Regulation of human tyrosine hydroxylase gene by neuron-restrictive silencer factor

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Abstract

Tyrosine hydroxylase (TH), the biosynthetic enzyme of catecholamine, is synthesized specifically in catecholaminergic neurons. Thus, it is possible that neuronal cell type-specific expression of this gene is coordinately regulated. One of the neuron-specific transcription regulators, neuron-restrictive silencer factor (NRSF)/repressor element 1 (RE1) silencing transcription factor (REST), represses the expression of neuronal genes in non-neuronal cells. To elucidate the molecular mechanisms that control catecholaminergic neuronal expression of human TH, we initially characterized the 5’ regulatory region. Previous studies have shown that a 3174 bp fragment of the human TH promoter confers specific expression to the reporter gene in dopaminergic neuron-like cell lines. Within this 5’ regulatory region, three putative neuron-restrictive silencer elements (NRSE)/RE1 were identified, which bound NRSF/REST in a sequence-specific manner, as confirmed using EMSA and ChIP assays. In transient transfection assays, deletion or mutation of NRSE/RE1 elements led to a 7-fold increase in activity of the 3.2 kb TH promoter in human neural stem cells (NSCs), but had no major effects on differentiated neuron-like cells. Suppression of NRSF/REST functions with either the histone deacetylase inhibitor, trichostatin, or DN-NRSF induced TH promoter activity. Our data strongly suggest that NRSF/REST functions as a repressor of TH transcription in NSCs via a mechanism dependent on the TH NRSE/RE1 sites.

Keywords: Tyrosine hydroxylase; Promoter; Repression; NRSF/REST; NRSE/RE1; Catecholaminergic; Neural stem cells

Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in the biosynthesis of catecholamines [1]. Therefore, control of the TH protein level and activity represents the central means for regulating the synthesis of these neurotransmitters. In the brain, TH is expressed in several cell groups, including dopaminergic (DAergic) neurons of the substantia nigra, ventral tegmental area, hypothalamus, and olfactory bulb, noradrenergic neurons of the locus coeruleus and lateral tegmental system, and adrenergic neurons of the brainstem. In the periphery, TH is expressed in sympathetic ganglia and adrenal chromaffin cells [2]. In view of this wide distribution, it is clear that distinct transcriptional control mechanisms account for regulation of expression of the TH gene in different cell populations.

The silencing transcription factor, neuron-restrictive silencer factor (NRSF)/repressor element 1 (RE1) silencing transcription factor (REST), is widely expressed during embryogenesis, and plays a strategic role in terminal neuronal differentiation [3]. In neural progenitor and non-neuronal cells, NRSF/REST actively represses a large array of neural-specific genes important for synaptic plasticity and remodeling, including synaptic vesicle proteins,
structural proteins, voltage-sensitive ion channels, and neurotransmitter receptors [4–11]. As neural progenitors differentiate and migrate out of the ventricular zone, NRSF/REST downregulation is essential for induction and maintenance of the neuronal phenotype [12]. NRSF/REST contains two main repressor domains [13–15]. Several reports suggest that NRSF/REST acts as a repressor, in part, through association of a domain in the N-terminus with the Sin3A-histone deacetylase 2 (HDAC2) complex [9,16,17]. Sin3A-HDAC2 is one of several HDAC complexes that down-regulate gene expression [17,18]. Histone deacetylation possibly triggers a “closed” conformation of chromatin surrounding a gene, thereby restricting access of the RNA polymerase II complex, leading to transcriptional repression [19,20]. Several studies using trichostatin A (TSA), an HDAC-specific inhibitor that disrupts histone deacetylation activity in cell cultures, have confirmed the importance of HDAC activity in the repressive function NRSF/REST [8,21]. The nuclear receptor corepressor (N-CoR), which interacts with Sin3A and other HDACs, including HDAC3, 4, 5, and 7, additionally mediates NRSF/REST repression in specific genetic contexts [22]. The C-terminal zinc finger of NRSF/REST associates with at least one other factor, CoREST, to mediate HDAC-independent repressor activity [23–25]. Moreover, NRSF/REST interacts with several other proteins, including TBP and LIM domain protein (RILP) [14,15,26]. Here, we report the identification of a functional NRSE/REI regulatory DNA element in the human TH gene promoter, and describe its potential importance in the regulation of neuronal cell type-specific TH expression.

**Materials and methods**

**Cell culture.** The immortalized human neural stem cell (NSC) line, HB1.F3, was established according to a previous report [27], and grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Hyclone). SH-SYSY (human DAergic neuroblastoma), C6 (rat glioma), C17.2 (mouse NSC), and HEK293 (human embryonic kidney) cell lines were grown in DMEM supplemented with 10% FBS. PC12 (rat adrenal pheochromocytoma) cells were cultured in DMEM with 5% FBS and 5% horse serum (Hyclone).

**Plasmid constructs and mutagenesis.** A 3301 bp human TH promoter fragment (−3174 to +127) was sub cloned directly upstream of luciferase gene to assay promoter activity. Within this region (pTH-3174), 3 consensus elements for NRSF/REST are located at positions −1907 to −1887 (NRSE-R), −1555 to −1535 (NRSE-I), and −204 to −184 (NRSE-II), respectively. For mutant analyses, 3 additional constructs were generated by serial deletion of pTH-3174, using unique restriction endonuclease sites. All constructs were verified by sequencing. The pSV-β-galactosidase plasmid (Promega, USA) was employed as an internal control. Site-directed mutagenesis of NRSE/REST binding sites (NRSEs) within the human TH promoter was performed using the QuickChange Mutagenesis kit (Stratagene), following the manufacturer’s instructions. The pGL3-basic-hTH expression vector was used as a template. Oligonucleotides containing the desired point mutations are shown below:

- **mut-NRSE I** 5'-GGGCACGGAGAGGAGGTGGTGAGCAGG-3'
- **mut-NRSE II** 5'-GAAGGACATTAGATCTTACGGACCCCTTTC-3'

The base codons for mutated residues are underlined. Mutations were confirmed by sequence analysis.

**Transfection and luciferase assay.** Transfections were performed using LipofectAMINE PLUS reagent (Invitrogen, USA) according to the manufacturer’s instructions. A total of 3 μg of DNA was used per transfection. Where necessary, 1 μg of dominant-negative (DN)-NRSF expression vector (kindly provided by Dr. G. Mandel, Stony Brook, NY) was co-transfected. All transfection reactions contained 1.5 μg of luciferase reporter plasmid and 0.5 μg of the internal control plasmid, pSV-β-galactosidase (Promega). Cells were transfected at 60–80% confluency in 35 mm 6-well plates and harvested after 48 h. All transfections were performed three times in triplicate. Promoter activity was determined using the Single-Luciferase® Reporter Assay System (Promega), following the manufacturer’s recommendations. Luciferase activities were normalized, based on β-galactosidase activity in each well.

**Western blot analysis.** To prepare nuclear proteins, cells were treated with cold buffer (10 mM Heps, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1% NP-40, 0.5 mM DTT, and 0.2 mM PMSF) and RIPA lysis buffer. After incubation for 30 min on ice, samples were centrifuged at 10,000 rpm for 10 min at 4 °C. Cell extracts (100 μg) were separated on a 10% SDS-PAGE gel. Following electrophoresis, gels were transferred to a PVDF membrane (Millipore, USA) for 1 h. The membrane was blocked for 1 h with 5% skimmed milk. This was followed by incubation with an anti-NRSE/REST antibody (2 μg) (12C11 MAb, kindly provided by Dr. D.J. Anderson, Howard Hughes Medical Institute, CA) with 3% BSA in TBS-T buffer overnight at 4 °C. The membrane was washed three times with 15 min in TBS-T, and incubated for 1 h with secondary antibody peroxidase-conjugated anti-mouse (1:4000, Sigma). Immunoblots were detected using an ECL (Amersham) kit and visualized after exposure to film.

**RNA preparation and RT-PCR.** Total RNA was extracted from cell lines, as described previously [28]. Total RNA (5 μg) was subjected to RT-PCR with superscript II reverse transcriptase, using random hexamers (pd[N6]) as primers. Amplification reactions were performed with 1/10 volume of the reverse-transcribed product in a final volume of 50 μl with recombinant Taq DNA polymerase (Gibco). An initial denaturation for 5 min at 94 °C was followed by 28 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, followed by 10 min extension at 72 °C. PCR products were separated on a 1.5% agarose gel with TAE buffer. Primer sets used for human, rat, and mouse NRSF/REST mRNA isoforms [29] are described in a report by Abderrahmani et al. [30].

**Electrophoretic mobility shift assay (EMSA).** Nuclear extracts from HB1.F3 and SH-SYSY cells were prepared essentially as described earlier [31], and protein concentrations were determined using the Bio-Rad Dc kit (Bio-Rad). Sense and antisense oligonucleotides containing each hTH-NRSE were annealed, and end-labeled with [γ-32P]ATP (Amersham) and T4 polynucleotide kinase, following a standard protocol [32]. All labeled probes were purified on 19% non-denaturing polyacrylamide gels. DNA-protein binding reactions were performed in a final volume of 20 μl reaction buffer containing 10 mM Tris, pH 7.6, 50 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 1 mM MgCl₂, 0.5% glycerol, and 250 μg of poly(dI-dC) per ml. The nuclear extract (10 μg of protein) was added to reaction buffer in the absence or presence of unlabeled competitor DNA, and pre-incubated for 15 min on ice. Radiosotope-labeled probes (40,000 cpm), were added, and the mixture incubated for a further 30 min at room temperature. To resolve DNA-protein complexes, electrophoresis was performed using a 5% non-denaturing polyacrylamide gel. Gels were fixed, dried, and visualized by Phosphoimager and autoradiography. The various oligonucleotides employed are specified below (only sense strands are presented):
NRSE-I 5'-CGAGGAGCCACGGAGGGGTGT-3',
NRSE-II 5'-TTAGATTCCACGGACGAGCCC-3',
mtNRSE-R(1) 5'-TGCCCCCTCACTTGGTGGCCC-3',
mtNRSE-R(2) 5'-TGCAAGAACCCAGGGGTTGGTGT-3',
mtNRSE-I(1) 5'-CGAGGAGCCACGGAGGGGTGT-3',
mtNRSE-I(2) 5'-TTAGATTCCACGGACGAGCCC-3',
Chromatin immunoprecipitation assay (ChIP). ChIP was performed according to a previous study (Upstate Biotechnology). HB1.F3 cells were subjected to protein DNA cross-linking by 1% formaldehyde exposure. Cells (1 x 10^6) were used for each assay, and split into three aliquots for immunoprecipitation in the presence or absence of NRSF/REST antibody. Genomic DNA was sheared to produce fragments of approximately 700 bp. The average size was verified by agarose gel electrophoresis. Complexes were de-cross-linked, and purified by organic extraction and isopropanol precipitation. Immunoprecipitated DNA were isolated, and dissolved in 50 l TE. A quantitative PCR assay to determine linearity of amplification was performed before further diluting the sample 10-fold. Immunoprecipitated genomic DNA samples (5 l) were applied as templates for PCRs. As a negative control, PCR was additionally performed on genomic DNA samples that were precipitated with unrelated anti-β tubulin antibodies.

Results

Expression of NRSF/REST in NSCs and neuronal SH-SY5Y cells

We previously reported the cloning and characterization of the promoter of the human TH gene [33]. Here, we investigate the pan-neuronal molecular mechanism by which tissue-specific TH expression is regulated. We identify three motifs of 21 nucleotides in the 5' flanking 3.2 kb sequence of the TH promoter region with considerable homology to the consensus sequence of NRSE/RE1, designated NRSE-R, NRSE-I, and NRSE-II (Fig. 1).

To our knowledge, this is the first documented investigation on the expression of NRSF/REST and NRSE/RE1 binding factor in human NSCs and neuron-like cells to clarify the mechanism of pan-neuronal regulation of TH gene expression. We compared the levels of NRSF transcript isoforms in various cell lines (human NSC HB1.F3, human DAergic neuroblastoma SH-SY5Y, human embryonic kidney HEK293, rat glioma C6, rat adrenal pheochromocytoma PC12, and rat NSC C17.2) by RT-PCR. Primers spanning exon N were designed to discriminate between the most abundant NRSF/REST mRNA isoforms in humans, rats, and mice [29,34]. RT-PCR analysis of RNA from HB1.F3 and HEK293 cell lines disclosed only one detectable PCR fragment. No PCR product was evident in the PC12 cell line (Fig. 2A). In contrast, SH-SY5Y, C17.2, and C6 cells expressed both full-length NRSF/REST and isoform NRSEs (REST4) (Fig. 2A). While REST4, the splicing variant form of NRSF/REST, does not bind to NRSE/RE1, it regulates the repressor activity of NRSF/REST via the antisilencer mechanism [35]. Therefore, we propose that in SH-SY5Y, C17.2, and C6 cells, NRSF/REST binds to NRSE/RE1, and transcriptional repression of the TH gene promoter does not occur as for PC12 cells. Moreover, Western blot data were consistent with RT-PCR findings (Fig. 2B). Specifically, in HB1.F3 and HEK293 cells, only the NRSF/REST protein was detected, while REST4 was additionally observed in

Fig. 1. NRSE-like sequences in the human TH promoter gene. Putative NRSE/RE1 motifs in the human TH promoter region are boxed. The transcription start site is designated ‘+1’.

Fig. 2. Expression of NRSF/REST mRNA and protein in HB1.F3, SH-SY5Y, C17.2 and C6, HEK293 and PC12 cells. (A) RT-PCR was performed with primer pairs specific for NRSF/REST. Primers were designed to span between exons 4 and 6, the region that differs in NRSF/REST mRNA isoforms. Lanes: 1, isoform of human NRSF; 2, human NRSF; 3, isoform of rat NRSF; 4, rat NRSF; 5, GAPDH. (B) Nuclear extracts (100 µg) were prepared from HEK293, HB1.F3, SH-SY5Y, C6, PC12, and C17.2 cells. Extracts were separated by SDS–PAGE (8%), and analyzed by immunoblotting with anti-NRSF/REST antibody.
SH-SY5Y, C6, and C17.2 cells. No bands were detectable in PC12 cells.

Sequence-specific binding of NRSF/REST to NRSEs of the human TH promoter

To examine NRSF/REST-binding activity of newly identified human TH NRSEs, we performed EMSA using nuclear extracts prepared from HB1.F3 and SH-SY5Y cells. We additionally designed two mutated NRSE oligonucleotides in which guanine or cytosine residues at the core of the element were replaced with thymine or adenine, respectively. Mutation of these bases alters the binding of NRSF/REST to its preferential NRSE/RE1 site [4,6]. As shown in Fig. 3A, DNA-binding complexes from HB1.F3 nuclear extracts were observed using all NRSEs of the TH promoter as labeled probes. The complex was supershifted in the presence of monoclonal NRSF/REST antibody. Competition experiments were conducted using 100-, 40-, or 20-fold molar excess of unlabeled wild type or mutated NRSE (Fig. 3B). Wild-type oligonucleotide competed for protein–DNA interactions, whereas the unre-

Fig. 3. Specific NRSF/REST binding to human TH NRSE-like elements in HB1.F3 cells. (A) Incubation of HB1.F3 nuclear extracts with 32P-labeled probes derived from three identified human TH NRSE/RE1-like sequences (NRSE-R, NRSE-I, and NRSE-II). The addition of an NRSF/REST antibody (12C11) specifically retarded the NRSF/NRSE shift (arrows). (B) Specific association of a nuclear protein complex with human TH NRSE-II element. Rat SCG10 NRSE/RE1 was employed as a positive control. Unlabeled TH NRSE-II primers were added at 20-, 40-, or 100-fold molar excess, as indicated. (C) EMSA was performed with wild-type NRSE-R/I/II or mutated mtNRSE-R/I/II oligonucleotides. The mutations introduced are specified in the bottom box. Unlabeled oligonucleotide (40-fold molar excess) was added as a competitor in the reaction mixture. The retarded complex is indicated with an arrow. (D) ChIP was applied to determine whether NRSF/REST bound to NRSE/RE1s of the TH promoter in HB1.F3 cells. Lane 1 is load control (1%); lanes 2 and 4 represent no antibody and anti-β tubulin, respectively.
lated NBRE did not affect binding of NRSF/REST to human NRSE-II. This DNA-binding complex was also detected in nuclear extracts of SH-SY5Y cells (data not shown). In EMSA with mutated NRSE-R oligonucleotides, mutant mt 1 and mt 2 competitor probes did not inhibit binding of NRSF/REST to NRSE-R (lanes 3 and 4), or form a DNA–NRSF complex (lanes 5 and 6) (upper panel of Fig. 3C). Similar results were obtained with other two NRSE/RE1 sites (mid and lower panels of Fig. 3C). The data strongly indicate that NRSF/REST binds human TH NRSE/RE1s in HB1.F3 cells.

To provide further support for the hypothesis that NRSF/REST regulates endogenous TH transcription, a ChIP assay was performed to confirm in vivo interactions of NRSF/REST with the human TH promoter region (Fig. 3D). ChIP PCR products of all NRSE/RE1 sites were detected with the NRSF/REST antibody in HB1.F3 cells (Fig. 3D). Thus, endogenous NRSF/REST specifically binds NRSEs of the TH promoter in HB1.F3 cells, consistent with the in vitro results of our gel retardation assays.

Cell-specific expression patterns of TH promoter constructs

We compared the expression levels of the reporter constructs in various cell lines (human NSC HB1.F3, human DAergic neuroblastoma SH-SY5Y, human embryonic kidney HEK293, rat glioma C6 and rat adrenal pheochromocytoma PC12). Constructs with increasing 5′-deleted portions of the human TH gene promoter, designed around the pGL3-basic reporter vector, were employed to determine the promoter activation levels (Fig. 4A). These cells, particularly human NSC HB1.F3, displayed varying levels of TH promoter activity (Fig. 4B). In undifferentiated HB1.F3 cells, 5′-deletion of 1010 bp (−3174 and

![Fig. 4](image-url). Cell-specific expression patterns of TH promoter constructs. (A) Representation of the reporter gene used for transient transfections from fragments of the cloned human TH gene promoter region. (B) Cells were transiently transfected with the corresponding reporter gene constructs specified above. Each experiment was performed in triplicate.
2164 bp constructs) did not significantly alter luciferase activity. However, further 5'-deletion of 954 bp (-1210 bp construct) resulted in a 7-fold increase in luciferase activity, indicating the presence of a silencing element(s) between -2164 and -1210 bp. This region included putative NRSE/RE1 sites, NRSE-R and NRSE-I. Accordingly, we assume that NRSE-R and NRSE-I confer repression of the TH gene promoter. Additional 5'-deletion of 763 bp (Fig. 4B) decreased luciferase activity by approximately 50%. In contrast, in DAergic neuroblastoma SH-SY5Y cells, no derepression effect of deletion mutants was evident, and luciferase activity of the TH promoter gradually decreased as the 5'-flanking region became shorter. The shortest 5'-deletion construct reduced promoter activity by 25% (Fig. 4B). Similar results were obtained with other cell lines. The data suggest that the region between -2164 and -1210 bp contains silencing element(s), which repress TH gene transcription in NSCs, but not other cells, including neuron-like cells.

Transcriptional activity conferred by TH NRSE-II and NRSF/REST

The NRSE/RE1 sites of the human TH promoter were modified by site-directed mutagenesis (Fig. 5A). Substitution of two guanine nucleotides at the core of the site to thymine led to loss of NRSF/REST binding to mutated NRSE, as shown in Fig. 3C [4,6]. While TH promoter activity mediated by mutant NRSE-II dramatically increased (5-fold), compared to wild-type in HB1.F3 cells, less than 2-fold increase in activity was observed with mutant NRSE-R (Fig. 5B). Moreover, TH promoter activity of the mutant NRSE-I construct was similar to that of wild-type. In contrast, no significant increase or decrease in transcriptional activity was observed for mutated NRSEs in SH-SY5Y cells. Data from Figs. 4 and 5 indicate that while all NRSE sites in the TH promoter contribute to the repression of TH promoter activity in NSCs and not neuronal cells, the effects of NRSE-II are distinct from those of NRSE-R and NRSE-I.

Derepression of TH promoter activity by DN-NRSF or TSA

Simultaneous deletion of NRSE-R and NRSE-I sites increased TH promoter activity, as shown in Fig. 4. However, single point mutations of either NRSE site did not lead to derepression of TH promoter activity (Fig. 5). To further study these differential effects of NRSE sites on TH promoter repression, we introduced an expression vector encoding DN-NRSF to evaluate whether the repression mediated by NRSF/REST could be relieved. DN-NRSF corresponds to human cDNA encoding the DNA-binding domain of NRSF/REST without the two repressor domains. Deletion mutants of TH promoter constructs were transiently transfected into HB1.F3 and SH-SY5Y cells in the presence or absence of DN-NRSF (Fig. 6A). In HB1.F3 cells, co-transfection of DN-NRSF led to up to 2- to 3-fold recovery of activity with -3174 and -2164 bp constructs. However, with the shorter -1210 and -447 bp TH promoter constructs, higher induction (more than 5-fold) of transcriptional activity was evident. In contrast, transcriptional activity of the TH promoter was not restored by DN-NRSF in SH-SY5Y cells. It is proposed that NRSF/REST-dependent repression is mainly mediated downstream of -1210 bp (possibly in NRSE-II), and repression by regions between -2164 and

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**Fig. 5.** Transcriptional activity conferred by human TH NRSE/RE1 and NRSF/REST in NSCs. (A) The nucleotide sequence of each putative NRSE/RE1 motif in the TH promoter gene. Nucleotides differing from the consensus are presented in small letters, and mutated nucleotides are underlined in bold. (B) Repression of TH promoter activity was mediated by NRSE/RE1. The schematic diagram represents the 5'-regulatory region of the human TH gene from nucleotide -3174 to +127 bp containing the translation start site, designated ‘+1’.

Each TH promoter construct (mNRSE-R, -I, and -II) containing mutated NRSE-R, NRSE-I or NRSE-II was cloned into the pGL3 basic vector. Error bars indicate the range of SE.
−1210 bp (NRSE-R and NRSE-I) is less dependent on NRSF/REST in HB1.F3 cells. To assess whether repression of TH promoter activity is HDAC-dependent, HB1.F3 and SH-SY5Y cells were transiently transfected with deletion mutants of the TH promoter, and incubated for 24 h with 100 nM TSA, a specific inhibitor of HDAC [17]. The luciferase activity was significantly increased (5- to 7-fold) in -3174 and -447 bp constructs, compared to -3174 and -2164 bp mutants (~2-fold) (Fig. 6B). Our data indicate that repression mediated by NRSF/REST and NRSE-II in the TH promoter involves NRSF and HDAC, while repression by the region between −2164 and −1210 bp is less dependent of NRSF/REST and HDAC (Fig. 6A and B).

Two types of repression of TH expression in human NSCs

To confirm whether NRSE/RE1-mediated repression is directed specifically through NRSE site, we introduced DN-NRSF or administered TSA into cells transfected with point mutants of the TH promoter (mR, mI, and mII). As shown in Fig. 7A, co-transfection of mutated NRSE-R (mR) or NRSE-I (mI) constructs with DN-NRSF increased the promoter activity of TH. In contrast, co-transfection of DN-NRSF with wild-type or mutated NRSE-II (mII) constructs did not affect TH promoter activity. This result suggests that if two NRSE-R and NRSE-I are simultaneously intact (as in wild-type TH promoter), it may prevent to derepress NRSF-dependent repression of TH promoter activity through NRSE-II by DN-NRSF in higher order. In contrast, inactivation of either NRSE site as in mR or mI constructs affect this type of repression function and permits to replace NRSF by DN-NRSF and increase TH promoter activity. Therefore, the second type of repression may simultaneously require two NRSE/RE1 sites, NRSE-R and NRSE-I.

Moreover, promoter activities of mR and mI constructs were particularly sensitive in increases in the TSA dose, in contrast to wild-type TH and mII constructs (Fig. 7B), similar to data from Fig. 7A. Our results collectively suggest that the NRSE-II region is related to the repression of TH mediated via NRSF/REST and HDAC, and that the repression by NRSE-R and NRSE-I is possibly mediated in a NRSF/REST- or HDAC-independent manner. In addition, although NRSE-R and NRSE-I sites may not directly mediate NRSF/REST-dependent repression, it may regulate NRSF-dependent repression/derepression of TH transcription through NRSE-II site in higher order.

Discussion

With the aid of TH deletion constructs that dramatically enhance levels of promoter activity, we previously showed that transcriptional repression activity of the human TH gene is confined to the region between −2164 and −1210 bp, which harbors two putative NRSE/RE1 elements [33]. In this investigation, data obtained with
defined deletion and point mutation reporter gene constructs further confirm that: (1) NRSE-II region is related to the repression of TH mediated via NRSF/REST and HDAC in human NSCs, and that the repression by NRSE-R and NRSE-I is possibly mediated in a NRSF/REST- or HDAC-independent manner, (2) although NRSE-R and NRSE-I sites may not directly mediate NRSF/REST-dependent repression, it may regulate NRSF-dependent repression/derepression of TH transcription through NRSE-II site in higher order, and (3) the second type of repression may simultaneously require two NRSE/RE1 sites, NRSE-R and NRSE-I.

In HB1.F3 cells, which contain a high NRSF/REST protein level and do not express TH, the NRSE/RE1 elements mediate potent transcriptional repression of the TH gene. This repression is substantially alleviated by either mutating or deleting NRSE/RE1 (Figs. 4 and 5). In SH-SY5Y cells, which express the TH gene, mutating or deleting NRSE/RE1 had no effects on expression, implying that the NRSE/RE1 element regulates TH expression in a cell type-specific manner. This NRSE element, which is responsible for silencing the activity of a number of neuronal genes in non-neuronal cells [4–11], has not been shown to be active for the TH gene locus. Our study demonstrates for the first time that NRSE/RE1 elements modulate expression of the human TH gene.

Several possibilities exist for these apparent differences in gene expression patterns, including requirement of cell type-specific factors and chromatin structure [36]. Results from knockout NRSF/REST in mice strongly suggest that both tissue/cell type, as well as genetic context, are important for determining the effects of NRSF/REST on gene expression [36]. Our results highlight that the neural stem cellular context is critical for understanding and defining the functions of the NRSF/NRSE system in TH expression.

Experiments with NT2 cells, which resemble human committed neuronal progenitor cells and express low levels of endogenous NRSF/REST activity, disclose that they express βIII tubulin, but not other differentiation genes, such as synapsin, neurofilament200 or GluR [37]. Similarly, countering NRSF/REST-dependent repression alone by REST.DBD expression in Daoy human medulloblastoma cells did not induce synapsin [13]. Thus, there appear to be two types of repression of neuronal differentiation genes in mammals. One is represented by βIII tubulin, whose expression depends mostly on the release of NRSF/REST-dependent repression. Various non-neuronal and neuronal cell types contain all other promoter/enhancer-specific activators required for their expression. The other type is by proteins such as synapsin, GluR, and neurofilament200, among others. In these cases, expression requires not only the absence of NRSF/REST-dependent...
repression, but also the presence of promoter/enhancer-specific activators. Our results suggest that both types of repression are mediated within one gene promoter. Second type of repression system involving NRSE-R and NRSE-I may act in concert to regulate the first type of repression by NRSE-II. These differences may originate from variations in the surrounding sequences, resulting in the recruitment of distinct sets of cofactors. The identification of regulatory binding proteins should aid in elucidating the specific combinations of factors required for stringent targeting of TH gene expression to DAergic neurons.

Several groups have reported that regulation of gene expression of NRSF is important for normal development and pathogenesis of several diseases in NSCs [38,39]. Abnormal expression of NRSE-REST in neuron cells/progenitor cells induces cerebellum-specific tumors by blocking neuronal differentiation and thus maintaining the “stemness” of these cells [38]. In addition, Calderone et al. [39] showed that global ischemia triggers REST mRNA and protein expression. Derepression of REST is possibly an important mechanism of insult-induced neuronal death. Thus, the human NSC line, HB1.F3, provides a valuable opportunity to analyze NRSE/REST action in a stem cell type.

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