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Expression of tyrosine hydroxylase is epigenetically regulated in neural stem cells

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ABSTRACT

Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in the biosynthesis of catecholamines, and its expression is regulated in a developmental stage- and cell type-specific manner. Our previous work suggested that the genetic elements responsible for cell type-specific expression of TH were in the repressor region of the TH promoter between −2187 and −1232 bp. To investigate the molecular mechanisms underlying the specificity of TH expression, the DNA methylation patterns of the CpG islands in the repressor region of the TH promoter were examined in human neural stem cells (NSCs) and dopaminergic neuron-like cells. Using a bisulfite sequencing method, we found that the cytosine residues of CpG islands within the NRSE-R site were specifically methylated in NSCs, but not in SH-SY5Y neuroblastoma cells. In NSCs, CpG methylation correlated with reduced TH gene expression, and inhibition of DNA methylation with 5-azacytidine restored TH expression. Furthermore, methyl-CpG binding domain proteins (MBDs) bound to the highly methylated X-1 and X-2 regions of the TH gene in NSCs. Taken together, these results suggest that region-specific methylation and MBDs play important roles in TH gene regulation in NSCs.

1. Introduction

Tyrosine hydroxylase (TH) catalyzes the initial, rate-limiting step in catecholamine biosynthesis, which is the conversion of tyrosine to dihydroxyphenylalanine [1]. Catecholamines function as neurotransmitters and hormones, affecting the peripheral and central nervous systems by regulating a multitude of visceral, motor, and cortical functions. Endogenous TH gene expression is essential during mid-gestational embryogenesis [2]. TH expression can be modified in adults at both the transcriptional and post-transcriptional levels by a variety of physiological and pharmacological conditions [1].

The mechanisms of gene expression involve both positive and negative regulation of promoter elements that control transcription. An increasing number of studies show that negative regulatory mechanisms play important roles in tissue-specific gene expression [3–6]. For example, methylation of CpG sites in genomic DNA is involved in establishing and maintaining cell type-specific gene expression [7–9] as well as gene silencing [10,11]. CpG methylation is thought to alter chromatin density and thus DNA accessibility by blocking the binding of transcription factors to their cognate DNA sequences and/or by increasing recruitment of methyl-binding proteins and histone deacetylases [12,13]. The methyl-CpG binding domain (MBD) family of proteins is present in most mammals, and the interaction of these proteins with methylated DNA has been characterized extensively [14]. The MBD proteins Mecp2, MBD1, MBD2, and MBD3 have been characterized as transcriptional repressors that bind specifically to methyl-CpG pairs; the exception is MBD3, which localizes to methylated DNA regions by associating with MBD2 [15–17]. Interestingly, the methyl-CpG binding proteins MeCP2, MBD2 and KAISO are dispensable for mouse embryogenesis, but play a redundant function in neural differentiation [18].

We reported previously that two types of repression are involved in TH gene silencing in human neural stem cells (NSCs). Specifically, both the NRSE-II site and a repressor region between −2164 and −1210 bp in the TH promoter act as cis-regulatory elements [19]. The silencing element in the −2164 and −1210 region represses TH expression in an NRSF-independent manner.

In the present study, we investigated whether this silencing element regulates TH expression via CpG methylation. We mapped CpG islands in the −2187 to −1232 region of the human TH promoter using EMBOSS CpGPlot. Based on the results of this analysis, we also analyzed the methylation pattern of the TH promoter in NSCs and dopaminergic (DAergic) neuron-like cells to identify the CpG sites related to TH gene expression. To investigate whether TH mRNA expression is regulated by DNA methylation status and histone H3 modification, we treated HB1.F3 (TH^-) or SH-SY5Y cells (TH^+) with a DNA methylation inhibitor, 5′-aza-2′-deoxycytidine (5-AzadC) and with a histone deacetylase inhibitor, trichostatin A

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(TSA). This report provides the first evidence that TH gene expression is tightly linked to DNA methylation in the distal promoter region.

2. Materials and methods

2.1. Cell culture

The immortalized human NSC cell line HB1.F3 was established as described previously [20], and cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10% fetal bovine serum (FBS, Hyclone). SH-SY5Y, a human DAergic neuroblastoma cell line, was grown in DMEM supplemented with 10% FBS.

2.2. Construction of TH deletion promoters using PCR

To examine the role of the −2187 to −1232 bp region of the human TH promoter in TH expression, we divided this region three parts termed X-1, X-2, and X-3 that contained the NRSE-R, NRSE-I, and NBRE-B sites, respectively. Each of these three parts of the promoter were cloned into a TK–luciferase plasmid construct. A 956-bp fragment between −2187 and −1232 bp in the TH promoter was amplified with PCR using the following oligonucleotide primer pairs:

X-1F: gggaagctttttctggggaccttgagg, X-1R: cccggatcccagaccctgtcctctctc
X-2F: gggaagcttcactgggtgctgagagac, X-2R: cccggatccaactgcttcagccatggc
X-3F: gggaagcttttaggaaaggtcccaggg, X-3R: cccggatccgagaagaaccgtttccga

A restriction enzyme site was added to the 5′-end of each primer to facilitate subcloning. The TK–luciferase reporter constructs containing the PCR products were constructed using direct cloning of the PCR products after restriction enzyme digestion. All constructs were sequenced to confirm their identity.

2.3. Transfection and luciferase assay

Transfections were performed using LipofectAMINE PLUS reagent (Invitrogen, USA) according to the manufacturer's instructions. All transfections contained 1.5 μg of luciferase reporter plasmid and 0.5 μg of internal control plasmid pSV-β-galactosidase (Promega). Cells were transfected at ~60–80% confluence in 6-well plates and harvested 48 h after transfection. All transfections were performed three times in triplicate. Promoter activity was determined using the Single-Luciferase™ Reporter Assay System (Promega) following the manufacturer's instructions. Luciferase activity was normalized based on the β-galactosidase activity in each well.

2.4. RNA isolation and RT-PCR

Total RNA was purified from the cell lines. For reverse transcription-PCR (RT-PCR), 2 μg of total RNA was reverse transcribed with SuperScript II reverse transcriptase using random hexamers [pd(N)6] as primers. PCR reactions were carried out using a 1/10 volume of the reverse-transcribed product in a final volume of 50 μl using recombinant Taq DNA polymerase (GIBCO). Each PCR cycle consisted of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, followed by a 10-min extension at 72 °C. PCR products were separated in a 1.5% agarose gel with TAE buffer. The primer sets used for amplifying human TH mRNA were described previously [21].

2.5. Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assays were performed as follows. Cells were cross-linked with formaldehyde, suspended in SDS lysis buffer, and sonicated with a Sonic Dismembrator (Model 500, Fisher Scientific). Immunoprecipitation was performed using anti-MeCP2 (Santa Cruz) or MBD1 (Santa Cruz) antibodies. A protein A agarose/salmon sperm DNA (50%) slurry was added to remove the immunocomplexes. The precipitate was washed and heated to reverse the formaldehyde cross-linking, and DNA fragments were recovered by ethanol precipitation following proteinase K digestion and phenol/chloroform extraction. PCR was performed using the following TH promoter-specific primers:

X-1FCh: tttctggggaccttgagg, X-1RCh: cagaccctgtcctctctc
X-2FCh: ttaggaaaggtcccaggg, X-2RCh: gagaagaaccgtttccga

The PCR conditions were as follows: 94 °C for 5 min for initial denaturing followed by 35 cycles of denaturing at 94 °C for 45 s, annealing at 65 °C for 45 s, and extension at 72 °C for 45 s. The PCR products were visualized after separation using 1.5% agarose gel electrophoresis and ethidium bromide staining.

2.6. Bisulfite genomic sequencing analysis

Genomic DNA was isolated using the Wizard® Genomic DNA Purification System (Promega). Bisulfite modification of genomic DNA was performed as follows. Bisulfite-modified genomic DNA (1 mg) was used as a template in a PCR reaction to amplify the TH gene promoter region. PCR amplification involved 30 cycles of 30 s at 95 °C, 1 min at 54 °C, and 1 min at 72 °C after the initial Taq activation step (10 min at 95 °C). The bisulfite sequencing-specific primers used were 5′-tttctggggaccttgagg-3′ (forward) and 5′-gagaagaaccgtttccga-3′ (reverse). The PCR products were cloned into the pGEM-T-easy vector (Promega) and sequenced.

3. Results

3.1. Cell type-specific transcriptional activities of the TH promoter

Our previous study showed that the region between −2164 and −1210 bp in the TH promoter contains one or more silencing elements that represses TH gene transcription in NSCs but not in other cells (including DAergic neuron-like cells) [19]. Although this region contains two binding sites for the pan-neuronal repressors NRSF, NRSE-R and NRSE-I, mutation of these sites or treatment with the histone deacetylase inhibitor TSA has no effect on the transcriptional activity of TH promoter.

To examine the putative repressor activity in the −2164 and −1210 region in greater detail, we divided this region into three parts (X-1, X-2, X-3) and cloned each part into a vector with a TK–luciferase promoter. These were called X-1-TK–luc, X-2-TK–luc, and X-3-TK–luc, and they included the NRSE-R, NRSE-I, and BBE sites, respectively (Fig. 1A). To assess the functional significance of each part using the luciferase reporter assay, we transfected the constructs into HB1.F3 and SH-SY5Y cells (Fig. 1B). Interestingly, while the X-1-TK-luc and X-2-TK-luc constructs showed marked transcriptional repression in NSCs, X-3 showed no such effect. In SH-SY5Y cells, however, X-1 and X-2 showed no transcriptional repression. In addition, the X-3 construct showed increased transcriptional activity in SH-SY5Y cells. These data indicated that the X-1 and X-2 regions, but not the X-3 region, are responsible for the repressor activity of the TH promoter in NSCs.