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（神経・筋疾患分野）

スプライシングを利用した筋強直性ジストロフィーの治療

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主任研究者　石浦　章一

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厚生労働科学研究費補助金（障害者対策研究事業)(神経・筋疾患分野)
総括研究報告書

スプライシングを利用した筋強直性ジストロフィーの治療

総括研究者　　石浦章一　東京大学大学院 総合文化研究科　教授

研究要旨

筋強直性ジストロフィー１型(DM1)は、スプライシング異常を主徴とする全身性疾患で、筋強直、精巣萎縮、白内障、耐糖能異常などが特徴である。私たちはその中で本症の特長である筋強直を治療すべく、既報のアンチセンスよりも効率よく正常型塩素チャネルをつくらせる配列を特定し、エクソンスキッピングを行った。平成22年度は、CTGリピートを300含むトランスジェニックマウスにアンチセンスオリゴを投与し、筋強直を防ぐ動物実験を行った。

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A．研究目的

筋強直性ジストロフィー１型(DM1)は、筋強直、精神遅滞、精巣萎縮、白内障、耐糖能異常などを特徴とする全身性疾患で、スプライシングが異常になって全身症状が出現すると考えられている。本症は我が国の筋ジストロフィーの中では一番多い疾患で、致死ではないものの、QOLの観点からも筋力低下やミオトニアなどの治療法開発が望まれている。

本症の責任遺伝子は、第19染色体にあるDMPKであり、その3’非翻訳領域にあるCTGリピートの伸長が病気の直接の原因である。また最近、筋強直性ジストロフィー２型(DM2)も発見されたが、これは第3染色体にあるZNF9遺伝子中のイントロン１にあるCCTGリピートの伸長であることが判明した。また海外の研究結果によれば、伸長したリピートだけを発現させたマウスでも同じ症状が見られ、リピートの伸長が病気の原因であるとする説が有力になっている。これらの事実により、伸長したRNAに特定のスプライシング因子が結合することで起こるという「RNA機能獲得説」が唱えられているが、これは私たちによる「スプライシング因子MBNL1がDM1とDM2の2つの異なる型の塩基リピートに結合することの発見」が証拠となった。また私たちは、塩素チャネル・インスリン受容体・トロポニンTなどの試験管内スプライシングアッセイ系を確立し、MBNL1をはじめとするRNA結合タンパク質（スプライシング調節因子）の特異性を明らかにした。

３年目の最後の年である平成22年度は、現在まで最も効率よくスプライシングを変化させたRNA結合タンパク質MBNL1に注目し、明らかになったその結合配列付近を標的としたエクソンスキッピングよるスプライシング機能正常化を目指した。前年度までの研究の結果、既報のアンチセンスよりも効率よく正常型塩素チャネルをつくらせる配列を確認しており、今年度は、効率の良いエクソンスキッピングを、モルフォリノオリゴヌクレオチドとモデル動物（CTGリピートを300含むトランスジェニックマウス)を使って行った。

B．研究方法（倫理面の配慮含む）

塩素チャネルミニ遺伝子（エキソン6、7a、7でできている短い遺伝子）を用いて、エキソン7aの有無を定量するスプライシングアッセイが可能である。エキソン7aがない成人（正常）型と、エキソン7aを含む幼若（異常）型の比率は、正常では圧倒的に前者が多く、DM筋では後者が増えている。前年度までに、エキソン7aの最初の塩基を１として、-15～10、0～25、26～50、51～75、76～+21をコードする各アンチセンスを、

塩素チャネル・ミニ遺伝子をトランスフェクトしたCOS-7細胞に添加して効果を見たところ、エキソン7aの0～25に対するアンチセンスを投与したときが最も有効であることがわかっていたので、今年度は、0～25のモルフォリノオリゴヌクレオチドの*in vivo*での効果を調べた。また、このモルフォリノをCTG300を持つトランスジェニックマウス前脛骨筋に3回注射し、最後の注射から2日後に筋強直を測定した。

（倫理面への配慮）

DM患者生検筋は、患者からインフォームドコンセントを取得し、国立精神・神経医療研究センター倫理委員会で承認を受けたものを用いた。

C．研究結果

生体内への保持能力の高いモルフォリノアンチセンスオリゴの効果は培養細胞系で確認ずみのため、このオリゴを使ってHSALR-マウスの前脛骨筋に注射し、オリゴの効果を、筋強直を指標に判定した。その結果、筋強直症状が改善されていることがわかった。

D．考察

本実験では、アンチセンスの配列を最も効率の良いものに決定し、それを用いて動物の治療実験を行ったものである。昨年までの研究で、塩素チャネル遺伝子上にMBNL1結合部位を同定し、その付近に的を絞ったアンチセンス設計により、効率の良い配列が得られたものである。これは2007年に発表されたThorntonらの配列よりも数段、エクソンスキップ効率が良かった。

以上のように、エクソンスキッピングを実際に行うときには、効率の良い配列を選ばないと*in vivo*の治療効果が望めないことがわかった。

E．結論

CTGリピートを300含むトランスジェニックマウスに対して、私たちが新しく開発したアンチセンスモルフォリノオリゴ配列は劇的な治療効果をもたらした。今後は、ドラッグデリバリーの効率を高めることと、実際のQOLに必要な筋萎縮を防ぐために、筋力低下に関わる新規遺伝子を同定することが必要となる。

F．健康危険情報

なし

G．研究発表

1．論文発表

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H．知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（障害者対策総合研究事業)(神経・筋疾患分野)
分担研究報告書

先天性筋強直性筋ジストロフィーとミオチュブラーミオパチーの病理学的鑑別に関する研究

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筋強直性ジストロフィーは、筋病理学的に多彩な所見を呈することが知られている。特に、先天性筋強直性筋ジストロフィーでは、乳児重症型ミオチュブラーミオパチーとの鑑別が困難なことがある。そこで先天性筋強直性筋ジストロフィーと乳児重症型ミオチュブラーミオパチーの病理学的鑑別点を明らかにするべく、3歳以下の先天性筋強直性ジストロフィー22例とミオチュブラーミオパチー32例について病理学的比較を行った。先天性筋強直性筋ジストロフィーの47.6%にperipheral haloを認め、その頻度は16.0±25.5%であったが、ミオチュブラーミオパチーでは全例にperipheral haloを認め、頻度は70.5±24.1%であった。Ring fiberは先天性筋強直性筋ジストロフィーで27.3%に認めたが、ミオチュブラーミオパチーで認めた例は1例もなかった。先天性筋強直性筋ジストロフィーでのタイプ2C線維の頻度は、33.5±33.%に対して、ミオチュブラーミオパチーでは2.8±2.8%であった。タイプ1線維優位と強萎縮タイプ2線維は先天性筋強直性筋ジストロフィーでは認めなかったが、ミオチュブラーミオパチーではそれぞれ87.5%、81.0%に認めた。以上の結果から、病理学的にも先天性筋強直性筋ジストロフィーとミオチュブラーミオパチーを鑑別できる可能性がある。

A.研究目的

先天性筋強直性ジストロフィーは、臨床的にはfloppy infantを、病理学的にはミオチュブラーミオパチー様の変化を呈し、乳児重症型のミオチュブラーミオパチーとの鑑別が困難であるとされている。そこで、遺伝学的にMTM1変異を確認した乳児重症型ミオチュブラーミオパチー例と同様に遺伝学的に診断を確定した先天性筋強直性ジストロフィー例の筋生検組織を比較検討し、病理学的な鑑別が可能かどうかを詳細に検討した。

B.研究方法

対象は、2010年までに国立精神・神経医療研究センター生検筋レポジトリーに登録された凍結筋検体の内、3歳以下の例で、DMPK遺伝子内のCTGリピート及びZNF9遺伝子内のCCTGリピート伸張を確認した先天性筋強直性ジストロフィー例17例および、同様に3歳以下でMTM1遺伝子変異を確認したミオチュブラーミオパチー例32例。全検体に対して、3列からなる連続切片を作製し、ヘマトキシリン・エオジン、ゴモリ・トリクローム変法、NADH-TR、ミオシンATPaseを含む各種組織化学染色を行い、病理学的変化を比較検討した。またミオチュブラーミオパチーの1例で電子顕微鏡的検索を行った。

（倫理面配慮）

全ての検体について、国立精神・神経医療研究センター倫理委員会で承認を受けた「「診断」と「保存/研究使用」に関する同意書」を用い、患者から

のインフォームド・コンセントを取得している。

C. 研究結果

先天性筋強直性ジストロフィー例では、47.6%の例に peripheral halo を認めた。頻度は、16.0±25.5%であった。Peripheral halo を認めた例は全例生後 12 ヶ月以下であった。中心核線維の頻度は、平均 4.6±6.1%であった。Ring fiber は 27.3%に認めた。タイプ 2C 線維の頻度は、33.5±33.%であった。タイプ 2C 線維は、新生児とくに生後 1 ヶ月以内の例に多く認められ、大半の例で筋線維全体の 50%以上がタイプ 2C 線維であった。一方、生後 1 年を過ぎると全例 20%以下となった。タイプ 1 線維優位を認めた例はなかった。また一部のタイプ 2 線維が強萎縮を示す所見はなかった。

一方、ミオチュブラーミオパチー例では、年齢と関係なく全例に peripheral halo を認め、その頻度は 70.5±24.1%であった。中心核線維の頻度は、平均 15.4±14.4%であった。Ring fiber を認めた例はなかった。タイプ 2C 線維の頻度は、0-11% (平均 2.8±2.8%) であった。その頻度に年齢的な変化は認められなかった。87.5%の例にタイプ 1 線維優位が認められた。また一部のタイプ 2 線維が強萎縮を示す所見が 81%の例に認められた。電子顕微鏡的検索を行った 1 例では、中心核は認めるものの、個々の筋原線維は正常に構成されており、筋原線維自体に幼若性は認めなかった。

D. 考察

ミオチュブラーミオパチーでは、タイプ 2C 線維の頻度が多くても 10%程度であるのに対して、先天性筋強直性ジストロフィーでは、33.5±33.%であった。特に、peripheral halo を示す 12 ヶ月以下の例では、大半が 50%を越えていた。従って、peripheral halo を呈する例では、タイプ 2C 線維の頻度を見ることにより、ミオチュブラーミオパチーと先天性筋強直性ジストロフィーを病理学的に鑑別することが出来る可能性を示している。さらに、先天性筋強直性ジストロフィーにおいて、タイプ 2C 線維が多数認められることは、peripheral halo を伴うような病理学的変化が恐らく筋線維の幼弱性を反映している可能性を示している。一方、ミオチュブラーミオパチーでは、タイプ 2C 線維が少なく、peripheral halo を伴う筋原線維の配列の変化が幼弱ではなく、myotubularin の機能喪失による細胞内膜輸送の異常を反映したものであることを強く示唆している。電子顕微鏡的検索で、筋原線維の幼若性が認められなかった事実も、この考え方を支持している。従って、一見同様に見える病理学的変化ではあるが、恐らく全く異なった病態を背景としている可能性が高い。

E. 結論

先天性筋強直性ジストロフィーでは peripheral halo を認めて、乳児重症型ミオチュブラーミオパチーと一見病理学的に酷似する場合があるが、タイプ 2C 線維が高頻度に認められる点、ring fiber を認めることが多い点、ミオチュブラーミオパチーとの鑑別が可能であると考えられる。さらに、ミオチュブラーミオパチーにおいては、強萎縮したタイプ 2 線維認められることがある点も鑑別に有用であると考えられる。

F. 健康危険情報

特になし

G. 研究発表

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H.知的財産権の出願・登録状況(予定を含む)

1．特許取得

特になし

2．実用新案登録

特になし

3．その他

特になし

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
石浦章一	筋強直性ジストロフィーの成因	武田伸一	生体の科学	金原一郎記念医学医療財団	日本	2011	印刷中
Ishiura, S., Kino, Y., Oma, Y., Sasagawa, N. & Nukina, N.	MBNL proteins regulate alternative splicing of the skeletal muscle chloride channel *CLCN1*.	Takeda, S.	Discovery of serum creatine kinase as a diagnostic marker of muscular dystrophy.	シュプリンガー	日本	2011	印刷中

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sasabe, T., Futai, E. & Ishiura, S.	Polypyrimidine tract-binding protein 1 regulates the alternative splicing of dopamine receptor D2.	J.Neurochem.	116	76-81	2011
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研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Iwabu M, Yamauchi T, Okada-Iwabu M, Sato K, Nakagawa T, Funata M, Yamaguchi M, Namiki S, Nakayama R, Tabata M, Ogata H, Kubota N, Takamoto I Hayashi YK, Yamauchi N, Waki H, Fukayama M, Nishino I, Tokuyama K, Oike Y, Ishii S, Hirose K, Shimizu T, Touhara K, Kadowaki T	Adiponectin and AdipoR1 regulate PGC-1alpha and mitochondria by Ca(2+)and AMPK/SIRT1.	**Nature.**	464	1313-1319	2010
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Ⅳ.研究成果の刊行物・別刷

Journal of Neurochemistry

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Polypyrimidine tract-binding protein 1 regulates the alternative splicing of dopamine receptor D_2

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Abstract

Dopamine receptor D_2 (DRD2) has two splicing isoforms, a long form (D2L) and short form (D2S), which have distinct functions in the dopaminergic system. However, the regulatory mechanism of the alternative splicing of *DRD2* is unknown. In this study, we examined which splicing factors regulate the expression of D2L and D2S by over-expressing several RNA-binding proteins in HEK293 cells. In a cellular splicing assay, the over-expression of polypyrimidine tract-binding protein 1 (PTBP1) reduced the expression of D2S, whereas the knockdown of PTBP1 increased the expression of D2S. We also identified the regions of *DRD2* that are responsive to PTBP1 using heterologous minigenes and deletion mutants. Our results indicate that PTBP1 regulates the alternative splicing of *DRD2*. Considering that DRD2 inhibits cAMP-dependent protein kinase A, which modulates the intracellular localization of PTBP1, PTBP1 may contribute to the autoregulation of DRD2 by regulating the expression of its isoforms.

Keywords: alternative splicing, dopamine, dopamine receptor D_2, PTBP1.

J. Neurochem. (2011) **116**, 76–81.

Dopamine is the predominant neurotransmitter in the CNS, where it plays a leading role in the regulation of such physiological functions as locomotor activity, cognition, positive reinforcement, and hormone secretion. The effects of dopamine are mediated by its binding to five G-protein-coupled receptors, which are divided into two subclasses: D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, and D_4). Dopamine receptor D_2 (DRD2) is the main autoreceptor of the dopaminergic system (Centonze *et al.* 2002); however, it is also critical for post-synaptic transmission (Usiello *et al.* 2000).

Alternative gene splicing generates two distinct isoforms of DRD2, a long form (D2L) and short form (D2S), which differ in the presence of a 29-amino-acid insert in the third cytoplasmic loop. D2L is expressed mainly in post-synaptic regions, whereas D2S is expressed mainly in pre-synaptic regions (Khan *et al.* 1998; Usiello *et al.* 2000). These isoforms differentially contribute to the pre-synaptic inhibition of glutamate and GABA transmission (Centonze *et al.* 2004); moreover, they exhibit specific G_i protein preferences (Senogles 1994; Guiramand *et al.* 1995; Senogles *et al.* 2004) and have distinct roles in the regulation of protein phosphorylation (Lindgren *et al.* 2003). Furthermore, behavioral studies of D2L-deficient mice have shown that D2L and D2S contribute differentially to the regulation of certain motor functions (Usiello *et al.* 2000; Wang *et al.* 2000) and emotional responses (Hranilovic *et al.* 2008). Similarly, human genetic studies have shown that the intronic single nucleotide polymorphism rs1076560, which has a significant effect on the expression ratio of the DRD2 isoforms, is associated with cognitive processing (Zhang *et al.* 2007) and emotional processing (Blasi *et al.* 2009). These results suggest that the expression ratio of the DRD2 isoforms is important for their functions.

However, little is known about the regulatory mechanism that mediates the alternative splicing of *DRD2*. Although it has been reported that haloperidol, sex steroid hormones, and ethanol affect the expression of splice variants (Arnauld *et al.* 1991; Guivarc'h *et al.* 1995, 1998; Oomizu *et al.* 2003), the molecular basis for these differences is unclear. In general, changes in splicing patterns are directed by regula-

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Abbreviations used: D2L, long form of DRD2; D2S, short form of DRD2; DRD2, dopamine receptor D_2; nPTB, neural PTB; PTBP1, polypyrimidine tract-binding protein 1; Tpm2, tropomyosin 2.

tory proteins that bind the pre-mRNA sequence and enhance or silence particular splicing choices (Li *et al.* 2007). Thus, in this study, we searched for proteins that regulate the alternative splicing of *DRD2* using a cellular splicing assay and identified the involvement of the splicing factor polypyrimidine tract-binding protein 1 (PTBP1).

Materials and methods

Plasmid construction

The region from exon 5 to exon 7 of *DRD2* was amplified from human genomic DNA and cloned into the *Xho*I-*Hind*III site of pEYFP-C1 (Clontech, Mountain View, CA, USA) (Fig. 1a). The open reading frames that encode SF2/ASF, PTBP1, nPTB, NOVA1, HuB, FOX2, hnRNP A1, and Tra2b were amplified by PCR from a human fetal brain cDNA library (Clontech) and cloned into pcDNA3.1/V5-His (Invitrogen, Carlsbad, CA, USA) using conventional biological techniques. Primer sequences are listed in Table S1. Plasmid constructions of NAPOR and FOX1 are gifts from Dr. Yoshihiro Kino, RIKEN Brain Science Institute, and hnRNP H from Dr. Kinji Ohno, Nagoya University. Heterologous minigenes were generated by inserting *DRD2* fragments containing exon 6, exon 7 and flanking regions into pEGFP-Tpm2-ex1-2 (a gift from Dr. Kino, RIKEN Brain Science Institute). *DRD2* deletion mutants were generated by inverse PCR from the wild-type plasmid using primers flanking the deleted regions. The nucleotide sequences of the DNA inserts were confirmed by sequencing.

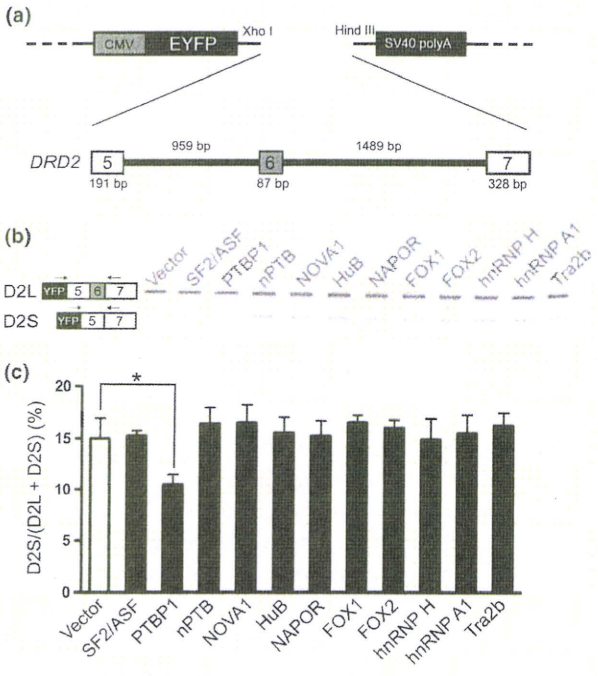

Fig. 1 The over-expression of PTBP1 reduced the alternative splicing of D2S. (a) Structure of the *DRD2* minigene. (b) Representative result from RT-PCR assays in which the *DRD2* minigene and plasmids for expressing RNA-binding proteins were transfected into HEK293 cells. The upper bands correspond to the splice product containing exon 6 (D2L), while the lower bands correspond to the splice product lacking exon 6 (D2S). (c) Bar chart showing the quantified percentage of D2S (Mean + SEM, $n = 3$). The statistical significance was analyzed by Dunnett's multiple-comparison test ($^*p < 0.05$).

Cell culture and transfection

HEK293 and SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and incubated at 37°C with 5% CO_2. For the minigene assays, HEK293 cells were transfected with plasmids for the expression of minigene and V5-tagged proteins using Fugene 6 (Roche Diagnostics, Basel, Switzerland). In our RNAi experiments, HEK293 cells were transfected with the minigene plasmids and an siRNA for *PTBP1* (Invitrogen, Stealth™ Select RNAi HSS143520, and Negative Control Hi GC) and *nPTB* (Invitrogen, Stealth™ Select RNAi HSS126818, and Negative Control Lo GC) using Lipofectamine 2000 (Invitrogen), and SH-SY5Y cells were transfected with the siRNA using Lipofectamine RNAiMAX (Invitrogen) and the Reverse Transfection protocol. The efficacy of the RNAi-mediated knockdown of endogenous PTBP1, nPTB, and actin expressions was determined by western blot analysis using anti-PTBP1 (Invitrogen, catalog No. 32-4800), anti-nPTB (Abnova, Taipei City, Taiwan, catalog No. H00058155-A01), and anti-actin (Sigma-Aldrich, St. Louis, MO, USA, catalog No. A2066) antibodies.

Identification of DRD2 splice variants

Forty-eight hours after transfection, total RNA was isolated from the cells using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). cDNA synthesis was performed using a PrimeScript First Strand cDNA Synthesis Kit (TAKARA BIO, Shiga, Japan) using oligo dT primer. The DRD2 minigene fragments were amplified by PCR (20 cycles) using a forward primer specific for the 3′ region of *EYFP* (AAGTCCGGACTCAGATCTCG) and a *DRD2*-specific reverse primer (DRD2-Ex7-Rv) that annealed to the 5′ region of exon 7 (CATCTCCATCTCCAGCTCCT). To detect endogenous *DRD2* fragments, a forward primer specific for exons 4 and 5 (CAATAACGCAGACCAGAACG) and DRD2-Ex7-Rv were used (40 cycles). For tropomyosin 2 (Tpm2)-based minigenes, primers green fluorescence protein (GFP)-Fw (CATGGTCCTGCTGGAGTTCGTG) and Tpm2-ex2-splicing-Rv2 (GGAGGGCCTGCTGCTCTTC) were used (Kino *et al.* 2009). The amplified products were resolved by 6% polyacrylamide gel electrophoresis and visualized using ethidium bromide. The intensities of the bands corresponding to the long and short forms were quantified by LAS-3000 and MultiGage software (Fuji Film, Tokyo, Japan). The quantified values were divided by the number of base pairs.

Results

PTBP1 regulates the alternative splicing of DRD2

To identify trans-acting factors that regulate the alternative splicing of *DRD2*, we used RT-PCR to detect splice variants. We constructed a gene fragment encompassing exons 5 through 7 of human *DRD2* in the vector pEYFP (Fig. 1a). This minigene was then transfected into HEK293 cells, and the expression ratios of D2L and D2S were analyzed by

RT-PCR. When the *DRD2* minigene was transfected with empty pcDNA3.1, the percentage of D2S was about 15% (Fig. 1b and c). Next, we expressed V5-tagged versions of several proteins known to regulate pre-mRNA splicing in the nervous system (SF2/ASF, PTBP1, nPTB, NOVA1, HuB, NAPOR, FOX1, FOX2, hnRNP H, hnRNP A1, and Tra2b); notably, SF2/ASF was previously proposed to regulate the alternative splicing of *DRD2* (Oomizu *et al.* 2003). Among the proteins tested, only when PTBP1 was transfected with the *DRD2* minigene was the percentage of D2S significantly reduced (to about 10%; Fig. 1b and c). We have confirmed the expressions of each RNA-binding proteins by western blot analysis and noted that the abundance of nPTB, NAPOR, and FOX1 are low (Figure S1). In addition, we showed the effects of PTBP1 were concentration dependent (Figure S2).

Next, we knocked down endogenous PTBP1 expression using an siRNA to confirm the effect of PTBP1 on *DRD2* splicing. We first confirmed the efficacy of the siRNA in modulating the expression of the target protein by western blot analysis (Fig. 2b). The presence of two PTBP1 bands rather than one is most likely because of phosphorylation. (Grossman *et al.* 1998). When the *DRD2* minigene was transfected with an siRNA for *PTBP1*, the percentage of D2S was significantly increased compared to transfection with a control siRNA (Fig. 2a). We also examined the effect of the knockdown of nPTB, a homologue of PTBP1, because it was reported that appearance of some exons are affected by both PTBP1 and nPTB (Boutz *et al.* 2007). The knockdown of PTBP1 increased the expression of nPTB (Fig. 2b), consistent with the previous reports (Boutz *et al.* 2007; Makeyev *et al.* 2007). While endogenous nPTB level was remarkably low and the knockdown of nPTB by siRNA was not observed, the increase in nPTB expression by the knockdown of PTBP1 was clearly inhibited by a siRNA for nPTB (Fig. 2b). Even when the increase in nPTB was inhibited, the knockdown of PTBP1 still increased the production of D2S splice variant (Fig. 2a), suggesting that the increase in nPTB has little or no effect on the alternative splicing of DRD2. Furthermore, we examined whether PTBP1 regulates the alternative splicing of endogenous *DRD2* in human neuroblastoma SH-SY5Y cells. When the siRNA for *PTBP1* was transfected into SH-SY5Y cells, the percentage of endogenous D2S fragments was also increased (Fig. 2c and d).

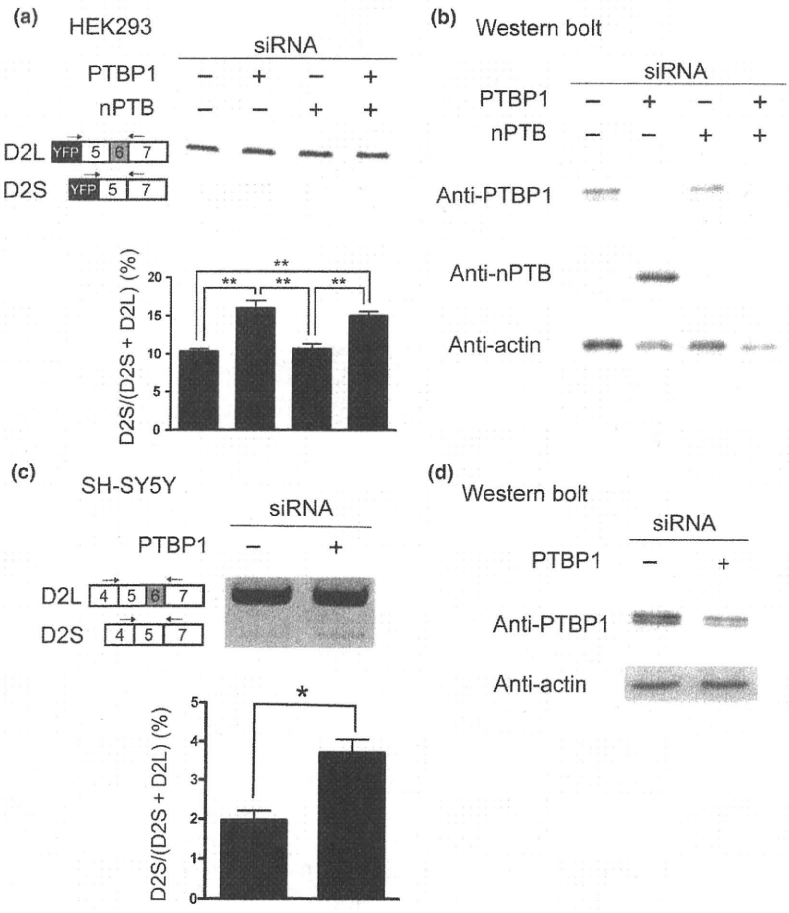

Fig. 2 The knockdown of PTBP1 increased the production of D2S splice variant. (a) Representative result from our cellular splicing assay using the *DRD2* minigene and siRNA for *PTBP1* and *nPTB* in HEK293 cells. Bar charts show the quantified percentages of D2S (Mean + SEM, $n = 3$). The statistical significances were analyzed using Tukey's multiple comparison test ($^{**}p < 0.01$). (b) Representative result of western blot analysis of PTBP1 and nPTB in HEK293 cells. (c) Representative result of endogenous *DRD2* splicing using a siRNA for *PTBP1* in SH-SY5Y cells. Bar charts show the quantified percentages of D2S (Mean + SEM, $n = 3$). The statistical significance was analyzed using *t*-tests ($^{*}p < 0.05$). (d) Representative result of western blot analysis of PTBP1 in SH-SY5Y cells.

Intronic regions flanking exon 6 are required for the PTBP1-mediated regulation of DRD2 splicing

To define the regions of *DRD2* that are required for its regulation by PTBP1, we utilized several previously generated heterologous minigenes (Kino *et al.* 2009). In these minigenes, the regions of interest were inserted in the context of constitutive exons of mouse *Tpm2*, which is distinct from *DRD2*. A reference fragment containing exon 9 of *Tpm2* and its flanking intronic regions or a *DRD2* fragment containing exon 6 or exon 7 and their flanking regions were inserted into a *Tpm2* fragment covering exons 1 and 2 (Fig. 3a). First, we predicted branch sites by a web-based program called ESEfinder 3.0 (Table S2, http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi). Then, using our *Tpm2*-based heterologous minigenes, we found that 14 bp upstream and 5 bp downstream of exon 6 are necessary for proper splicing (Fig. 3b). When exon 7 of DRD2 was inserted into Tpm2 cassette, a shorter exon 7 lacking the first 140 nucleotides was the main product. It was shown that 17 bp upstream of exon 7 is necessary for the splicing of full-length exon 7 (Fig. 3c). Because the primary elements regulating alternative splicing are thought to be located up to 200–300 nucleotides upstream and/or downstream of the regulated exon (Cooper 2005), a *DRD2* fragment stretching from 200 bp upstream of exon 6 (760 bp downstream of exon 5) to 200 bp downstream of exon 6 was used to examine the binding sequence of PTBP1 (Fig. 4a). PTBP1 had no effect on the inclusion of *Tpm2* exon 9 in HEK293 cells (Fig. 4b, left). In contrast, PTBP1 repressed *DRD2* exon 6 inclusion of the heterologous minigene, demonstrating that the inserted fragment of *DRD2* was sufficient for the response to PTBP1 (Fig. 4b, right). Next, to examine which region is necessary for the gene's responsiveness to PTBP1, we constructed *DRD2* deletion mutants lacking 200 bp upstream of exon 6 (Δint5_760–945), downstream of exon 6 (Δint6_6–200) or upstream of exon 7 (Δint6_1290–1487) (Fig. 4c). These deletion mutants were designed to include the regions that are necessary for splicing of exon 6 and exon 7. As shown in Fig. 4(d), Δint5_760–945 and Δint6_6–200 mutations altered the basal splicing pattern. Both deletion mutants exhibited markedly increased exclusion of exon 6 (from 15% to about 60% with vector transfection), suggesting the presence of elements in the deleted regions that enhance the inclusion of exon 6. Further, the over-expression of PTBP1 had no effect on either deletion mutant, indicating that both mutants had impaired responsiveness to PTBP1 (Fig. 4d). On the other hand, the over-expression of PTBP1 reduced D2S in the Δint6_1290–1487 mutant as well as a wild-type minigene, suggesting that PTBP1 affects the alternative splicing of DRD2 in regions other than the 3′ end of intron 6.

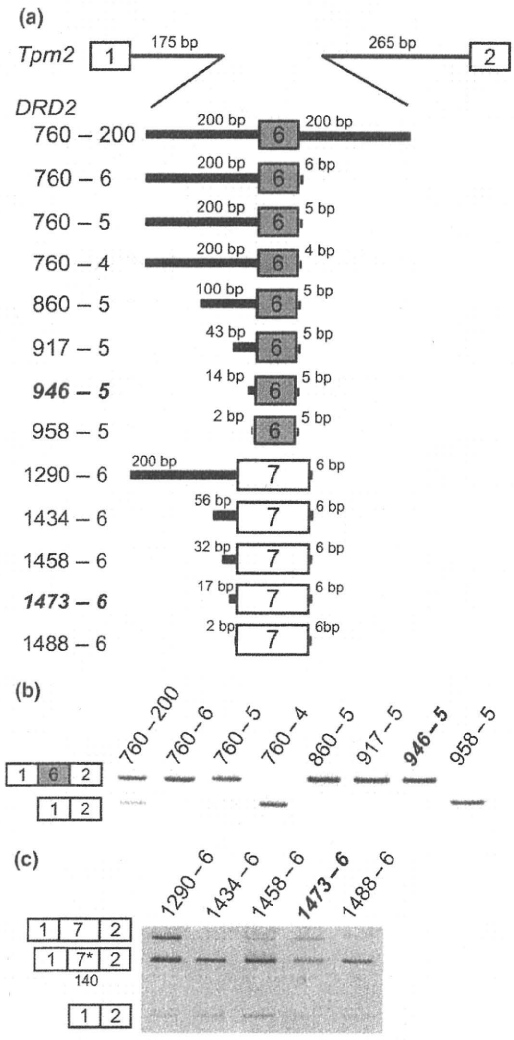

Fig. 3 Identification of DRD2 intronic regions which are necessary for the splicing of exon 6 and exon 7. (a) Structure of the *Tpm2*-based heterologous minigenes. The positions of the inserted nucleotides in introns 5, 6 and 7, as well as the numbers of base pairs in the fragments, are indicated. (b, c) Representative results from identification of splice variants using *Tpm2*-based heterologous minigenes in HEK293 cells. The white box 7* shows a shorter exon 7 lacking the first 140 nucleotides.

Discussion

Previous studies have shown that the functions of two splice variants of DRD2, D2L and D2S, differ in their biochemical properties and physiological functions (Senogles 1994; Guiramand *et al.* 1995; Khan *et al.* 1998; Usiello *et al.* 2000; Wang *et al.* 2000; Centonze *et al.* 2002, 2004; Lindgren *et al.* 2003; Senogles *et al.* 2004; Hranilovic *et al.* 2008); however, it is unclear what regulates the expression ratio of these isoforms. In this study, we identified PTBP1 as a splicing regulatory protein that reduces the expression of the D2S isoform.

Among the eleven proteins that we over-expressed with the *DRD2* minigene in HEK293 cells, only PTBP1

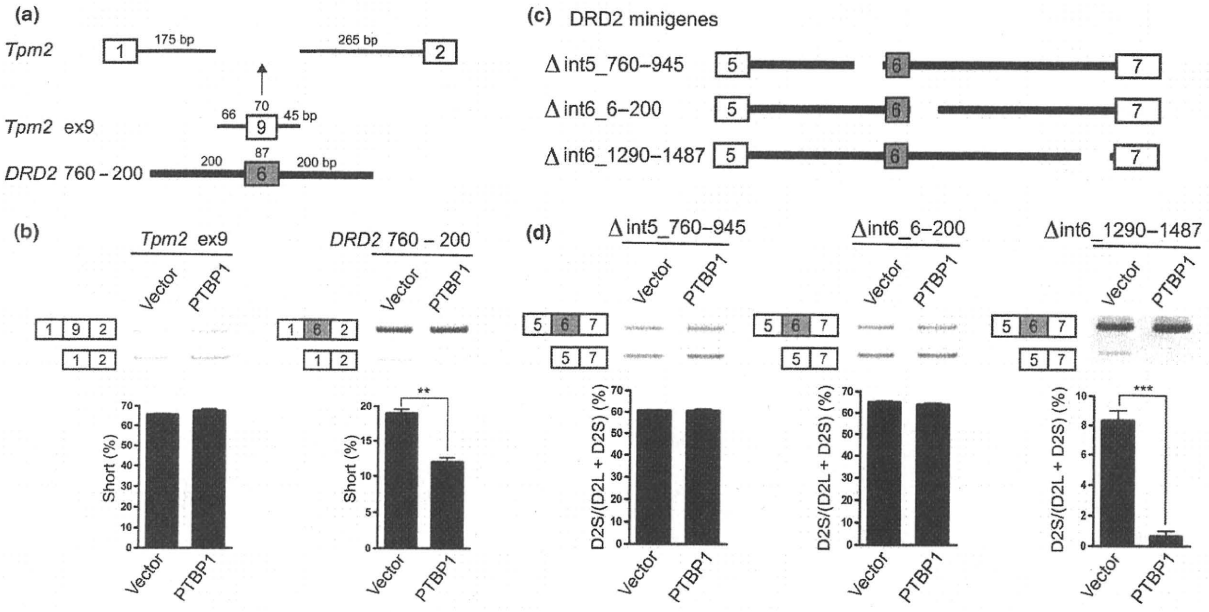

Fig. 4 Splicing regulation by PTBP1 in heterologous minigenes and *DRD2* deletion mutants. (a) Structure of the *Tpm2*-based heterologous minigene. Intronic fragments derived from *DRD2* are indicated by thick lines, whereas those derived from *Tpm2* are indicated by thin lines. (b) Splicing assay results using *Tpm2*-based heterologous minigenes and PTBP1 in HEK293 cells. Bar charts show the quantified percentages of exon exclusion (Mean + SEM, $n = 3$). (c) Structure of the *DRD2* deletion mutants. The positions of the inserted nucleotides in introns 5 and 6 are indicated. (d) Splicing assay results using the *DRD2* deletion mutants and PTBP1 in HEK293 cells. Bar charts show the quantified percentages of D2S (Mean + SEM, $n = 3$). The statistical significance was analyzed using *t*-tests (**$p < 0.01$, ***$p < 0.001$).

produced an altered splicing pattern (Fig. 1b and c). The reduction in the percentage of D2S suggests that PTBP1 enhances the inclusion of the alternative exon 6. Although the effect of PTBP1 was relatively small, this effect was shown to be concentration dependent (Figure S2). We also demonstrated that endogenous PTBP1 regulates DRD2 splicing by knockdown experiments in HEK293 cells with the *DRD2* minigene and in SH-SY5Y cells with the endogenous *DRD2* gene (Fig. 2a and c). Even though the effect of PTBP1 was statistically significant, it was quantitatively small in our splicing assay. Therefore, some other splicing factors may be involved in the splicing regulation of *DRD2*. In addition, the double knockdown of PTBP1 and nPTB suggested that nPTB, a homolog of PTBP1, has little or no effect on the alternative splicing of DRD2 (Fig. 2a). However, because the expression levels of exogenous and endogenous nPTB were remarkably lower than PTBP1 in HEK293 cells, it is still unclear whether nPTB regulates the splicing of DRD2.

Next, we identified the regions responsive to PTBP1, using *Tpm2*-based heterologous minigenes and *DRD2* deletion mutants. Using our heterologous minigenes, the splicing of a *DRD2* fragment containing exon 6 as well as 200 bp-upstream and -downstream intronic regions was altered by PTBP1 (Fig. 4b), similar to the results obtained using the *DRD2* minigene (Fig. 1b and c). In the *DRD2* deletion mutants, PTBP1 had no effect on the splicing of deletion mutants lacking exon 6-flanking regions in intron 5 or 6, whereas PTBP1 still affected the splicing of a deletion mutant lacking the 3′ end of intron 6 (Fig. 4d). These results indicate that exon 6-flanking regions are sufficient for the response to PTBP1, and that both regions in introns 5 and 6 are necessary.

Although PTBP1 is known to bind cytosine and uracil (CU)-rich intronic elements flanking an exon and repress splicing (Wagner and Garcia-Blanco 2001; Sharma *et al.* 2008), in this study PTBP1 appeared to enhance the inclusion of *DRD2* exon 6 rather than repressing the splicing from exon 5 to exon 7. It is noted that intron 5 contains UCUCU (849–853) and intron 6 contains UCUUUCU (32–38) sequences, but we have no evidence that PTBP1 directly binds the pre-mRNA of *DRD2*. Therefore it is possible that PTBP1 may indirectly affect the alternative splicing of *DRD2*.

It was reported that a DRD2 antagonist, haloperidol, increased the expression of D2S (Arnauld *et al.* 1991). The activation of DRD2 is coupled to the inhibition of adenylyl cyclase and cAMP-dependent protein kinase A, and cAMP-dependent protein kinase A has been shown to modulate the nucleocytoplasmic translocation of PTBP1 (Xie *et al.* 2003; Knoch *et al.* 2006). Together with these reports, our results suggest that DRD2 regulates the expression of its isoforms by modulating the localization of PTBP1.

Acknowledgements

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1. The expressions of RNA-binding proteins were confirmed by western blot analysis.

Figure S2. The concentration dependency of the PTBP1 effects.

Table S1. Primer sequences used for cloning.

Table S2. Branch site prediction by ESEfinder 3.0.

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Differential Effects of the HESR/HEY Transcription Factor Family on Dopamine Transporter Reporter Gene Expression Via Variable Number of Tandem Repeats

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The 3′-untranslated region (UTR) of the human dopamine transporter (*DAT1*) gene contains a variable number of tandem repeats (VNTR) domain, which is thought to be associated with dopamine-related psychiatric disorders, personality, and behavior. However, the molecular and neuronal functions of polymorphisms within the VNTR domain are unknown. We previously identified the transcription factor HESR1 (HEY1) as a VNTR-binding protein. *Hesr1* knockout mice exhibit DAT up-regulation in the brain and low levels of spontaneous activity. Other members of the HESR (HEY) family, including HESR2 (HEY2) and 3 (HEYL), have similar DNA-binding domains. In this study, we analyzed the effects of HESR1, -2, and -3 on *DAT1* expression in human neuroblastoma SH-SY5Y cells using luciferase reporter assays. We found that the VNTR domain played an inhibitory role in *DAT1* reporter gene expression and that HESR1 and -2 inhibited expression via both the core promoter and the VNTR. The inhibitory effects of HESR family members on *DAT* reporter gene expression differed depending on the number of repeats in the VNTR domain. We also found that each Hesr was expressed in the dopaminergic neurons in the mouse midbrain. These results suggest that the HESR family is involved in *DAT* expression via the VNTR domain.

Key words: dopamine; genetic polymorphism; VNTR; luciferase reporter assay

The dopaminergic nervous system plays important regulatory roles in locomotion, cognition, reward, affection, and hormone release (Bannon et al., 2001; Jackson and Westlinddanielsson, 1994; Missale et al., 1998; Uhl, 2003). Thus, dopamine and its related genes are thought to be involved in neuropsychiatric disorders and behavioral traits. The human dopamine transporter (*DAT1*) gene is involved in many dopamine-related disorders. DAT levels 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; Brunswick et al., 2003; Krause et al., 2003). In addition, several psychoactive drugs, including cocaine, amphetamine, and methylphenidate, are known to inhibit dopamine reuptake by DAT (Giros et al., 1991, 1992; Kilty et al., 1991; Shimada et al., 1991; Giros and Caron, 1993).

A functional genetic polymorphism has been described in the 3′-untranslated region (UTR) of exon 15 in *DAT1* (Michelhaugh et al., 2001). This 3′-UTR contains a 40-bp-long variable number of tandem repeats (VNTR) domain (Fig. 1; Vandenbergh et al., 1992; Michelhaugh et al., 2001). The polymorphism within this region is known to be associated with such neuropsychiatric disorders as ADHD, PD, alcoholism, and drug abuse (Cook et al., 1995; Ueno et al., 1999; Vandenbergh et al., 2000; Ueno, 2003; D'souza and Craig, 2008) and with modified gene expression depending on the genotype in vivo (Heinz et al., 2000; Jacobsen et al., 2000; Mill et al., 2002; D'souza and Craig, 2008) and in mammalian cell lines (Fuke et al., 2001, 2005; Inoue-Murayama et al., 2002; Miller and Madras, 2002; Greenwood and Kelsoe, 2003; Mill et al., 2005; VanNess et al., 2005; D'souza and Craig, 2008). It is expected that this region contains binding sites for interacting proteins, but, because these factors have not been described, the molecular and neuronal functions of the polymorphism are unknown (Michelhaugh et al., 2001).

We previously identified and characterized HESR1 (HEY1) as a *trans*-acting repressor of gene expression

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that acts through the 3′-UTR of *DAT1* via a yeast one-hybrid system (Fuke et al., 2005). In addition, we also showed that HESR1 binds directly to the region by electrophoretic mobility shift assay (EMSA) and represses the expression of the endogenous *DAT1* gene in the HEK293 cell line by RT-PCR assay (Fuke et al., 2006). In *Hes1* knockout (KO) mice, *DAT1* expression was enhanced, and the mice exhibited a reduction in spontaneous locomotor activity and exploration to novelty (Fuke et al., 2006). These findings suggest that the human *HESR1* gene and the polymorphisms could be related to many psychiatric disorders and behavioral traits. However, it is possible that other factors affect *DAT1* expression via the VNTR domain, insofar as more than one interacting factor is expected to bind this region (Michelhaugh et al., 2001). Conflicting results have been reported from studies using different cell lines, which may express different transcription factors (Fuke et al., 2001, 2005; Inoue-Murayama et al., 2002; Miller and Madras, 2002; Greenwood and Kelsoe, 2003; Mill et al., 2005; VanNess et al., 2005; D'souza and Craig, 2008). These results suggest that *DAT1* expression can be altered by cell-specific factors depending on the VNTR alleles present.

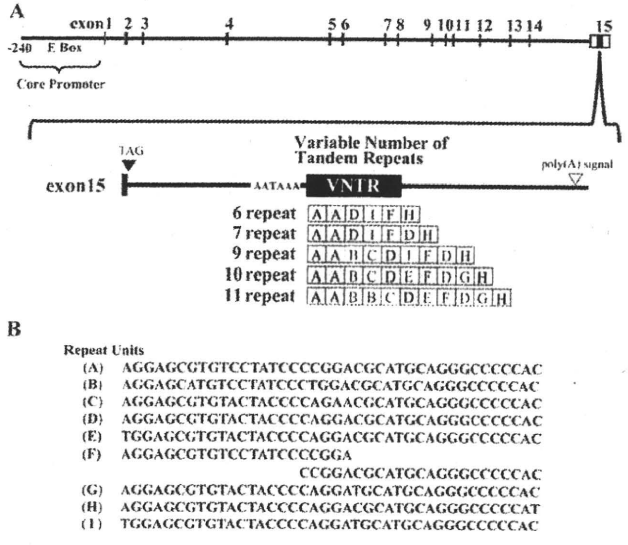

Fig. 1. Genomic structure of *DAT1* and allelic variants of the VNTR domain in exon 15. **A:** The coding region (black box), noncoding region (open box), VNTR domain, and constant parts of the repeat units (gray box) are shown. Exon 15 of *DAT1* contains a stop codon (solid arrowhead) and polyadenylation signal (open arrowhead). Upstream of the VNTR domain are six nucleotides (AATAAA) that resemble a polyadenylation signal. The allelic variants of the VNTR indicate the repeat unit type (A–I) for each allele. **B:** Nucleotide sequence of each unit of the VNTR polymorphism in the 3′-UTR of *DAT1*.

The Hesr family genes *Hesr1*, *-2*, and *-3* (*Hey1*, *Hey2*, and *HeyL*) were identified as the hairy/enhancer split-type basic helix-loop-helix (bHLH) genes. They have been shown to be direct transcriptional targets of the Notch signaling pathway, which is essential for neural development (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999; Henderson et al., 2001; Iso et al., 2001, 2003; Wang et al., 2002; Sakamoto et al., 2003). HESR family genes carry a bHLH domain essential for DNA binding as well as an Orange domain and YRPW motif, which mediate interaction with proteins and affect dimerization or recruitment of corepressors (Fischer and Gessler, 2007; Fig. 2). HESR proteins repress the expression of target genes by binding to E- or N-box bHLH-binding consensus sites (Nakagawa et al., 2000; Iso et al., 2001, 2003). The bHLH domain is highly conserved among HESR family members (Steidl et al., 2000), and Hesr1 and -2 repress gene expression via the same sequence (Kokubo et al., 2007). Thus, HESR1 along with HESR2 and -3 may be candidate factors regulating *DAT* expression via the VNTR. However, any roles of HESR2 and -3 in *DAT* gene expression have not yet been characterized.

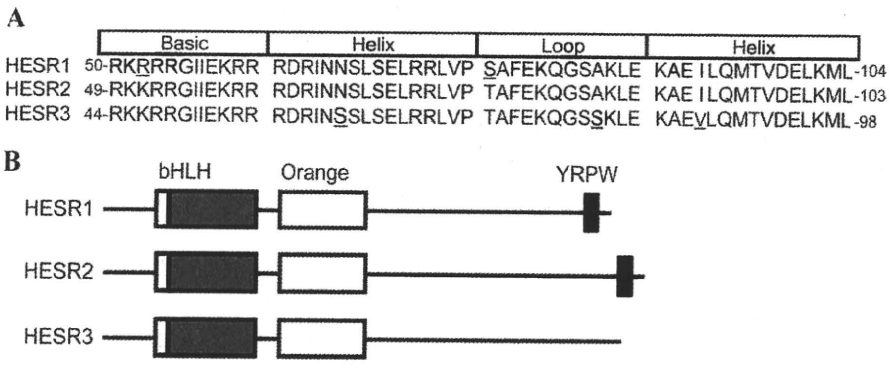

Fig. 2. Structure of HESR family members. **A:** Comparison of the primary sequences of the bHLH domain among human HESR family members (HESR1, 50–104 amino acids; HESR2, 49–103 amino acids; and HESR3, 44–98 amino acids). Those residues that differ among the family members are underscored. The primary sequences of the bHLH domain in the mouse Hesr family are identical to those in the human protein. **B:** Structure of the HESR family with the three major domains noted: bHLH domain, Orange domain, and YRPW motif.

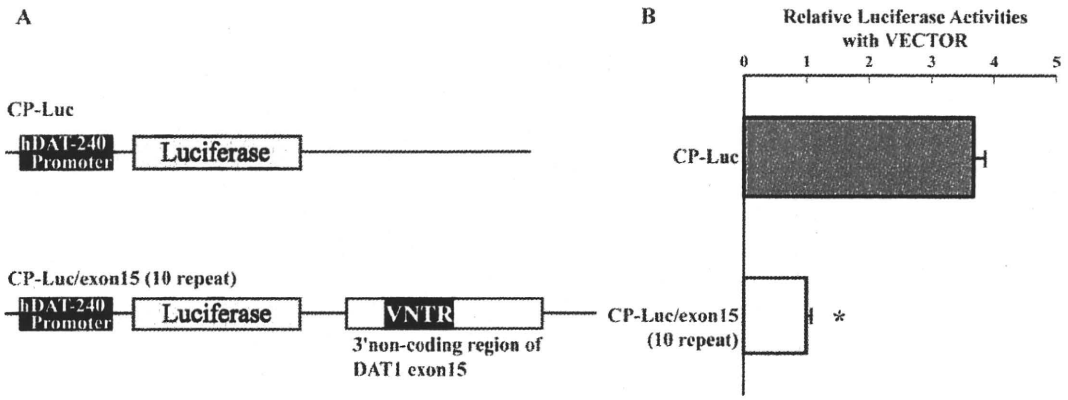

Fig. 3. Luciferase reporter vector and its activity in SY-SH5Y cells. **A:** Schematic diagram of the luciferase reporter vectors CP-Luc and CP-Luc/exon 15. CP-Luc contains only the *DAT* core promoter, whereas CP-Luc/exon 15 contains both the core promoter and the 3′-UTR with 10r, which is the most common allele. **B:** Negative regulation of gene expression through the 3′-UTR of *DAT* in SY-SH5Y cells. CP-Luc or CP-Luc/exon 15 and empty vector were transfected into the cells. Relative luciferase activity is expressed as the mean ± SEM. $^*P < 0.0001$, Student's *t*-test.

In this study, we sought to clarify whether HESR2 and -3, as well as HESR1, also affect *DAT1* expression in human neuroblastoma cells via the 3′-UTR, including the VNTR region. Luciferase reporter constructs were made containing the endogenous *DAT1* core promoter and the VNTR with 6, 7, 9, 10, or 11 repeats, which is consistent with the sequences reported previously (Fuke et al., 2001). The differential effects on VNTR among HESR1, -2, and -3 were also characterized. Furthermore, we conducted immunohistochemistry for Hesrs and tyrosine hydroxylase (TH), a marker of dopaminergic neurons, in mouse midbrains to analyze localization of Hesr proteins for the investigation of HESR family function in the brain dopamine systems, because localization of HESR proteins in the adult brain has not been reported, although dopaminergic neurons localize in the specific brain regions (Bjorklund and Dunnett, 2007).

MATERIALS AND METHODS

General Procedure

First, to characterize the role of the 3′-UTR in *DAT* expression, a CP-Luc or CP-Luc/exon15 (10r) reporter vector and empty vector (control) were expressed in cells. Ten repeats is the most common allele in the population (Fuke et al., 2005; D'souza and Craig, 2008). CP-Luc contained only the *DAT* core promoter, whereas CP-Luc/exon15 contained both the core promoter and the 3′-UTR (Fig. 3). Next, the empty vector or each HESR was cotransfected with CP-Luc or CP-Luc/exon15 (10r). Finally, to compare the VNTR alleles and HESRs, each repeat (6, 7, 9, 10, or 11r) of CP-Luc/exon15 and the HESR or vector were cotransfected. The relative luciferase activity was standardized to that of the vector. In the experiment examining the repeat effect, the relative luciferase activity was standardized to that of 10r in each group. In addition, the localization of Hesrs in the mouse midbrain was detected by immunohistochemistry.

Cloning and Construction

Two kinds of luciferase reporter vectors were prepared: CP-Luc and CP-Luc/exon 15 (see Fig. 3A). CP-Luc contained the human *DAT* core promoter (–240 to +2; Fig. 1A; Kouzmenko et al., 1997; Sacchetti et al., 1999) cloned from the DAT1-8317 plasmid (Sacchetti et al., 1999), a gift from Dr. Michael J. Bannon (Wayne State University, School of Medicine, Detroit, MI), upstream of the firefly luciferase site in the modified pGL3 vector (Promega, Madison, WI). CP-Luc/exon 15 contained the human *DAT* core promoter and 3′-UTR region including the VNTR domain downstream of the luciferase site in CP-Luc. There are five kinds of VNTR alleles (6, 7, 9, 10, and 11r), which is consistent with the sequence (Fig. 1B) reported in our previous study (Fuke et al., 2001). These reporter vectors are the same as the constructs used in our previous study (Fuke et al., 2005); schematic structures of the reporter vectors are shown in Figure 3A. Additional information on these constructs is described in our previous work (Fuke et al., 2005).

All of the HESR family expression vectors were made by cloning the cDNA into myc-pcDNA modified from pcDNA 3.1+ (Invitrogen, Carlsbad, CA), an expression vector for mammalian cell lines. The Myc-tag is located upstream of the multicloning site. Myc-pcDNA was also used as a control vector (the vector described in Figs. 3–6). Myc-pcDNA and Myc-HESR1 (human) are the same as the constructs described in our previous study (Fuke et al., 2005). Myc-Hesr1 (mouse) was a gift from Dr. Hiroki Kokubo (Division of Mammalian Development, National Institute of Genetics, Mishima, Japan). Human HESR2 and -3 cDNAs were amplified from a fetal brain cDNA library (Clontech, Palo Alto, CA) by PCR with oligonucleotide primers and the high-fidelity DNA polymerase PrimeStar (TaKaRa, Shiga, Japan). Mouse Hesr2 and -3 cDNAs were amplified from cloned cDNAs in pBluescript (gifts from Dr. Hiroki Kokubo) by PCR with oligonucleotide primers and PrimeStar (TaKaRa). Each oligonucleotide primer was designed to

contain a restriction site (italicized). The primer sequences are as follows: human HESR2, Fw (XhoI) 5′-TTA*CTCGA*GAT GAAGCGCCC-3′ and Rv (ApaI) 5′-TTA*GGGCCC*T TAAAAAGCTCCAAC-3′; human HESR3, Fw (XhoI) 5′-TTA*CTCGA*GATGAAGCGACCC-3′ and Rv (XhoI) 5′-TTA*CTCGA*GTCAGAAAGCCCC-3′; mouse Hesr2, Fw (XhoI) 5′-TTA*CTCGA*GATGAAGCGCCCT-3′ and Rv (ApaI) 5′-ATA*GGGCCC*TTAAAAGCTGGCTCC-3′; and mouse Hesr3, Fw (XhoI) 5′-TTA*CTCGA*GATGAA GCGGCC-3′ and Rv (ApaI) 5′-TTA*GGGCCC*TCAGAAA GCC-3′. These amplified fragments were cloned into T-Vectors (Promega p-GEM T Easy Kit) and sequenced by the dideoxy chain termination method with CEQ DTCS and CEQ8000 (Beckman Coulter, Brea, CA). Finally, the fragments were digested with each restriction enzyme and subcloned into myc-pcDNA.

Cell Culture and Transient Transfection: Luciferase Reporter Assay

The methods used for culture, transfection, cell harvesting, and luciferase activity measurements followed the standard methods of the Dual-Luciferase Reporter Assay System (Promega). SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% FBS at 37°C in an atmosphere of 5% CO_2. SH-SY5Y cells were plated into 24-well plates and cultured until they grew to 80% confluence before transfection. The cells were transfected with 1 μg total plasmid using Lipofectamine 2000 reagent (Invitrogen). The firefly luciferase reporter gene (0.5 μg) and each HESR or vector (0.5 μg) were coexpressed in the cells. Plasmid pRL (Promega) containing the sea pansy luciferase gene was cotransfected (20 ng) as an internal control to normalize the transfection efficiency in all experiments. After 48 hr, the cells were harvested and stored at −80°C. Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega). The firefly and sea pansy luciferase activity was measured using a Centro LB 960 (Berthold, Bad Wildbad, Germany) for 10 sec after a 2-sec delay, and then the value of each sample was calculated as light units of firefly luciferase per that of sea pansy. Each HESR group and its controls were measured at the same time on a Centro LB 960.

Animals

Adult (9-week-old) male C57BL6/J mice (CLEA Japan, Tokyo, Japan) were kept under a controlled temperature (23–25°C) and photoperiod (LD 14:10, lights off at 22:00 hr). Food and water were available freely. All experiments were conducted according to the Regulations for Animal Experimentation at the University of Tokyo (Tokyo, Japan).

Tissue Preparation

The mice were deeply anesthetized using sodium pentobarbital (50 mg/kg body weight) and then perfused intracardially with 0.05 M phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in 0.05 M phosphate buffer (PB). The brains were removed and postfixed with the same fixative for 2 hr and immersed in 30% sucrose in 0.05 M PB for several days at 4°C. Serial coronal brain sections (20 μm) including the midbrain ventral tegmental area (VTA) and substantia nigra (SN; −3.04 to −3.49 to the bregma) were made with a cryostat and collected according to a brain map (Franklin and Paxinos, 2008). Five animals were used in this experiment.

Immunohistochemistry

Immunoperoxidase staining. Free-floating sections of the midbrain were incubated with 0.6% H_2O_2 in 10 mM PBS for 30 min at room temperature (RT) before and after rinsing with 10 mM PBS. Next, the sections were incubated with 5% normal goat serum (NGS; Vector Laboratories, Burlingame, CA), 0.4% Triton X-100, and 10 mM PBS for 1 hr at RT and then with a primary rabbit antibody against Hesr1 (working dilution 1:500; Chemicon, Temecula, CA), Hesr2 (1:1,000; Chemicon), or Hesr3 (1:500; Chemicon) containing 5% NGS, 0.4% Triton X-100, and 10 mM PBS for 3 nights at 4°C. After washing with 10 mM PBS, the sections were reacted with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories) in 5% NGS, 0.4% Triton X-100, and 10 mM PBS overnight at 4°C. The sections were rinsed with 10 mM PBS three times and then incubated in avidin-biotin-peroxidase complex (ABC) solution (Vectastain Elite ABC Kit; Vector Laboratories). Next, the sections were reacted with 0.05% 3,3′-diaminobenzidine (DAB) in 0.01% H_2O_2 and 100 mM Tris-HCl to visualize Hesr1-, -2-, or -3-immunoreactive (-ir) cells.

For single staining of the TH-ir (a marker of dopaminergic neurons) cells, similar steps were taken, except for the steps involving incubation with the primary or secondary antibody and visualization. The sections were incubated with a mouse anti-TH antibody (1:10,000; Chemicon) overnight at 4°C. The sections were then reacted with biotinylated goat anti-mouse IgG (1:200, Vector Laboratories) in 5% NGS, 0.4% Triton X-100, and 10 mM PBS overnight at 4°C after washing with 10 mM PBS three times. The sections were rinsed with 10 mM PBS three times and then incubated in ABC solution (Vector Laboratories). Next, the sections were reacted with 0.02% DAB solution to visualize TH-ir cells.

For double labeling, the same staining steps as for TH were performed after staining for each Hesr. Instead of DAB, a Vector SG Kit was used for visualization of TH after the ABC reaction. Immunostained sections mounted on slides were dehydrated through a graded ethanol series, cleared with xylene, and then coverslipped with an embedding agent.

Fluorescence immunohistochemistry for Hesrs and TH. Free-floating sections were rinsed with 10 mM PBS. The sections were then incubated with 5% normal donkey serum (NDS; Jackson Immunoresearch, West Grove, PA), 0.4% Triton X-100, and 10 mM PBS for 1 hr at RT and then with the primary rabbit antibody against Hesr1 (1:500; Chemicon), Hesr2 (1:1,000; Chemicon), or Hesr3 (1:500; Chemicon) and the primary mouse antibody for TH (1:10,000; Chemicon) in 5% NDS, 0.4% Triton X-100, and 10 mM PBS for 2 nights at 4°C. After washing with 10 mM PBS, the sections were reacted with donkey anti-rabbit IgG (1:200; Jackson Immunoresearch; Cy3) and donkey anti-mouse IgG (1:200; Jackson Immunoresearch; Cy2) in 5% NDS, 0.4% Triton X-100, and 10 mM PBS overnight at