fuke et al., 2005). In this study, we found that the expression of the human *HESR1* gene represses the expression of the endogenous *DAT1* gene (Fig. 1a). Furthermore, we showed that DNA-protein complexes are formed by incubation of in vitro-translated HESR1 proteins with part of the 3′ non-coding region of the *DAT1* gene containing the VNTR polymorphism region (Fig. 1b). Moreover, incubation with an anti-HESR1 antibody caused a super shift. However, incubation with an anti-*myc* antibody inhibited the formation of complexes. Therefore, the dimerization of HESR1 seems to be inhibited by the interaction between the anti-*myc* antibody and the Myc epitope tag located at the N-terminus close to the bHLH domain. These findings support the notion that HESR1 binds to the target element directly. However, the target element region used in the binding assay has no E-box or N-box known to be bHLH binding consensus sites. To our regret, no binding sites or sequences have been identified. It has been reported that there is no E-box or N-box in the mouse *HRT2/Hesr2* gene repressed by HRT/HESR proteins (Nakagawa et al., 2000). Therefore, it is likely that HESR1 recognizes a novel binding site different from that of other bHLH family members. Interestingly, the expression of *HESR1-12nt* showed lower repression activity, and the in vitro-translated HESR1-12nt proteins had little DNA-binding activity (Fig. 1a,b). According to the finding that the HESR1-12nt protein has an insertion of four amino acids in the loop region of the bHLH domain (Fuke et al., 2005), the intact bHLH domain of the HESR1 protein is required for binding to the 3′ non-coding region of the *DAT1* gene. These findings suggest that HESR1 re-

presses the expression of the *DAT1* gene by binding directly to the 3′ non-coding polymorphic region of the *DAT1* gene through the bHLH domain of the molecule.

Loss of the *Hesr1* Gene in the Dopaminergic Nervous System

In this study, we showed that a deficit of the *Hesr1* gene causes an enhancement of the expression of the *DAT* gene in vivo (Table I). On the other hand, it remains unsettled whether the increase in the expression of the *DAT* gene depends on overexpression or spatial misexpression. The deficit enhances the expressions of several dopamine receptor genes, *D1*, *D2*, *D4*, and *D5*, the main targets of synaptic dopamine responsiveness. The repression of dopamine receptor genes *D1* and *D2* has been reported in mice lacking the *DAT* gene (Giros et al., 1996; Jaber et al., 1999; Fauchey et al., 2000; Gainetdinov et al., 2002). In this study, the enhancements in the expression of dopamine receptor genes might occur in compensation for the reduction in dopamine during synaptic transmission depending on the overexpression of the *DAT* gene. These results indicate that the increase in the expression of the *DAT* gene occurs in the dopaminergic nervous system. Furthermore, the change in expression may occur in confined dopaminergic neuronal cells because the change was unremarkable, and no significant expression of the *D3* gene was observed. The expressions of other dopamine-related genes, *TH*, *nurr1*, and α-*synuclein*, as well as β-*actin*, did not differ. This suggests that the deficit in the *Hesr1* gene does not affect the total amount of dopamine or dopaminergic neurotransmission in the whole brain. The data suggest that the *Hesr1* gene regulates the expression of the *DAT* gene and affects the dopaminergic nervous system in vivo.

Function of the *Hesr1* Gene in Behavior

Although members of the *Hesr* family are thought to play important roles in the development of several tissues, heart, vascular, somitic, and brain (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999, 2000; Iso et al., 2001, 2003; Henderson et al., 2001; Wang et al., 2002; Sakamoto et al., 2003), it has been shown that *Hesr1* single knockout mice are viable and fertile, and show no major developmental or obvious functional impairments (Fischer et al., 2004; Kokubo et al., 2005). In the present study, we assessed some behavioral consequences as a novel phenotype of the *Hesr1* deficient mice. We found that mice lacking the *Hesr1* gene exhibit decreased total distance, long non-locomotor time, and slow movement in the open-field test. The decrease of ambulation was detected also in the Y-maze spontaneous alternation test and elevated plus-maze test. Furthermore, the inhibition of DAT activity by the treatment with cocaine produced no difference in locomotor activities between genotypes. According to this observation, the decrease of ambulation was caused by overexpression of the *DAT* gene. The results of our experiments are consistent with the spontaneous hyper-locomotion reported in *DAT* deficit mice (Giros et al., 1996). Moreover, the enhanced expressions of the *D1*, *D2*, and *D5* gene were observed in both heterozygous and homozygous mice. Therefore, it is assumed that the decrease in spontaneous locomotor activity is caused by overexpression of the *DAT* gene and the depletion of dopamine in the synapses on the dopamine neurons that have expressed the *D1*, *D2*, or *D5* gene. In particular, it has been reported that the *D2* knockout mice exhibit a lower locomotor activity than the wild-type mice (Vallone et al., 2002).

The *Hesr1* knockout mice showed a tendency to make fewer entries and spend less time in the center but significantly more time in the corners of the open-field compared to wild-type mice. Furthermore, *Hesr1* knockout mice spent less time in open arms in the elevated plus-maze test. These findings may imply the fact that *Hesr1* knockout mice show altered emotional states under novel environments. Interestingly, these alterations were observed in homozygous mice but not heterozygous mice, it suggests that the possible altered emotional states are independent of a lower locomotor activity observed in both heterozygous and homozygous mice. Furthermore, these results are consistent with the increase in the expression of the *D4* gene in homozygous mice but not heterozygous mice. This suggests that the depletion of dopamine caused by the overexpression of the *DAT* gene in dopaminergic synapses, which depends on the function of the *D4* gene, triggers a reduction in behavioral responses to novelty. The results of our experiments are also consistent with the reduced exploration of novel stimuli reported in *D4* deficit mice (Dulawa et al., 1999). It is interesting to investigate the emotional state of *Hesr1* knockout mice in detail for understanding the function of the *Hesr1* gene in behavioral alterations.

HESR1 Gene and Human Behavior

We have shown a novel behavioral phenotype of *Hesr1* knockout mice. Moreover, it is likely that the overexpression of the *DAT* gene is a principal factor in the behavioral alterations of *Hesr1* knockout mice. These results strongly indicate that the *HESR1* gene and its polymorphism(s) might be related in dopamine-related neuropsychiatric disorders and behavioral traits, according to the observation that the dopaminergic nervous system is involved in neuropsychiatric disorders such as bipolar disorder, schizophrenia, Tourette's syndrome, drug abuse, drug-induced paranoia, alcoholism, PD, and ADHD. Therefore, the fact that there are many polymorphisms in the coding and non-coding regions of the human *HESR1* locus should not be overlooked. Several polymorphisms may affect the function of the *HESR1* gene in vivo. Indeed, L94M that is caused by the C386A candidate SNP of the human *HESR1* gene (Buetow et al., 1999), in the second helix of the bHLH

domain. This leucine residue was conserved among other members of the bHLH family (Kokubo et al., 1999; Iso et al., 2003; Fuke et al., 2005). Moreover, we showed that C386A SNP causes a loss of function of the HESR1 protein (Fuke et al., 2005). This suggests that an individual with C386A SNP in the *HESR1* gene will show a phenotype similar to that of *Hesr1* knockout mice. However, no evidence for a direct relationship between the *HESR1* gene and neuropsychiatric disorders has been reported. It is known that the Notch signaling pathway is involved in early neurodevelopment, learning and memory, and human disease. The *Notch4* gene, which induces expression of the *Hesr1* gene, has been identified as a susceptibility gene for schizophrenia, which is a complex mental illness with multiple phenotypic presentations (Wang et al., 1995; Taylor et al., 2002). It is possible that the *HESR1* gene is an important risk factor in dopamine-dependent neuropsychiatric disorders and behavioral traits.

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Association analysis of the dopamine receptor D2 (DRD2) SNP rs1076560 in alcoholic patients

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Abstract

The dopamine system plays a well-established role in alcoholism. In this study, we examined the association between the single-nucleotide polymorphism (SNP) rs1076560 of the dopamine receptor D2 (DRD2) gene and susceptibility to alcoholism. SNP rs1076560 (C/A) is located in intron 6 of *DRD2*, where it is 1.4 kb downstream from alternative exon 6 and 83 bp upstream from exon 7. A total of 248 alcoholic patients and 322 healthy controls, all Japanese males, were genotyped for rs1076560 polymorphism by direct sequencing and allele-specific PCR. Data were analyzed using standard χ^2 statistics and a backwards logistic regression approach to adjust for the contribution of aldehyde dehydrogenase-2 (ALDH2) genotype status. The *DRD2* risk allele A was more prevalent in the alcoholic patients (40.1%) than in the healthy controls (34.0%) ($P = 0.034$, odds ratio = 1.300, 95% confidence interval = 1.020–1.657). These data identify SNP rs1076560 as a potentially important variable in the development of alcoholism.
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Keywords: Dopamine; DRD2; SNP; Alcoholism

Human twin and adoption studies support a strong hereditary component in susceptibility to alcoholism. For example, the genotype of the ethanol-metabolizing enzyme aldehyde dehydrogenase-2 (ALDH2) affects the risk of alcoholism [3]. The dopaminergic system, which plays an important role in brain reward mechanisms [1,10] is a strong candidate for an additional hereditary component for risk of alcoholism risk. Previous analysis of the dopamine receptor subtypes (D1–D5) and the dopamine transporter protein has clearly demonstrated that the dopamine receptor D2 (DRD2) is involved in susceptibility to alcoholism [9]. In mice, both deficiency and overexpression of DRD2 reduced alcohol preference [5,8], suggesting that DRD2 has an optimal level in the brain.

Alternative mRNA splicing generates two isoforms of DRD2, the long (D2L) and short (D2S) isoforms, which differ in the presence of a 29-amino-acid insert in the third cytoplasmic loop. Both the D2S and D2L receptors have been shown to couple with members of the Gi family of G proteins, and each isoform exhibits a specific Gi protein(s) preference [6]. Therefore, we hypothesized that variations in DRD2 RNA splicing affect dopamine function and also the risk of substance abuse. In the present study, we sought to identify the *DRD2* polymorphism(s) associated with alcoholism; in particular, we focused on the single-nucleotide polymorphisms (SNPs) located near the alternative exon that might influence alternative splicing.

Genomic DNA samples of known *ALDH2* genotypes were provided by the Kurihama Alcoholism Center of the National Hospital Organization and these were part of the samples previously studied [3]. This study was approved by the Ethics Committee of the National Institute on Alcoholism, and written informed consent was obtained from all of the subjects. The study population consisted of 248 Japanese alcoholic males (mean age ± S.D., 50.6 ± 8.8 years) who were diagnosed as having either DSM-III-R alcohol dependence or alcohol abuse based on the Structured Clinical Interview for DSM-III-R (SCID) assessment. Unrelated nonalcoholic control subjects were matched for age ($n = 322$; mean age ± S.D., 48.4 ± 15.0 years). Samples with homozygous ($2^*2/2^*2$) *ALDH2* genotypes were excluded from the study because the *ALDH2* genotype $2^*2/2^*2$ is a strong negative risk factor for alcohol abuse.

We searched for SNPs in the regions surrounding *DRD2* exon 6, which undergoes alternative splicing. We identified three

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SNPs in intron 5 and six SNPs in intron 6; these SNPs have been submitted to the SNP database dbSNP (www.ncbi.nlm.nih.gov/projects/SNP). Since SNP rs1076560 was the only one of these SNPs with reported allele, we selected this SNP for genotyping.

A 543-bp *DRD2* gene fragment containing SNP rs1076560 was amplified by PCR using the forward primer 5′-atgag gattg ccatg ggaaa aagga-3′ and the reverse primer 5′-AGAAC AATGG CGAGC ATCTG AG-3’. These primers were designed to anneal at 65 °C. The PCR reactions were performed in a total volume of 10 μl containing 200 mM dNTP mix, 500 nM of each primer, 1 × reaction buffer, and 0.25 U Ex-Taq DNA polymerase (TaKaRa). Amplification conditions consisted of denaturation at 96 °C for 2 min, 40 cycles of denaturation at 96 °C (20 s), annealing at 65 °C (20 s), and extension at 72 °C (45 s), and a final extension at 72 °C for 10 min. The amplified DNA products were visualized using ethidium bromide under UV light after electrophoresis in 2% SeaPlaque GTG agarose gels (Cambrex). The DNA bands were excised from the gels, purified, and sequenced directly using the dideoxy chain-termination method with dye-terminator cycle sequencing on a CEQ8000 Genetic Analysis System for Sequencing and Fragment Analysis (Beckman Coulter), which employs capillary array electrophoresis (Fig. 1).

To confirm these results, we performed allele-specific PCR using allele-specific forward primers designed to amplify specific genotypes. These primers were 5′-TGCAG GAGTC TTCAG AGGA**G** G-3′ (allele-specific forward primer C) and 5′-TGCAG GAGTC TTCAG AGGA**T** G-3′ (allele-specific forward primer A). These primers were identical, with the exception of the second nucleotide from the 3’ end (shown in bold), which matched one of the two biallelic SNP bases. The third nucleotide (underlined) was a mismatch to the SNP. The reverse primer sequence was 5′-GGAAG GACAT GGCAG GGAAT GGGAC-3′. The allele-specific PCR reactions were performed as described above. Amplification was confirmed by agarose gel electrophoresis (Fig. 2). The samples yielding allele-specific PCR results that did not match the original SNP sequence results were sequenced using an automated DNA sequencer (DSQ-2000L; Shimadzu), which uses a universal sequencing primer labeled with the fluorescent dye FITC. The results of direct sequencing and the allele-specific PCR were identical in 493 samples. Then, direct sequencing of the allele-specific products genotyped 77 samples.

Differences in allele and genotype frequencies of the *DRD2* SNP and the *ALDH2* genotype between cases and controls were evaluated with the χ^2-test (with one and two degrees of freedom, respectively). Initially, differences were considered significant at a nominal *P*-value of < 0.05. The significance of the odds ratios (ORs) was determined using a *z*-statistic.

To test the association of the *DRD2* genotype with the diagnosis of each patient, we used the program JMP (Release 6.0.0; SAS) to apply a backwards elimination logistic regression approach while adjusting for the impact of *ALDH2* genotype status. The JMP Backward Wald procedure fits all of the variables into the model and then removes the insignificant variables, one-by-one, based on the probability of the Wald test statistic (removal if $P > 0.10$).

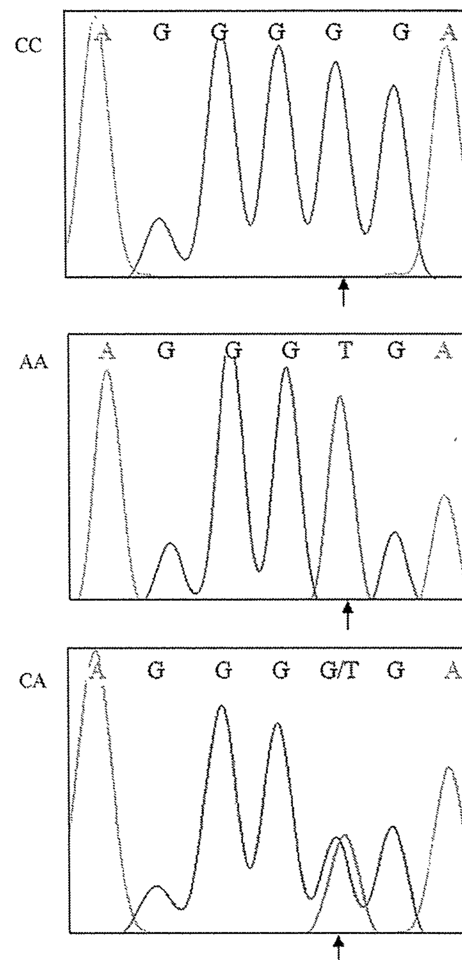

Fig. 1. DNA sequencing chromatogram region showing the variant bases of the antisense sequence of SNP rs1076560, resulting in three rs1076560 genotypic patterns. The chromatogram was obtained using CEQ dye-terminator cycle sequencing on a CEQ8000 capillary array (Beckman Coulter). The position of the SNP is indicated by arrows.

In this study, the SNP rs1076560 was genotyped in a case-control group of 248 alcoholic patients and 322 unrelated controls (Table 1, a and b). The *DRD2* genotypes exhibited a Hardy–Weinberg equilibrium in the patients and the controls. In the χ^2-test, significant allelic and genotypic associations were detected between rs1076560 and a diagnosis of alcoholism (allele $P = 0.034$, genotype $P = 0.035$). The A allele and the CA + AA genotype were more prevalent in the

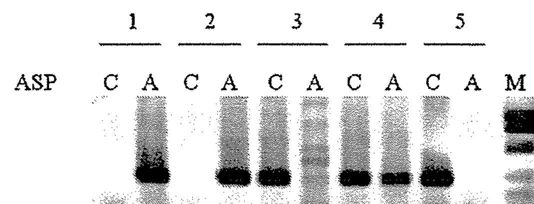

Fig. 2. Amplification of SNP rs1076560 samples with allele-specific primers C and A produces three different electrophoretic patterns corresponding to three different genotypes, homozygous AA (lanes 1 and 2), homozygous CC (lanes 3 and 5), or heterozygous CA (lane 4).

Table 1
Total counts of the alleles and genotypes with percentages

Allele	Allele C		Allele A		P-value (χ_1^2)	$OR_{A/C}$ (95% CI)
	n	%	n	%		
a.						
Alcoholic	297	59.9	199	40.1	**0.034**	1.300
Control	425	66.0	219	34.0		(1.020–1.657)

Genotype	CC		CA		AA		P-value (χ_2^2)	$OR_{CA+AA/CC}$ (95% CI)
	n	%	n	%	n	%		
Alcoholic	80	32.3	137	55.2	31	12.5	**0.035**	1.575
Control	138	42.9	149	46.3	35	10.9		(1.115–2.226)

Allele	Allele C		Allele A		P-value (χ_1^2)	$OR_{A/C}$ (95% CI)
	n	%	n	%		
b.						
ALDH2 2*1/2*1						
Alcoholic	232	58.9	162	41.1	**0.025**	1.397
Control	256	66.7	128	33.3		(1.043–1.870)
ALDH2 2*1/2*2						
Alcoholic	65	63.7	37	36.3	0.431	1.057
Control	169	65.0	91	35.0		(0.656–1.704)

Genotype	CC		CA		AA		P-value (χ_2^2)	$OR_{CA+AA/CC}$ (95% CI)
	n	%	n	%	n	%		
ALDH2 2*1/2*1								
Alcoholic	61	31.0	110	55.8	26	13.2	**0.048**	1.662
Control	82	42.7	92	47.9	18	9.4		(1.096–2.519)
ALDH2 2*1/2*2								
Alcoholic	19	37.3	27	52.9	5	9.8	0.172	1.275
Control	56	43.1	57	43.8	17	13.1		(0.655–2.479)

Significance was assessed using the χ^2-test of independence. Differences with a nominal P-value < 0.05 (bold) are considered significant. ORs and 95% CIs are shown relative to the C allele and the CC genotype. a. Calculated jointly for *ALDH2* genotypes 2*1/2*1 and 2*1/2*2. b. Calculated separately for *ALDH2* genotypes 2*1/2*1 and 2*1/2*2.

alcoholic patients than in the controls (A allele: OR = 1.300, 95% confidence interval (CI) = 1.020–1.657; CA + AA genotype: $OR_{(CA+AA)/CC}$ = 1.575, 95% CI = 1.115–2.226; Table 1a). When the allele and genotype frequency analyses were limited to samples with the 2*1/2*1 *ALDH2* allele, a statistically significant association was observed (allele *ALDH2* 2*1/2*1, P = 0.025; *ALDH2* 2*1/2*2, P = 0.431; genotype *ALDH2* 2*1/2*1, P = 0.048; *ALDH2* 2*1/2*2, P = 0.172; Table 1b).

Backward logistic regression analysis was performed to eliminate the effect of the interaction between rs1076560 and ALDH2 genotype status, and the difference between rs1076560 genotypes CA and AA. The results of this analysis confirmed that the rs1076560 A allele might be a risk factor for alcohol abuse, independently of the *ALDH2* genotype status.

Pair-wise comparison of the ORs of the rs1076560 genotypes suggested that the ORs of genotype CA + AA and CA were significantly different from that of genotype CC when CC was set as the reference genotype; that is, OR = 1 (z-test P = 0.005 and P = 0.006, respectively), and the difference between the ORs of genotypes AA and CC tended toward significance (P < 0.10) when CC was set as the reference genotype (z-test P = 0.068). However, the OR of AA was not significantly different from that of CA (z-test P = 0.554), suggesting a dominant mode of inheritance (AA + CA versus CC), which, when tested using the χ^2-test, revealed a strong association between SNP rs1076560 and the risk of alcoholism (P = 0.010, OR = 1.575, 95% CI = 1.115–2.226).

Although previous animal and human studies have provided much evidence for an association between the dopamine receptor D2 gene and alcoholism [1,10], the present study is the first demonstration of an association between SNP rs1076560 of *DRD2* and a diagnosis of alcoholism. We found that the A allele of this SNP is a positive risk factor for alcoholism. Although the interaction between rs1076560 and ALDH2 genotype status was eliminated in this study, since alcoholism is multifactorial, DRD2 probably interacts with other genes.

The frequency of this allele varies in different ethnic populations; in dbSNP, 11.6, 6.2, and 30.5% of Caucasians, Africans/African-Americans, and Hispanics studied, respec-