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Full Length Research Paper

In vitro cloning and cell type-specific regulation of tyrosine hydroxylase promoter in C57BL/6J mice

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In order to investigate the ability of mouse tyrosine hydroxylase (TH) promoter regulating target genes *in vitro*, high-fidelity PCR was employed to amplify 450 bp, 1500 bp, 2000 bp, 2400 bp and full-length fragments of mouse TH promoter, which were then independently inserted into pcDNA3.0 plasmid as a promoter to regulate EGFP in two different cell lines [MN-9D (TH+) and ECV (TH-) cells]. The results showed that all the five cloned fragments of TH promoter contained the basic promoter elements of eukaryotic cells and the EGFP expression could be regulated by these 5 fragments in MN-9D cells to a similar extent. But, in ECV (TH-) cells, the capacity of 3650 and 2400 bp fragments of TH promoter regulating EGFP was markedly compromised when compared with that of the remaining three fragments. These findings suggested that, in the sequence between -2000 bp ~ -2400 bp of TH promoter, some dominant elements determined the ability of TH promoter regulating target genes in specific cells.

Key words: Tyrosine hydroxylase, promoter, regulation, cloning.

INTRODUCTION

Apoptosis of dopaminergic (DA) neurons in midbrain substantia nigra is the most common direct cause of Parkinson's disease (PD) (Baimbridge et al., 1992). It has been demonstrated that apoptosis of DA neurons is correlated with the expression of intracellular calcium-binding proteins, glia cell-line derived neurotrophic factors (GDNF), aromatic amino acid enzyme (AADC) and tyrosine hydroxylase (TH) (Howard, 2003; Jeffrey et al., 2000; Meyer et al., 1999; Mimoun et al., 2002). Studying the regulation of these genes, the above mentioned has provided directions for research on PD (Gardanch and O'Malley, 2004).

Tyrosine hydroxylase (TH) is a rate-limiting enzyme of catecholamine biosynthesis. The expression and activity of TH directly determine the amount of catecholamine in cells and further affect many important cellular physiological functions (Ernsberger, 2001). During the development of mammalian nervous system, TH is expressed tissue-specifically due to multiple mechanisms (Black et al., 1987). TH is predominantly expressed in several cell groups in substantia nigra, ventral tegmental area, hypothalamus, olfactory bulb dopaminergic neurons and noradrenergic neurons in locus coeruleus (Kumer and Vrana, 1996). TH expression in the specific neurons of brain is indicative of the specific regulation activity of TH promoter, which has been applied in gene therapy and purification of dopaminergic neurons (Cao et al., 2003; Hedlund et al., 2007).

It was reported that the two upstream sequences of rat TH gene, 272 and 200 bp, were used as promoters for cell transfection *in vitro* and preparation of transgenic animals *in vivo*. However, the results did not support that the downstream report gene was specifically regulated by these two assumed promoters (Trocmé et al., 1998). Mark et al. (2003) produced transgenic mice bearing an 11-kb fragment of human TH promoter and results revealed its downstream genes were specifically expressed in the dopaminergic cells.

Therefore, there might be specific regulatory elements in different regions of TH gene upstream sequence which determined the regulation of TH promoter. To confirm the hypothesis above, the present study was designated to

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clone several fragments of mouse TH gene promoter and detect the abilities of these fragments in regulating target genes. Our study will provide a specific promoter for over-expression of some interested genes in DA neurons and new strategy for the treatment of PD.

MATERIALS AND METHODS

Ethics statement

All animal experiments were approved by the Administrative Committee of Experimental Animal Care and Use of Xuzhou Medical College (XZ2007-0003), and conformed to the National Institute of Health guidelines on the ethical use of animals.

TH promoter cloning

The specific PCR primers were designed according to the DNA sequence of TH promoter in Genebank and the overlap PCR method was employed. The two pairs of primers were as follows:

5'-CATGGATCCAATGATTGGTATGGCTGGGGT-3', 5'-GTTGCTGACCCAGGAAGAGTTC-3',
5'-AGCAAGCTTAGCTGGTGGTCCCGAGTTCTGTC-3', 5'-GAACTCTTCCTGGGTCAGCAAC-3'.

The whole genomic DNA of C57BL/6J mice were used as templates to amplify upstream 1870 bp and downstream 1800 bp sequences of TH promoter, which were then amplified to TH promoter full-sequence by overlap PCR with PrimeSTARTM HS DNA Polymerase system (TaKaRa Company). The amplified fragments were retrieved by 1% agarose gel (QIAquick Gel Extractio Kit).

Construction of recombinant pTH-EGFP plasmid with TH promoter

pEGFP-N1 and pcDNA3.0 plasmids were double-digested with restriction enzymes Hind □ and Not □, and then EGFP fragment and the linear fragment of pcDNA3.0 were retrieved with QIAquick Gel Extractio Kit (Qiagen Corporation). The two fragments above were connected using a T4 DNA Ligase reaction system (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.) to obtain pcDNA3.0-EGFP, which was used to transfect competent DH (5a) and seeded in plates. After inoculation, a colony in 1 ml of liquid LB medium was obtained and the pcDNA3.0-EGFP plasmid was extracted with SV Minipreps DNA purification system (Promega Corporation). Restriction enzymes Hind β and Not – were independently used to confirm plasmid pcDNA3.0-EGFP.

Then, the anticipated pcDNA3.0-EGFP plasmid was digested with restriction enzymes, Hind β and Bglα, to retrieve 5400 bp fragment. TH promoter sequences were digested with restriction enzymes Hind β and BamH - at the same time, and the recovered fragments and the 5400 bp fragment were connected with T4 DNA Ligase reaction system to establish a plasmid entitled pTH-EGFP, which was identified by restriction endonuclease Hind β digestion and PCR, amplifying the upstream sequences of TH promoter.

Lipofection and expression of the recombinant pTH-EGFP plasmid in cells

A large number of pTH-EGFP, pcDNA3.0-EGFP and pcDNA3.0 plasmids were prepared with EndoFree Plasmid Maxi Kit (Qiagen Corporation). The materials required were as follows, MN-9D cells (hybridoma dopaminergic neurons, and TH+ cells, provided by Professor Qunyuan Xu, Capital Medical University, Beijing, China), ECV cells (ECV304 cell line, human umbilical vein endothelial cells, TH- cells provided by Professor Jianfeng Chen, the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences, Shanghai, China) Cells were plated in a 6-well plate one day prior to transfection. MN-9D cells were maintained in DMEM/F12 (Gibco Inc.) containing 10% fetal bovine serum and ECV cells were grown in 1640 medium supplemented with 10% of calf serum (Gibco Company).

After 16 h of incubation, the three plasmids were independently diluted with serum-free medium (4 μg of plasmid in 250 μl of medium in each well). The liposomes were diluted with antibiotics and serum free medium (10 μl of liposomes in 250 μl of medium in each well). Cell transfection was performed with Lipofectamin TM 2000 Reagent (Invitrogen Company). Following transfection, the transfected cells were incubated at 37℃ for 5 h in humidified air with 5% CO_2 and then the medium was refreshed for continous incubation for 24 h, which was repeated twice. Two types of cells were used for transfection with three different plasmids. The EGFP expression was observed under a fluorescence microscope (wavelength 480~490 nm) 48 h later.

Meanwhile, three fields of each well were randomly selected and photographs were captured at a magnification of x200, followed by cell counting. The total number of cells and the number of EGFP positive cells after transfection with different plasmids were determined. The experiment was repeated at least 3 times.

Sequence analysis of TH promoter

Four pairs of specific primers were designed according to the sequence of TH promoter in Genebank with THr as the downstream (antisense) primer among these primers. The anticipated length of amplified fragments was 2400 bp, 2000 bp, 1500 bp and 450 bp, respectively.
Primers were as follows:

TH1 f 5-CT AGGATC CGATCTTGGCACAGTGTGGTCTAC-3,
TH2 f 5-CT AGGATC CGTGGTGGGCATGCAGACCTAGG-3,
TH3 f 5-CT AGGATC CGAAGAGGGCTGGGTCTGGTAAG-3,
TH4 f 5-CT AGGATC C CGACA GTGGATGCAATTAGATC-3,
THr 5-AGC AAGCTT AGCTGGTGGTCCCGAGTTCTGTC-3.

Recombinant plasmids pTH1-EGFP, pTH2-EGFP, pTH3-EGFP and pTH4-EGFP were constructed with the method in constructing pTH-EGFP. The plasmid pTH5-EGFP (pTH-EGFP) and the four plasmids above, were utilized to transfect MN-9D cells and ECV cells with Lipofectamin TM 2000 reagent. The cells were rinsed twice with PBS, 48 h after transfection and harvested after digestion by trypsin. Then, cells were re-suspended in 400 μl of PBS in each well. The EGFP-positive cells were determined by BD FACalibur flow cytometry.

The expression of Calbindin D-28k gene was regulated by TH promoter

Based on Calbindin D-28k open reading frame sequence in Genebank, a pair of primers were designed:

5'-AGCAAGCTTACCATGGCAGAATCCCACCTGCAGTC-3', 5'-ACGCGGCCGCCTAGTTGTCTCCAGCAGAAAGAATAAG-3'

Calbindin D-28k gene cDNA sequence was amplified with RT-PCR and was integrated into pTH-EGFP plasmids, after DNA sequencing. EGFP sequence was replaced with the cDNA

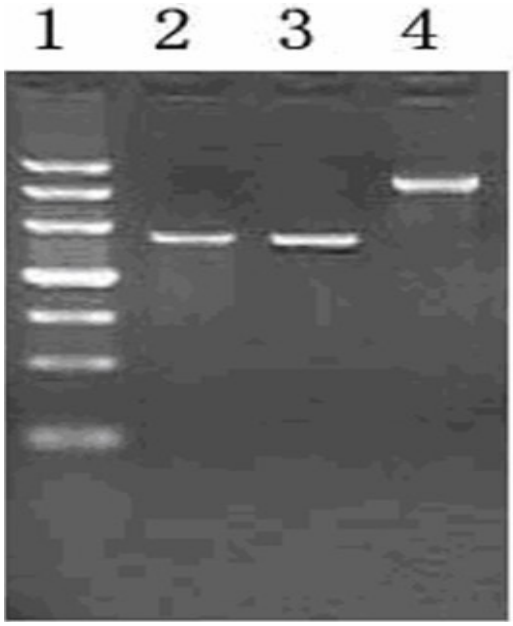

Figure 1. PCR amplification products of TH promoter. Lane 1: 4500 bp maker; lane 2: 1870 bp upstream fragment; lane 3: 1800 bp downstream fragment; lane 4: 3650 bp full-fragment.

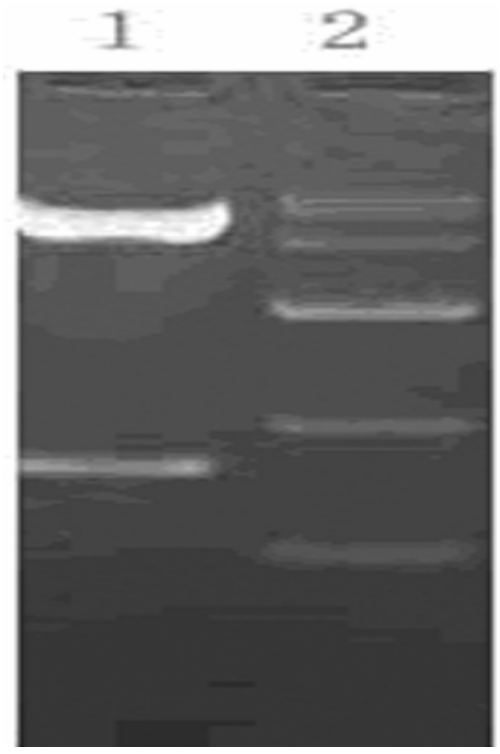

Figure 2. Double restriction digestion of pcDNA3.0-EGFP. Lane 1: products of double-digestion (5400 bp fragment and 718 bp fragment); lane 2: 7500 bp marker.

sequence and the consequent recombinant plasmid was named as pTH-CB which was applied to transfect MN-9D cells by lipofection. Calbindin D-28 gene cDNA expression was determined by immunoprecipitation and finally the viable cells after pTH-CB transfection were determined after Hoechst staining. pTH-CB and pTH-EGFP were used to transfect MN-9D cells for 24 h to which 6-OHDA were added followed by incubation for 30 min. Then, these cells were washed with PBS and stained with hoechst33258 (Sigma Corporation) for 10 min. Finally, representative photographs of these cells were captured under a fluorescence microscope followed by hoechst33258 positive cell counting.

Statistical analysis

Five to six independent animals were sacrificed at each point in time for western blot and histological examination. Semi-quantitative analysis of the bands was performed with the Image J analysis software (Version 1.30v; Wayne Rasband, NIH, USA). Data were expressed as the means ± SD. Comparisons were carried out with one-way analysis of variance (ANOVA) followed by the least significant difference test or Newman-Keuls test. A value of $P < 0.05$, was considered statistically significant.

RESULTS

Amplification of TH promoter sequence

The sequence of TH gene promoter was 3650 bp and a pair of complementary primers was designed at 1870 bp, from which the upstream and downstream genes were amplified independently. Then, the full-sequence of TH promoter was amplified by overlap PCR (Figure 1) and the full-fragment was identical to the mouse TH gene promoter in Genebank.

Identification of recombinant pcDNA3.0-EGFP plasmids

pEGFP-N1 was digested with restriction enzymes Hind III and Not I, to obtain EGFP fragment, which was integrated into plasmid pcDNA3.0 after recovery and purification. Positive clones were identified by digestion with restriction enzymes Hind III and Not I, and the 5400 bp and 718 bp fragments were obtained (Figure 2). The results showed EGFP fragments were cloned into pcDNA3.0 plasmids.

Identification of recombinant pTH-EGFP plasmids

Recombinant pTH-EGFP plasmids were digested with Hind III endonuclease to obtain the 8800 bp linear fragment, which was consistent with what we expected. The pTH-EGFP plasmids were further amplified by PCR and the 1870 bp fragment upstream of the TH gene promoter was harvested. Results showed TH promoter was cloned into pcDNA3.0-EGFP plasmids (Figure 3).

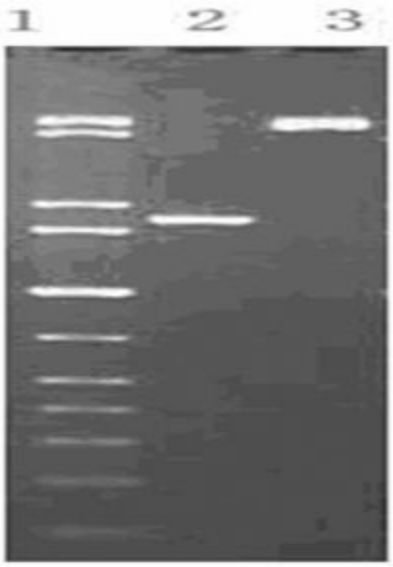

Figure 3. Restriction digestion of pTH-EGFP. Lane 1: 10000 bp marker; lane: 2 1870 bp fragment upstream TH promoter after PCR amplification; lane 3: 9160 bp fragment after Hind □ digestion

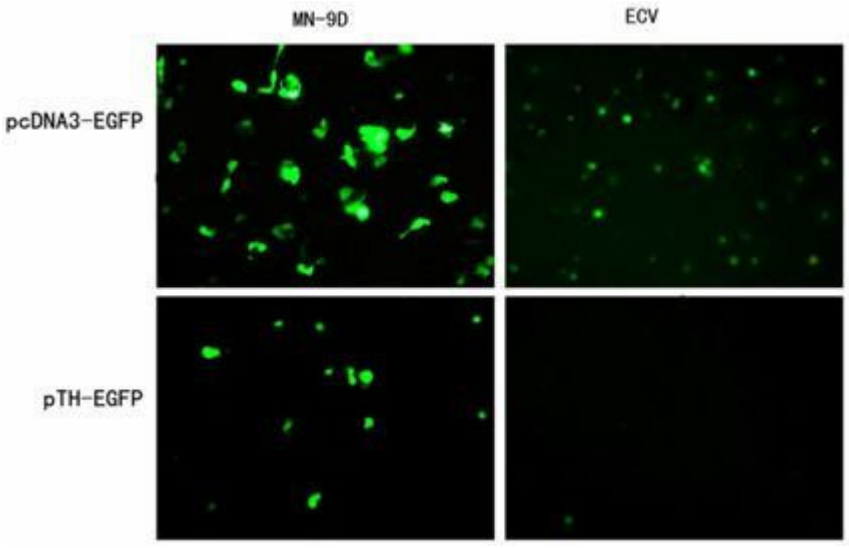

Figure 4. Proportion of EGFP-positive MN-9D cells and ECV cells transfected pTH-EGFP or pcDNA3.0-EGFP.

Expression of recombinant pTH-EGFP plasmids in cells

Plasmid lipofection is the most common method in *in vitro* cell transfection. After MN-9D cells were transfected with pcDNA3.0-EGFP plasmids, the percentage of EGFP-positive cells was 53.71 ± 3.2%, while this proportion varied from different cell types. However, the proportion of EGFP-positive ECV cells was 46.6 ± 1.1% after transfection with pcDNA3.0-EGFP plasmid (Figure 4). pTH-EGFP plasmids were applied to transfect MN-9D cells, achieving the proportion of EGFP-positive cells of 7.71 ± 0.3%, while the proportion of EGFP-positive ECV cells was 0.74 ± 0.1%, after pTH-EGFP transfection.

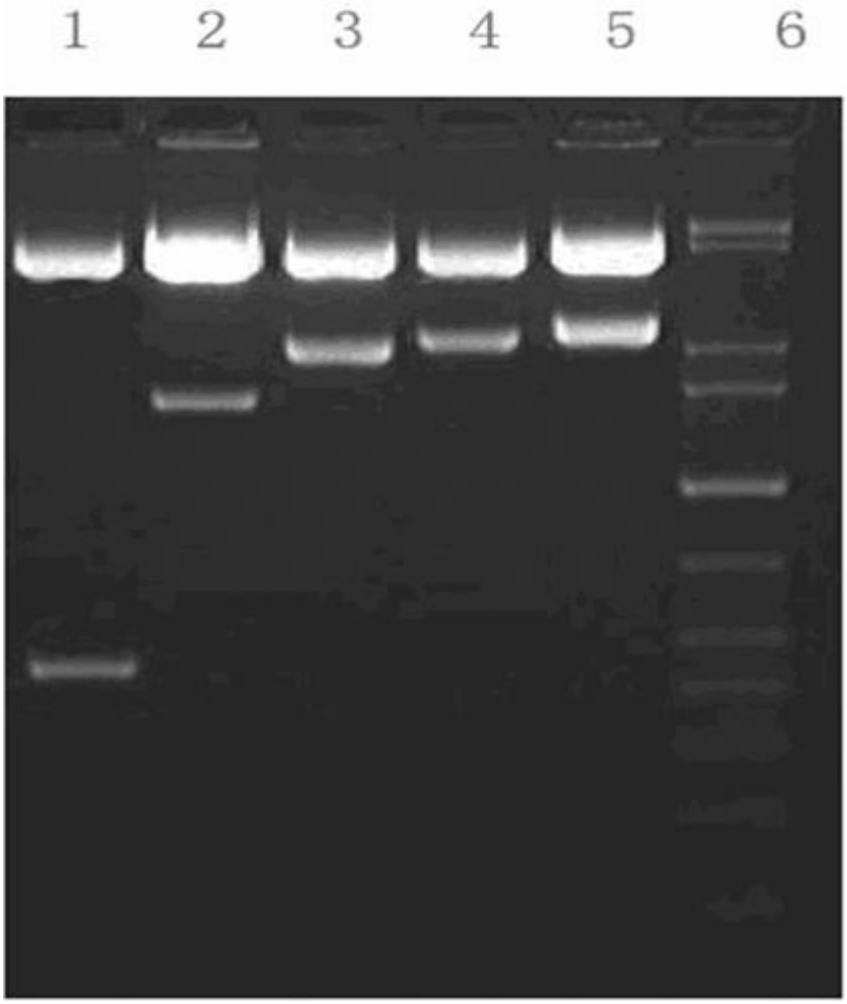

Figure 5. Recombinant pTH-EGFP, pTH1-EGFP, pTH2-EGFP, pTH3-EGFP, pTH4-EGFP plasmids after double restriction digestion. Lane 1: 580 bp fragment and 4400bp fragment of pTH1-EGFP after digestion; lane 2: 1630 bp fragment and 4400 bp fragment of pTH2-EGFP after digestion; lane 3: doule-digestion fragment 2130 bp fragment and 4400 bp fragment of pTH3-EGFP after digestion; lane 4: 2530 bp fragment and 4400 bp fragment of pTH4-EGFP after digestion; lane 5: 3900 bp fragment and 4400 bp fragment of pTH5-EGFP after digestion; lane 6: 10000 bp marker.

Sequence analysis of TH promoter

Construction of pTH1-EGFP, pTH2-EGFP, pTH3-EGFP, pTH4-EGFP, pTH5-EGFP plasmids and identification by double restriction digestion

The five recombinant plasmids were digested with Ssp and Hind β restriction endonucleases resulting in pTH1-EGFP 580 bp and 4400 bp fragments, pTH2-EGFP 1630 bp and 4400 bp fragments, pTH3-EGFP 2130 bp and 4400 bp fragments, pTH4-EGFP 2530 bp and 4400 bp fragments, pTH5-EGFP 3900 bp and 4400 bp fragments. The length of all fragments obtained from double restriction digestion of recombinant plasmids were identical to what we expected (Figure 5).

Expression of EGFP in transfected cells

The five types of recombinant plasmids above were applied to transfect MN-9D cells independently and the proportion of EGFP positive cells was: 8.01 ± 0.11%, 7.97 ± 0.23%, 7.85 ± 0.18%, 7.72 ± 0.25% and 7.74 ± 0.23%, respectively. The proportion of EGFP positive cells was not significantly different among five types of plasmids (Figure 7). After transfection, the proportion of EGFP positive ECV cells was 6.51 ± 0.26%, 6.35 ± 0.28%, 6.71 ± 0.26%, 0.89 ± 0.08% and 1.02 ± 0.17%, respectively.

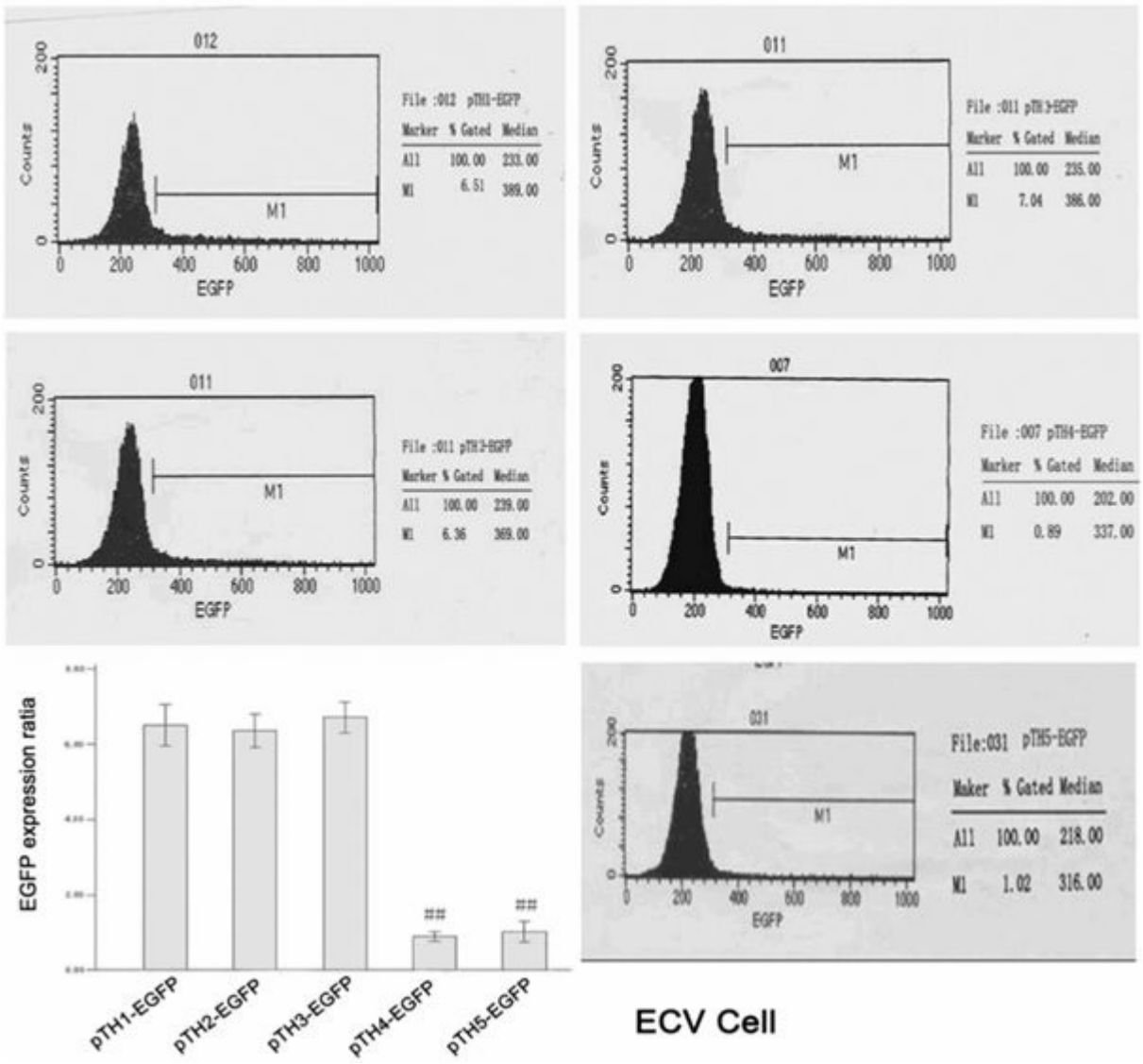

Figure 6. Proportion of EGFP positive ECV cells transfected with 5 different types of recombinant plasmids. Proportion of EGFP positive ECV cells transfected with pTH 4-EGFP or pTH 5-EGFP was significantly lower than that of EGFP positive ECV cells transfected with pTH 1-EGFP, pTH 2-EGFP or pTH 3-EGFP. However, no significant difference was noted between ECV cells transfected with pTH 1-EGFP, pTH 2-EGFP, pTH 3-EGFP (n=3).

The proportion of EGFP-positive cells transfected with pTH4-EGFP or pTH5-EGFP was significantly decreased (Figure 6) when compared with EGFP-positive cells trasfected with other plasmids.

TH promoter regulated mouse Calbindin D-28k gene expression

pTH-CB was used to transfect MN-9D cells and the expression of CaBP protein was markedly increased in cells, the x-bar stands for the portion between CaBP and β-actin; semi-quantitative analysis of the bands was performed with the Image J analysis software, which suggested that TH promoter could regulate the expression of exogenous Calbindin D-28k gene in MN-9D cells (Figure 8).

Both normal cells and early apoptotic cells can be stained with Hoechst but the nucleus of normal cell was round and light blue, after Hoechst staining and that of apoptotic cells was bright blue. 6-OHDA was used to treat MN-9D cells inducing apoptosis and Hoechst positive cells were bright blue. The results showed that the number of Hoechst positive cells was profoundly increased. CaBP over-expression could markedly reduce the number of apoptotic cells (Figure 9).

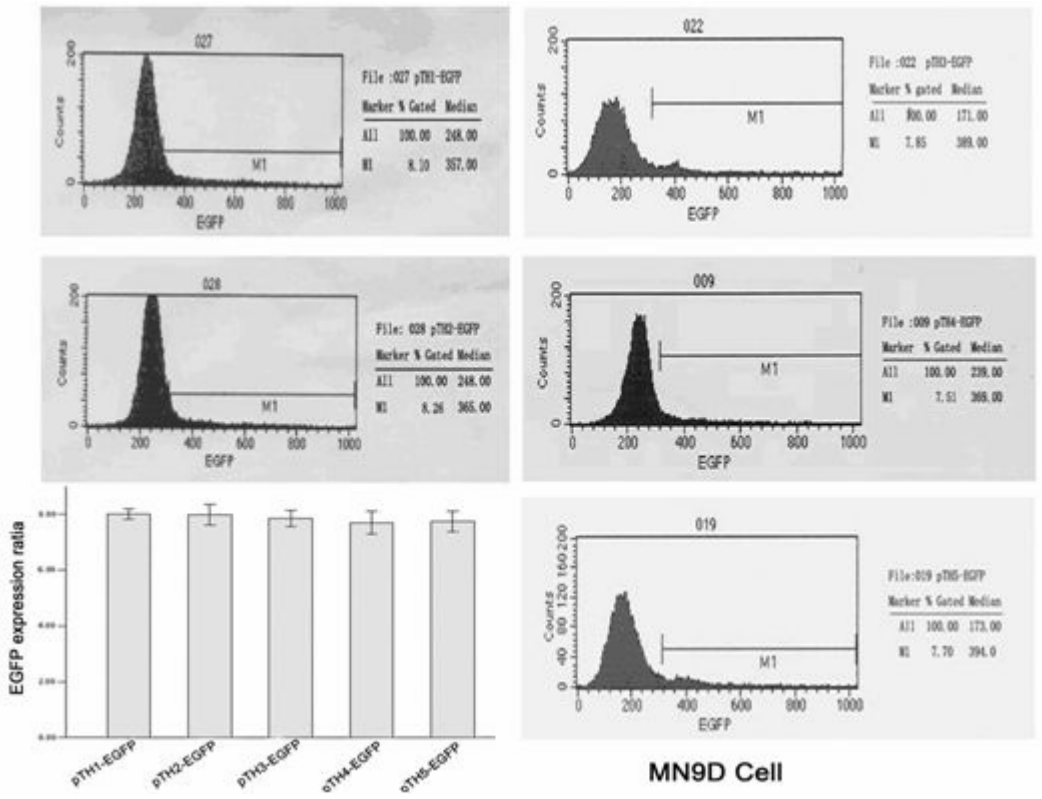

Figure 7. Proportion of EGFP-positive MN-9D cells transfected with 5 different types of recombinant plasmids. Proportion of EGFP positive MN-9D cells was not markedly different among MN-9D cells transfected with different plasmids (n = 3).

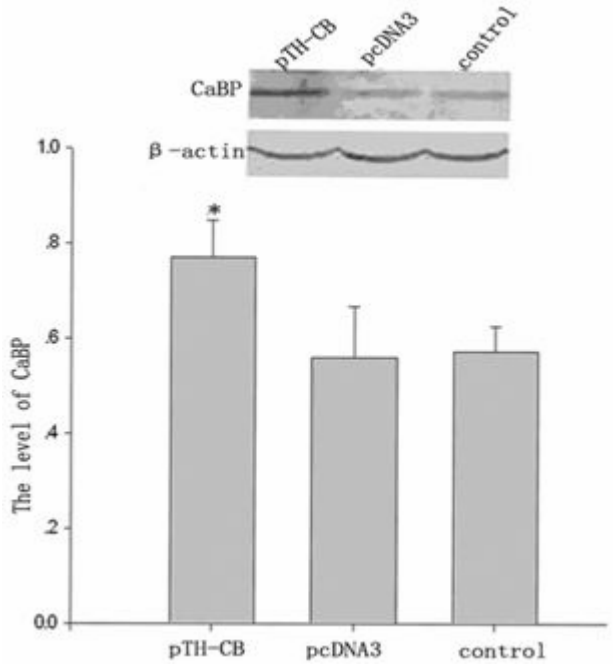

Figure 8. CaBP expression in MN-9D cells. In MN-9D cells transfected with pTH-CB, the expression of Calbindin D-28k was markedly increased when compared with the control cells and those transfected with pcDNA3 .0 ($P<0.05$, n=3).

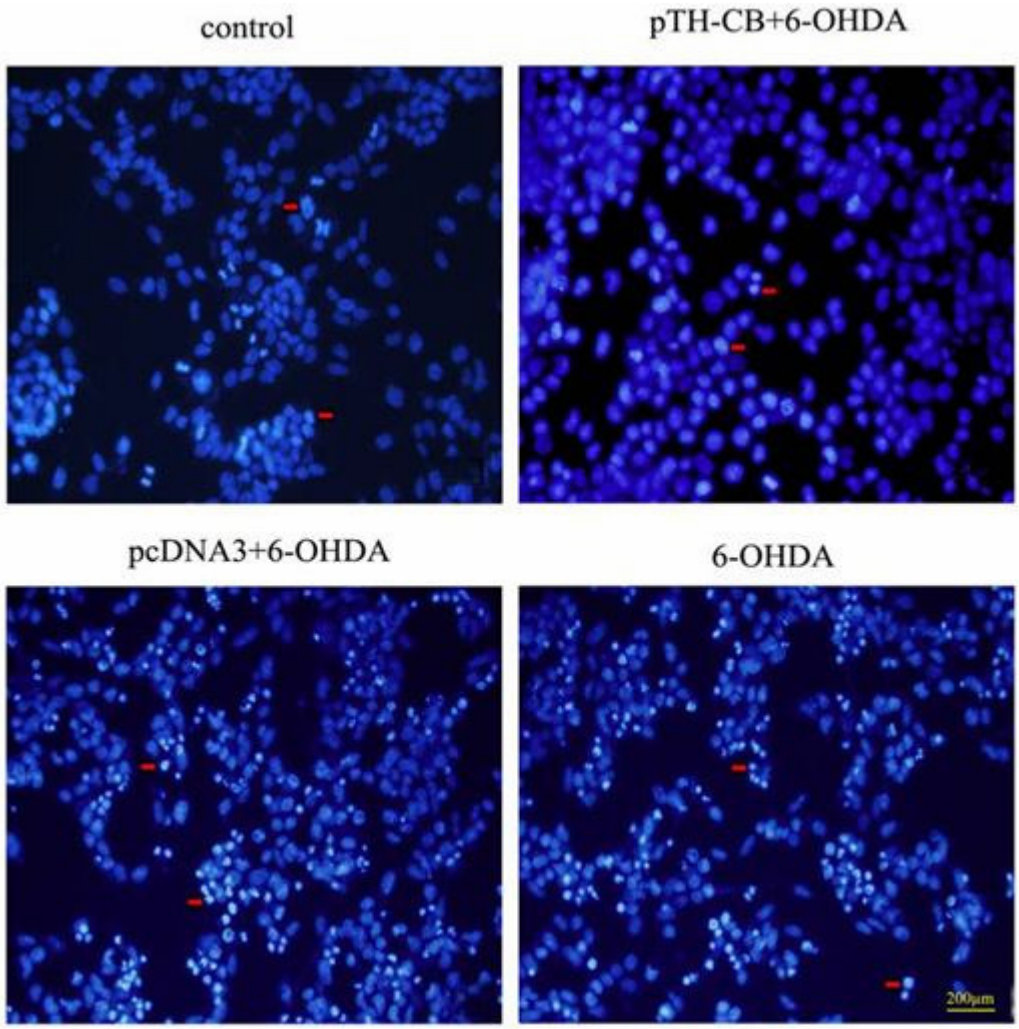

Figure 9. Analysis of apoptotic cells after Hoechst staining. The apoptotic cells were blue (red arrows). According to cell counting, 6-OHDA could cause apoptosis of MN9D cells. While, the number of apoptotic cells transfected with pTH-CB was significantly decreased when compared with those transfected with pcDNA3.0 or those without transfection (6-OHDA treatment alone) ($P<0.01$, $n=6$).

DISCUSSION

Promoter is an important cis-element in gene expression and regulation, as well as an important component of expression vector in genetic engineering. There are numerous strategies for promoter cloning, some of which are commonly used such as promoter probe vector filter (Fodor et al., 1990), foot printing (Ganley et al., 2005), PCR cloning, etc. Moreover, several branches of PCR cloning have been developed including the standard PCR cloning, thermal asymmetric interlaced PCR (TAIL-PCR) (Xiao et al., 2002), single specific primer PCR (SSP-PCR), Y-shaped adaptor dependant extension (YADE), etc (Luo and Shi, 2003).

In the present study, we designed specific primers based on the sequence of C57BL/6J mouse TH gene promoter in Genebank, followed by amplification with standard PCR. The results demonstrated that, TH promoters could specifically regulate the *in vitro* expression of EGFP. This method is simple and effective and may be widely applied. Eukaryotic gene promoter contains core components (Gershenzon and Ioshikhes, 2005), among which TATA box can mediate the formation of transcription initiation complexes including RAN pol II and initiate basic transcription. It can also mediate its upstream/downstream components to regulate the transcription and determine the initiation location of RAN pol II transcription (Montanuy et al., 2008).

Based on TFSEARCH analysis, the sequence from -58 bp to -62 bp in cloned TH promoter is a TATA box. One or more regulatory elements (UPE), besides TATA box, are required to perform the function of eukaryotic gene promoter. CAAT box and GC box are common regulatory elements in eukaryotic promoter. The sequence from

– 193 bp to - 201 bp is CAAT-box and CAAT box is the main binding site recognized by transcription factor CTF/NF-1, C/EBP, CP1, etc. While the sequence from -146 bp to -154 bp is the GC box, which is rich in GC, is the main sequence bound by transcription factor SP1 activating basic transcription in eukaryotic cells. In addition, sequence in -63 bp ~ -78 bp is the binding domain for activation transcription factor (ATF) which can promote gene transcription activation and determine the frequency of transcription initiation (Diamond et al., 1990).

In short, the cloned TH promoter has basic features of eukaryotic genes. In our study, 7.7% of MN-9D cells transfected with pTH-EGFP recombinant plasmid, could express fluorescent protein, which suggested the promoter was active. To further confirm the regulatory ability of the cloned TH promoter, TH promoter was integrated into mouse Calbindin D-28 gene. The results showed that TH promoter could regulate the expression of exogenous Calbindin D-28 gene in cells demonstrated by Hoechst staining (Whiteside et al., 1998). Furthermore, the CaBP positive cells were anti-apoptotic (Choi et al., 2008; Alexi et al., 2000; Yenari et al., 2001), which further confirmed the regulatory ability of TH promoter.

TH is a highly specific rate-limiting enzyme and can regulate catecholamine synthesis in neurons. During development and differentiation of neurons, TH expression is tissue-specific. In brain, it is expressed in substantia nigra, ventral tegmental area, hypothalamus, olfactory bulb, adrenaline and adrenergic nerve cells. It is also found in sympathetic ganglion, adrenal medulla and pheochromocytoma (Kumer and Vrana, 1996). TH locates in specific tissues and cells, implies that the promoter has the ability to regulate the expression of target genes in specific cells. The TH promoter contains many positive and negative-regulatory elements which ensure the specificity of TH expression (Yang et al., 1998). In order to study the specific regulation of TH promoter, we amplified 3650 bp, 2400 bp, 2000 bp, 1500 bp and 450 bp fragments of TH gene promoter, which were applied to regulate the EGFP expression in different cells after transfection. In MN-9D cells, all the fragments can stimulate EGFP expression and the proportion of EGFP positive cells was not statistically different among cells transfected with different plasmids.

However, in ECV cells, the regulatory activity of 3650 bp and 2400 bp fragments, was significantly inhibited and the proportion of EGFP positive cells transfected with 3650 bp or 2400 bp fragments, was significantly lower than that of EGFP positive cells transfected with 2000 bp, 1500 bp or 450 bp fragments. These results implied that there were several specific elements in the region of -2400 bp ~ -2000 bp in TH promoter. These specific elements could inhibit the regulation by promoter in the TH- cells. However, in TH+ cells, there are some special cytokines, which can bind to certain binding sites in the promoter, enhancing the inhibition of promoter function (Kim et al., 2003).

Furthermore, the gene sequences of human, rat and mouse TH promoters in Genebank were compared with TFSEARCH software and the results showed that the TH promoter in human, rat and mouse have two conservative sequences (CR1 and CR2) (Figure 10). CR1 is a 60 bp sequence containing activator protein -1 (AP-1, transcription factor), hepatocyte nuclear factor -3 (HNF-3) and Nurr1 binding sites. Mouse CR1 was located at -2400 bp ~ -2000 bp of TH gene promoter. TFSEARCH analysis of mouse TH promoter revealed that the -2274 bp ~ -2284 bp region, was highly homologous with NBRE (AAATCCACAT), which is the Nurr1 binding site. Nurr1 gene is mainly expressed in the ventral midbrain dopaminergic neurons (Kim et al., 2003; Park et al., 2006) and plays an important role in the development of midbrain dopamine neurons. Further studies are required to investigate whether the regulation of target genes by TH gene promoter in specific cells, was related to Nurr1 and whether these important components are located in CR1.

In this study, two kinds of mouse promoter sequences were identified in the Genebank, version-X53503 (BABL/c) and version -AF415235 (C57BL/6J). With the blast analysis, we found that, the sequences of two promoters are identical in -2400 bp ~ -1 bp region, while no homology was found in -3650 bp ~ -2400 bp region. As a housekeeping gene, the promoter of TH gene should have the specific-regulation ability in different strains of animals. From the evolutionary perspective, a crucial element of the promoter should be located in the conservative region (Domènec et al., 2007). However, C57BL/6J and BABL/c mouse promoter sequences were not homologous in the -3650 bp ~ -2400 bp, implying that there may be no crucial elements in this region.

CR2 contains TATA box, GC box, cAMP responsive element (CRE), stress response element (STRE), ADR1 (transcription-activating factor), MZF1 (zinc finger protein) and activator protein -4 (AP-4) binding sites. Romano et al. (2005) who studied CR2 of human TH promoter used CR2 as a promoter and found that reporter-gene expression was absent of tissue-specificity. Meanwhile, our study showed that the regulatory activity of 450 bp fragment of TH gene promoter, was also not tissue-specific. Mouse CR2 involves 450 bp fragment of TH gene promoter. Our results were consistent with that previously reported by Romano (2005).

Conclusion

Taken together, we have successfully cloned TH promoter with PCR, which could regulate the expression of target genes in specific cells. Then, we further analyzed the sequence of TH gene promoter with TFSEARCH and the results demonstrated that, it had the

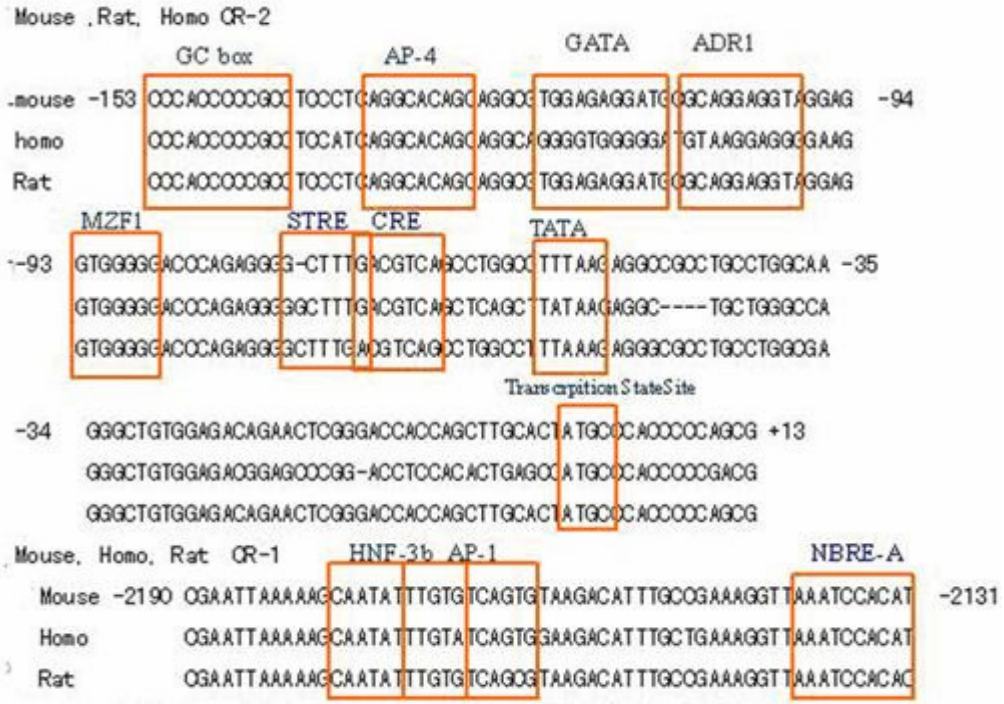

Figure 10. Analysis of conserved sequence of mouse, human and rat TH promoter.

basic characteristic of eukaryote TH promoter. Furthermore, the key regions exerting regulatory effects were also identified, which might shed light on the treatment of PD in the future.

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REFERENCES

Alexi T, Borlongan CV, Faull RL, Williams CE, Clark RG, Gluckman PD, Hughes PE (2000). Neuroprotective strategies or basal ganglia degeneration:Parkinson's and Huntington's diseases. Prog. Neurobiol., 60: 409-470.

Baimbridge KG, Celio MR, Rogers JH (1992). Calcium-binding proteins in the nervous system . Trends Neurosci. 15(8): 303-308.

Black IB, Adler JE, Dreyfus CF, Friedman WF, LaGamma EF, Roach AH (1987). Biochemistry of information storage in the nervous system. Sci., 236: 1263-1268.

Cao HY, Zhang GR, Wang XD, Kong LX, Geller AI (2008). Enhanced nigrostriatal neuron-specific, long-term expression by using neural-specific promoters in combination with targeted gene transfer by modified helper virus-free HSV-1 vector particles. BMC Neurosci., 9: 1186-1198.

Choi WS, Lee E, Lim J, Oh YJ (2008). Calbindin-D28K prevents drug-induced dopaminergic neuronal death by inhibiting caspase and calpain activity . Biochem. Biophys. Res. Commun., 371: 127-131.

Diamond MI, Miner JN, Yoshinaga SK, Yamamoto KR (1990). Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. Sci., 249: 72-78.

Domènec Farré,Nicolás Bellora,Loris Mularoni,Xavier Messeguer,M Mar Albà (2007). Housekeeping genes tend to show reduced upstream sequence conservation.Genome Biol., 8(7): R140.

Ernsberger U (2001). The development of postganglionic sympathetic neurons: coordinating neuronal differentiation and diversification . Auton Neurosci., 94(1-2): 1-13.

Fodor I, Krasnikova OV, Berets E, Shubochkina EA, Boĭsar D (1990). Cloning structure and features of a Saccharomyces cerevisiae DNA fragment causing the expression of reporter genes. MOl BioI., 24: 1411-1418.

Ganley AR, Hayashi K, Horiuchi T, Kobayashi T (2005). Identifying gene-independent noncoding functional elements in the yeast ribosomal DNA by phylogenetic footprinting. Proc. Natl. Acad. Sci., 102(33): 11787-92.

Gardanch M, O'Malley KL (2004). Rat tyrosine hydroxylase promoter directs tetracycline-inducible foreign gene expression in dopaminergic cell types . Brain Res. Mol. Brain Res., 26(2): 173-180.

Gershenzon NI, Ioshikhes IP (2005). Synergy of human Pol II core promoter elements revealed by statistical sequence analysis. Bioinformatics, 21(8): 1295-300.

Hedlund E, Pruszak J, Ferree A, Viñuela A, Hong S, Isacson O, Kim KS (2007). Selection of embryonic stem cell derived eGFP dopamine neurons using the tyrosine hydroxylase promote is confounded by reporter gene expression in immature cell populations. Stem cells, 18: 321-327.

Howard K (2003). First Parkinson gene therapy trial launches . Nat. Biotech., 21(10): 1117-1118.

Jeffrey HK, Marina EE, Jocelyne B, Shuang YM, Yaping C, Liza LI, Jodi McB, Er-Yun C, Stéphane P, Ben Zion R, Douglas WB, James EH, Robert P, Michael DT, Paul C, ZaoDung L, Didier T, Philippe H, Nicole D, Patrick A (2000). Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate modeles of Parkinson's disease. Sci., 290(5492): 767-773.

Kim TE, Lee HS, Lee YB, Hong SH, Lee YS, Ichinose H, Kim SU, Lee MA (2003). Sonic hedgehog and FGF8 collaborate to induce dopaminergic phenotypes in the Nurr1-overexpressing neural stem

cell. Biochem. Biophys. Res. Commun., 305: 1040-1048.
Kim TE, Park MJ, Choi EJ, Lee HS, Lee SH, Yoon SH, Oh CK, Lee BJ, Kim SU, Lee YS, Lee MA (2003). Cloning and cell type-specific regulation of the human tyrosine hydroxylase gene promoter. Biochem. Biophys. Res. Commun., 312(4): 1123-31.
Kumer SC, Vrana KE (1996). Vrana Intricate regulation of tyrosine hydroxylase activity and gene expression . Neurochem. 67: 443-462.
Luo LJ, Shi JS (2003). TAIL-PCR: A Simple and Efficient Method to Isolate DNA Segments Adjacent to Known Sequence . J. Nanjing For. University, 27(4): 87-89.
Mark AK, Ming Y, Kandace LG, Hao J, Lorraine L (2003). The human tyrosine hydroxylase gene promoter. Mol. Brain Res., 112: 8-23
Meyer M, Zimmer J, Seiler RW, Widmer HR(1999). GDNF increases the density of cells containing calbindin but not of cells containing calretinin in cultured rat and human fetal nigral tissue. Cell Transplant, 8: 25-36.
Mimoun A, Enca M-R, Robert DB, Kyriacos AM, Emma EC, Jonathan BR, Susan MK, Alan JK, Nicholas DM (2002). Multicistronic lentiviral vector mediated striatal gene transfer of aromatic L-amino acid decarboxylase I induces sustained transgene expression dopamine production and functional improvement in rat model of Parkinson's disease. J. Neurosci., 22: 10302-10312.
Montanuy I, Torremocha R, Hernández-Munain C, Suñé C (2008). Promoter influences transcription elongation:TATA-box element mediates the assembly of processive transcription complexes responsive to cyclin-dependent kinase 9. J. Biol. Chem., 283(12): 7368-7378.
Park CH, Kang JS, Shin YH, Chang MY,Chung S, Koh HC, Zhu MH, Oh SB, Lee YS, Panagiotakos G, Tabar V, Studer L, Lee SH (2006). Acquisition of in vitro and in vivo functionality of Nurr1-induced dopamine neurons. FASEB J., 20: 1910-1923.
Romano G, Suon S, Jin H, Donaldson AE, Iacovitti L (2005). Characterization of Five Evolutionary Conserved Regions of the Human Tyrosine Hydroxylase (TH) Promoter: Implications for the Engineering of a Human TH Minimal Promoter Assembled in a Self-Inactivating Lentiviral Vector System. J. Cell Physiol., 204(2): 666-677.
Trocmé C, Sarkis C, Hermel JM, Duchateau R, Harrison S, Simonneau M, Al-Shawi R, Mallet J (1998). CRE and TRE sequences of the rat tyrosine hydroxylase promoter are required for TH basal expression in adult mice but not the embryo. J. Neurosci., 10: 508-521.
Whiteside G, Cougnon N, Hunt SP, Munglani R (1998). An improved method for detection of apoptosis in tissue sections and cell culture, using the TUNEL technique combined with Hoechst stain. Brain Res. Protoc., 2(2): 160-164.
Xiao Y H, Luo M, Fang WG, Luo KM, Hou L, Luo XY, Pei Y (2002). PCR Walking in Cotton Genome Using YADE Method. ACTA GENETICA SINICA, 29(1): 62-66.
Yang C, Kim HS, Seo H, Kim CH, Brunet JF, Kim KS (1998). Paired-like homeodomain proteins Phox2a and Phox2b, are responsible for noradrenergic cell-specific transcription of the dopamine beta-hydroxylase gene. Neurochem., 71: 1813-1826.
Yenari MA, Minami M, Sun GH, Meier TJ, Kunis DM, McLaughlin JR, Ho DY, Sapolsky RM, Steinberg GK (2001). Calbindin D28K Overexpression Protects Striatal neuronsf rom transient focal cerebral ischemia. Stroke, 32: 1028-1035.