Journal of Neurochemistry, 2003, **85**, 622–634 doi:10.1046/j.1471-4159.2003.01671.x

Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner

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Abstract

Tyrosine hydroxylase (TH) catalyzes the first and rate-limiting step of catecholamine synthesis and its expression is necessary for neurotransmitter specification of all catecholaminergic neurons, while dopamine β-hydroxylase (DBH) is essential for the noradrenergic phenotype. In the present study, we show that Nurr1, an orphan nuclear receptor critical for dopaminergic (DA) neuron development, directly transactivates the promoter activity of the *TH* gene in a cell type-dependent manner, while it does not regulate the DBH promoter. Consistent with these results, only the TH promoter contains multiple sequence motifs homologous to the known Nurr1-binding motif, NBRE. TH promoter deletional analysis indicates that < 1.0 kb upstream sequences, encompassing three NBRE-like motifs (i.e. NL1, NL2 and NL3) are mostly responsible for the effects of Nurr1. Among these potential motifs, site-directed mutational analysis showed that NL1, residing from − 35 to − 28 bp, was most critical for mediating the transactivation by Nurr1. Strikingly, however, both DNase I footprinting and electrophoretic mobility shift assays showed that NL3, but not NL1 or NL2, has high binding affinity to Nurr1. To determine whether the proximity of these motifs may be important for transactivation by Nurr1 in the transient transfection assay, we generated reporter gene constructs in which NL3 is immediately proximal to the TATA box. Indeed, NL3 was more efficient in this position than NL1 or NL2 for mediating the transactivation by Nurr1. Our results suggest that Nurr1 may play a direct role for specification of DA neurotransmitter identity by activating *TH* gene transcription in a cell context-dependent manner.

Keywords: *cis*-acting element, dopaminergic neurons, neurotransmitter phenotype, Nurr1, promoter, transcription, tyrosine hydroxylase.

J. Neurochem. (2003) **85,** 622–634.

During the development of the mammalian nervous system, determination of neurotransmitter identity is one of the critical steps because it determines the nature of the chemical neurotransmission it will mediate, and its specific neural circuitry with target neurons. Dopamine (DA) is one of the most important neurotransmitters and controls movement, reward, and other cognitive functions (Cooper *et al*. 1996). As a corollary, abnormal regulation of DA neurotransmitter system is implicated in major pychiatric and neurological disorders. For instance, specific degeneration of nigrostriatal DA pathway underlies the pathophysiology of Parkinson's disease. DA is synthesized by two enzymes, tyrosine hydroxylase (TH) (Nagatsu *et al*. 1964) and aromatic L-amino acid decarboxylase (AADC) (Jaeger *et al*. 1983). Therefore, expression of these two enzymes is essential for phenotypic specification of DA neurons during brain development. DA is converted to another neurotransmitter, noradrenaline (NA), by dopamine β-hydroxylase (DBH), whose expression is essential for the neurotransmitter

Received October 30, 2002; revised manuscript received January 3, 2003; accepted January 7, 2003.

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Abbreviations used: AADC, aromatic L-amino acid decarboxylase; CA, catecholaminergic; DA, dopaminergic; DBH, dopamine β-hydroxylase; NA, noradrenaline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TH, tyrosine hydroxylase.

phenotype of NA neurons. Thus, differential expression of TH and DBH is a critical mechanism for subspecification of the neurotransmitter identities of DA and NA neurons.

Specification of neurotransmitter phenotypes is primarily regulated at the transcriptional level by key transcription factors (reviewed in (Goridis and Brunet 1999; Goridis and Rohrer 2002). Transcriptional regulation of the *TH* gene has been extensively investigated in numerous laboratories. *In vivo* transgenic mice experiments have been performed to address whether different promoter regions of the human, rat, and mouse *TH* gene can confer cell type-specific gene expression (Gandelman *et al*. 1990; Kaneda *et al*. 1991; Banerjee *et al*. 1992; Min *et al*. 1994; Wong *et al*. 1995; Morgan *et al*. 1996). Despite some variations between such studies, in general they established that 5′ upstream sequences (> 5 kb) of the *TH* gene are able to drive reporter gene expression in most catecholaminergic (CA) neurons and endocrine cells, with some ectopic expressions. Transient transfection approaches along with mutational analysis of the TH promoter have also been extensively used to delineate molecular mechanisms of *TH* gene regulation. Although these *in vitro* cell culture techniques have provided important information including critical *cis*-regulatory elements, e.g. AP1, dyad/E box, CRE, AP2 sites and their corresponding transcription factors (reviewed in Kumer and Vrana 1996, also see Yang *et al*. 1998a; Kim *et al*. 2001), molecular mechanisms underlying the cell specificity of *TH* gene expression are still poorly understood.

Recently, a few transcription factors have been implicated in DA phenotype specification and/or maintenance. Among these, a homeodomain-containing factor Ptx3 is restrictively expressed in midbrain DA neurons (Smidt *et al*. 1997) and appears to be involved in activation of *TH* gene and/or maintenance of DA phenotype (Cazorla *et al*. 2000; Smidt *et al*. 2000). In addition, an orphan member of the nuclear receptor superfamily, Nurr1, was isolated and found to be coexpressed with TH in CNS DA neurons such as substantia nigra and olfactory bulb (Law *et al*. 1992; Zetterstrom *et al*. 1996). Gene inactivation studies showed that Nurr1 is essential for the later stages of DA cell development (Zetterstrom *et al*. 1997; Castillo *et al*. 1998; Saucedo-Cardenas *et al*. 1998). Taken together, Ptx3 and Nurr1 are key candidate transcription factors that my determine, regulate and/or maintain the cell fate of DA neurons. However, their immediate target genes and molecular mechanisms of action are not defined. Interestingly, expression of DA was down-regulated in the midbrain area of the Nurr1$^{+/-}$ heterozygote mice, compared with that of the wild-type animal (Zetterstrom *et al*. 1997), providing the first *in vivo* clue that Nurr1 may directly or indirectly regulate DA synthesis. Consistent with this idea, Wagner *et al*. (1999) have recently shown that overexpression of Nurr1 in combination with factors derived from local type 1 astrocytes resulted in induction of the DA phenotype in an immortalized multipotent neural stem cell line. In addition, Nurr1 was able to activate *TH* gene transcription in the adult hippocampus-derived progenitor cells (Sakurada *et al*. 1999). More recently, two groups independently demonstrated that exogenous expression of Nurr1 in mouse embryonic stem cells robustly increased the proportion of TH^{+} neurons after *in vitro* differentiation (Chung *et al*. 2002; Kim *et al*. 2002).

The purpose of this study is to delineate the mechanisms of action of Nurr1 in specification of the DA phenotype. Using promoter mutation and transient transfection assays, we show that the *TH* gene, but not the DBH gene, is a direct target of Nurr1. It appears that transactivation of the TH promoter activity by Nurr1 requires a specific cellular context as transactivation occurs in a cell-specific manner. Based on deletional analysis of the TH promoter showing that < 1.0 kb can mediate full responsiveness to Nurr1 transactivation, we analyzed the functional role of three NBRE-like motifs residing within this 1.0 kb upstream region by electrophoretic mobility shift, DNase I footprinting, and site-directed mutational analyses.

Experimental procedures

Cell culture, transient transfection and reporter gene assays

Human neuroblastoma SK-N-BE(2)C, HeLa, C6 and 293T cell lines were maintained as described previously (Kim *et al*. 1998). Drosophila SL2 and COS-7 cell lines were obtained from ATCC and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Hyclone), 100 μg/mL streptomycin and 100 units/mL penicillin. Transfection was performed by the calcium phosphate coprecipitation method as previously described (Yang *et al*. 1998a). Plasmids for transfection were prepared using Qiagen columns (Qiagen Co., Santa Clarita, CA, USA). For the SK-N-BE(2)C cell line, each 60-mm dish was transfected with an equimolar amount (0.5 pmol) of each reporter construct, 1 μg of pRSV-βgal, varying amounts of the effector plasmid, and pUC19 plasmid to a total of 5 μg DNA. For the HeLa and other cell lines, twice as much DNA was used in transfection. In control experiments, the effector plasmid was substituted for an equimolar amount of the empty vector. To correct for differences in transfection efficiencies among different DNA precipitates, cells were cotransfected with 1 μg of pRSV-βgal and CAT activity was normalized to that of β-galactosidase. CAT and β-galactosidase activities were assayed as previously described (Yang *et al*. 1998a).

Plasmid constructs

The TH2400CAT and DBH978CAT reporter constructs contain the 2.4 kb upstream sequences of the rat *TH* gene and the 978 bp upstream sequences of the human DBH gene, respectively, fused to the bacterial chloramphenicol acetyltransferase (CAT) gene (Yang *et al*. 1998a). Additional TH-CAT reporter constructs containing different lengths of the 5′ upstream sequences were described previously (Yang *et al*. 1998a). Base substitutions in the NBRE-like motifs on TH promoter were introduced into TH5600CAT using the Transformer Mutagenesis kit (Clontech, Palo Alto, CA, USA)

according to the manufacturer's instructions. The following oligonucleotides were used in the mutagenesis procedure: selection primer; 5′-TACTGAGAGTGCACCCGCGGCGGTGTGAAATACC-3′, NL1 mutant primer; 5′-GGGCTTTGACGTCAGCCGACAATTTAAAGAGGGCGCCTGC-3′, NL2 mutant primer; 5′-CTTCTAGATTTGTCTCCACCTTTTATAGTTCTAACATGAG-3′, and NL3 mutant primer; 5′-ATGCTAACTGGAAAACAAGGAATCACTTACTGTTAGACCTC-3′ (underscores indicate the mutated bases). pNL1(C) which had a consensus NBRE motif at NL1 position of pTH2400CAT was constructed by changing G at the third position of NL1 to A. The mutant primer used for this was 5′-GGCTTTGACGTCAGCCTGACCTTTAAAGAGGGCGCCT-3′ (underscore indicates the mutated base, and the selection primer, 5′-TACTGAGAGTGCACCCGCGGCGGTGTGAAATACC-3′. TATA-CAT has been described previously (Hwang *et al*. 2001). The plasmids containing a single copy of NL1 or NL3 upstream of the minimal promoter were generated as follows. Sense and antisense oligonucleotides for NL1 and NL3 were annealed first to generate double-stranded DNAs (NL1 sense primer; 5′-GATCTTCAGCCTGGCCTTTAAAGA-3′, NL1 antisense primer; 5′-GATCTCTTTAAAGGCCAGGCTGAA-3′, NL3 sense primer; 5′-GATCGAAAACAAAAGGTCACTTAC-3′, NL3 antisense primer; 5′-GATCGTAAGTGACCTTTTGTTTTC-3′). These annealed DNAs retained a 5′ overhang sequence (GATC) at both ends, and were inserted into the *Bgl*II site upstream of minimal promoter of TATA-CAT. Plasmids containing a single copy of NL1 or NL3 sequences in both orientations were obtained. The site-directed mutant constructs were verified by sequence analysis. A Nurr1-expression plasmid, pSV40Nurr1 as well as an empty vector pSV40 were kindly provided by Dr Orla M. Conneely at Baylor College of Medicine (Houston, TX, USA) (Murphy *et al*. 1996).

In vitro transcription and translation of Nurr1 protein

The TNT coupled Wheat Germ Extract Transcription/Translation System (Promega, Madison, WI, USA) was used to generate *in vitro* translated Nurr1 proteins according to the manufacturer's protocol. The construct, pT7Nurr1, containing the Nurr1 gene (from Dr Conneely) was digested with either the *Xho*I restriction enzyme for Nurr1 transcription/translation or the *Nco*I restriction enzyme for empty vector expression. The digested constructs were *in vitro* transcribed and translated with or without [^{35}S]methionine. Expressed proteins were analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by autoradiography.

Electrophoretic mobility shift assays (EMSA) and DNase I footprinting

Sense and antisense oligonucleotides corresponding to the sequences of Nurr1 binding sites of the rat *TH* gene were synthesized with the following sequences: NL1; 5′-GTCAGCCTGGCCTTTAAAGA-3′ and 5′-CTCTTTAAAGGCCAGGCTGA, NL2; 5′-GTCTCCAAAG GTTATAGTTC-3′ and 5′-AGAACTATAACCTTTGGAGA-3′, NL3; 5′-AAAACAAAAGGTCACTTACT-3′ and 5′-CAGTAAGTGACC TTTTGTTT-3′, NBRE; 5′-GATCTCGAAAAGGTCACGGGA-3′, 5′-ATCCCGTGACCTTTTCGAGAT-3′. A consensus CRE was described in previous studies (Seo *et al*. 1996). The sense and antisense oligonucleotides were annealed, gel-purified, ^{32}P-labeled with T4 DNA kinase, and used as probes in EMSA.

EMSA was performed using 40 000 cpm of the labeled probe (approximately 0.05–0.1 ng) and *in vitro* translated Nurr1 protein in a final volume of 20 μL of 12.5% glycerol, 12.5 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol with 1 μg of poly(dI-dC) as described previously (Seo *et al*. 1996). Competition binding assays were performed by adding non-radioactive competitor oligonucleotides in a molar excess prior to adding ^{32}P-labeled oligonucleotides.

The DNase I footprinting assay was performed to determine the DNA binding site of the *in vitro* translated Nurr1 protein on the TH promoter. For NL1 and NL2, pTH150CAT and pTH365CAT plasmids were used as templates for PCR with a ^{32}P-labeled oligonucleotide primer, which represents the non-coding nucleotides 15–31 of the CAT gene, 5′-CGGTGGTATATCCAGTG-3′ and an unlabeled oligonucleotide primer which represents a coding strand sequence from 388 to 406 bp of pBLCAT3, 5′-GGCCAGTGCCAAGCTTGC-3′ (Kim *et al*. 1993a). For NL3, pTH2.4CAT was used as a PCR template with a ^{32}P-labeled 5′-CTTCCATGGTACCCCGAG-3′, which represents upstream from − 843 to − 861 bp of the *TH* gene and an unlabeled 5′-ATGGGCCCAGCACAACTC-3′, which represents upstream − 1003 to − 985 bp of the *TH* gene. Approximately 30 000 cpm of the labeled probe was incubated with 27 μL of the *in vitro* translated Nurr1 protein, and subjected to DNase I digestion in 1 × binding buffer, which contains 20 mM HEPES (pH 7.9), 2 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol, as previously described (Seo *et al*. 1996). The amount of DNase I was adjusted empirically for each reaction to produce an even pattern of partially cleaved DNA fragments. The DNase I reaction was stopped by adding 100 μL of stop buffer (50 mM Tris (pH 8.0), 1% SDS, 10 mM EDTA (pH 8.0), 0.4 mg/mL proteinase K and 100 mM NaCl). The DNA sample was ethanol-precipitated and resuspended in sequencing stop buffer (0.05% xylene cyanol, 0.05% bromophenol blue, 10 mM Na_2EDTA and 90% deionized formamide) and incubated at 95°C for 3 min. An aliquot of sample was then loaded onto a 6% polyacrylamide-8 M urea sequencing gel. The location of each band was determined by Maxam–Gilbert sequencing reactions of the labeled probes.

Results

Nurr1 transactivates the promoter activity of the *TH* gene but not that of the DBH gene, in the SK-N-BE(2)C and HeLa cell lines

To determine whether Nurr1 is able to directly transactivate the promoter activity of the *TH* gene in different cell lines, we transiently transfected TH2400CAT reporter construct along with Nurr1-expressing plasmid to SK-N-BE(2)C, HeLa, and COS-7 cell lines. These cell lines have been previously used as the positive [SK-N-BE(2)C] and negative (HeLa and Cos7) model systems to study control mechanisms of *TH* and other CA neuron-specific gene expression (Yang *et al*. 1998a; Kim *et al*. 2001). We also tested the DBH978CAT construct that harbors the 978 bp upstream sequences of the DBH gene in front of the reporter gene

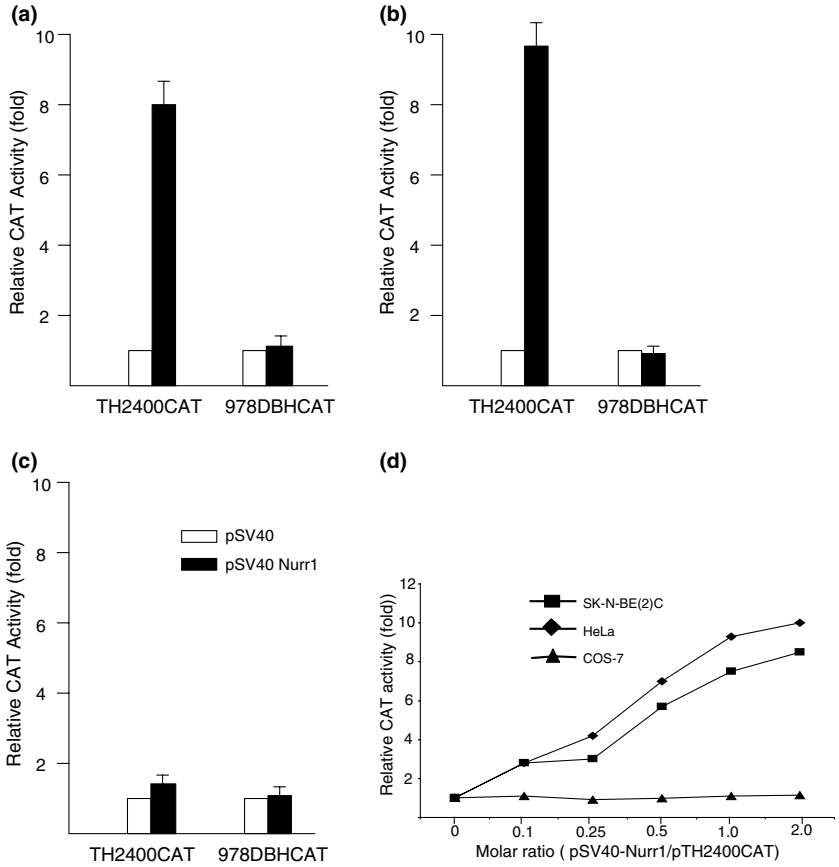

Fig. 1 Nurr1 transactivates the TH, but not DBH, promoters in a cell-specific manner. The effector plasmid pSV40-Nurr1 was cotransfected with the reporter plasmid TH2400CAT or DBH978CAT into SK-N-BE(2)C (a), HeLa (b), or Cos7 (c) cell lines. The molar ratio of effector plasmid to reporter plasmid used for transfection was 0.5 in each experiment. To compare the fold transactivation by Nurr1 directly, the CAT activity driven by each reporter construct in the presence of empty vector (pSV40) was set to 1. Cells were harvested 48–72 h after transfection. CAT activity was determined and normalized to the activity of the β-galactosidase, and the means ± SEM of six samples are presented. Exogenous Nurr1 expression robustly transactivated reporter gene expression driven by TH2400CAT in SK-N-BE(2)C and HeLa, but not in Cos7 cells. In contrast, the DBH promoter activity was not altered at all by Nurr1 expression. (d) Different amounts of the effector plasmid, shown at the bottom as the molar ratio of effector plasmid to reporter plasmid, were used in cotransfection assays. This result shows that Nurr1 can transactivate the TH promoter activity in a dose-dependent manner in SK-N-BE(2)C and HeLa, but not in Cos7 cells.

(Kim *et al*. 1998). When an Nurr1-expressing plasmid was cotransfected in a 1 : 0.5 molar ratio, reporter gene expression driven by TH2400CAT was robustly up-regulated by approximately eight- and 10-fold in SK-N-BE(2)C and HeLa cell lines, respectively (Figs 1a and b). In contrast, forced expression of Nurr1 did not affect the TH promoter function in the COS-7 cell line (Fig. 1c), suggesting that activation of the TH promoter by Nurr1 may depend on specific cellular contexts. Notably, forced expression of Nurr1 did not affect the DBH gene promoter in any of the three cell lines used. Therefore, it appears that Nurr1 can transactivate the promoter activity of the *TH* gene, but not that of the DBH gene in SK-N-BE(2)C and HeLa cell lines. Additional cotransfection experiments showed that Nurr1 transactivates the TH promoter activity in a dose-dependent manner both in SK-N-BE(2)C and HeLa cells (Fig. 1d). In contrast, there was no transactivation observed in all the range of doses in COS-7 cell line. Furthermore, in cotransfection experiments using other cell lines such as Drosophila SL2, rat glioma C6 and 293T, Nurr1 was shown to activate the TH promoter only marginally or not at all (data not shown).

The upstream promoter region of the *TH* gene, mediating full responsiveness to transactivation by Nurr1, contains multiple NBRE-like sequence motifs

In order to localize the specific sequence regions critical for such cell-specific transcriptional responsiveness, we tested Nurr1-mediated transcriptional activation against a series of TH-reporter constructs containing different lengths of the TH upstream sequences, by cotransfection assays in SK-BE(2)C and HeLa cell lines. Based on previous TH promoter studies indicating that 5.0–9.0 kb upstream sequences can drive the

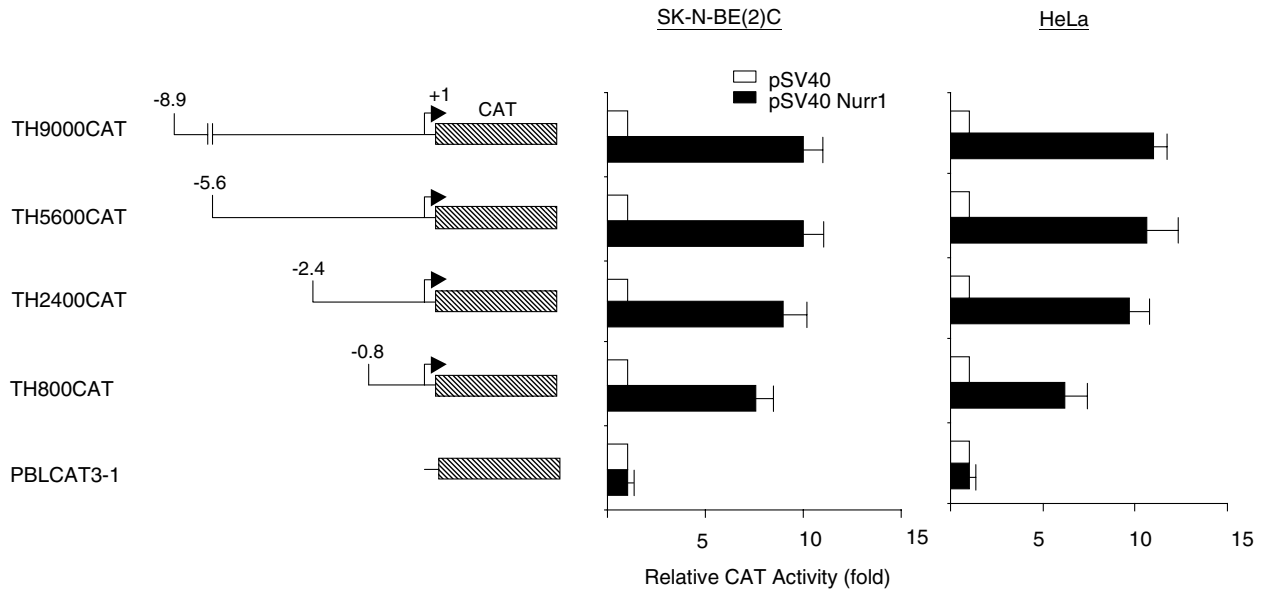

Fig. 2 Deletional analysis of the TH promoter for its transactivation by Nurr1 in the SK-N-BE(2)C and HeLa cell lines. Each of TH-CAT deletional constructs was cotransfected with pSV40Nurr1 or pSV40, and the means of relative CAT activity (induction fold) ± SEM is presented as the average value from four to six independent samples. The molar ratio of effector plasmid to reporter plasmid used for transfection was 0.5 in each experiment. To compare the fold transactivation by Nurr1 directly, the CAT activity driven by each reporter construct in the presence of empty vector (pSV40) was set to 1. Please refer to our previous report (Yang *et al.* 1998a) for comparison of the basal activities.

reporter gene in the catecholaminergic neurons (Banerjee *et al*. 1992; Min *et al*. 1994), we employed various reporter constructs containing 9.0 kb or shorter upstream sequences of the *TH* gene. As shown in Fig. 2, forced expression of Nurr1 caused an approximately 10- and 12-fold increase in reporter expression by the 9.0 kb upstream region in SK-N-BE(2)C and HeLa cell lines, respectively. Deletion of nucleotides to − 5.6 or − 2.4 kb upstream regions did not alter levels of transactivation by Nurr1 in either cell line. TH800CAT retained most (approximately 80%) of responsiveness to transactivation by Nurr1 in both cell lines, suggesting that the upstream 800 bp may contain most of the genetic information necessary for functional transactivation by Nurr1.

Nurr1 was originally characterized by binding as a monomer to a sequence motif named the NGFI-B response element (NBRE, 5′-AAAGGTCA-3′) (Wilson *et al*. 1991; Murphy *et al*. 1996). In addition, Nurr1 can form heterodimers with retinoid X receptor (RXR) and bind to a related sequence motif DR5 (5′-AGGTCANNNAAAGGTCA) in the presence of 9-*cis* retinoic acid (Perlmann and Jansson 1995). Finally, Nurr1 can bind to another related sequence with the palindromic structure, 5′-TGACCTTTNNNN-NAAAGGTCA-3′ as a dimer (Maira *et al*. 1999). To determine whether Nurr1 transactivates the TH promoter activity via interacting with binding motif(s), we searched the 5′ flanking sequences for the potential Nurr1-binding sequence motifs. There were no DR5-like or palindromic sequence motifs in the TH promoter region. Remarkably, however, 13 NBRE-like motifs (no more than one base deviation from the consensus NBRE) were identified within the 5.6 kb upstream region (Table 1). Among these, two

Table 1 Potential Nurr1-interacting sequence motifs residing in the 5′ upstream flanking region of the *TH* gene

Location	Sequence	Orientation
from − 34 to − 27	TGgCCTTT	R
from − 350 to − 343	AAAGGTtA	F
from − 872 to − 865	AAAGGTCA	F
from − 972 to − 965	AAAGGTCc	F
from − 1151 to − 1144	TGtCCTTT	R
from − 1494 to − 1487	gAAGGTCA	F
from − 2086 to − 2079	AAAGGTtA	F
from − 2202 to − 2195	AAAGGTCt	F
from − 2475 to − 2468	AAAGGgCA	F
from − 2579 to − 2572	TGAgCTTT	R
from − 3454 to − 3447	TGgCCTTT	R
from − 4916 to − 4909	TGgCCTTT	R
from − 5541 to − 5534	TGACaTTT	R

The 5′ flanking sequences were searched for the potential Nurr1-binding sequence motifs. Thirteen sequence motifs were identified to have no more than one base deviation from the consensus NBRE within the 5.6 kb upstream region. The deviated base is shown in lower case. The orientation (R; reverse, F; forward) and location relative to the transcription start site of each motif are shown.

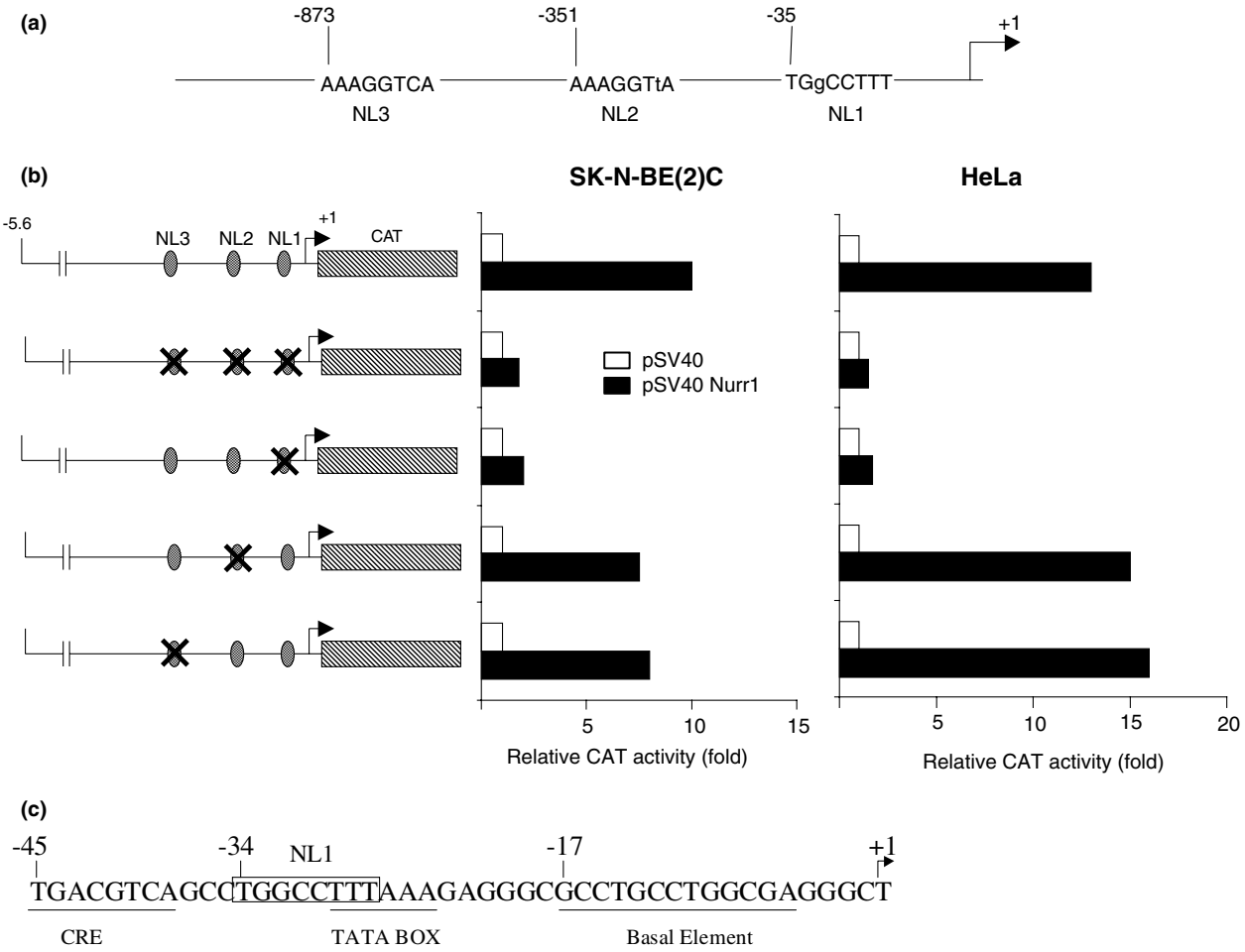

Fig. 3 Effect of site-directed mutation of putative Nurr1-binding motifs on transactivation of the TH promoter activity by Nurr1 in the context of the upstream 5.6 kb sequence. (a) Schematic diagram of the TH promoter encompassing three putative Nurr1-binding sites (NL1-3). Nucleotide sequences of each motif are shown with small case letters representing the nucleotides deviating from the consensus NRSE motif. (b) Effect of mutations on transactivation of the TH promoter activity by Nurr1 in SK-N-BE(2)C and HeLa cell lines. The effect of each mutation on transactivation by Nurr1 was examined by cotransfecting each TH-CAT construct with either Nurr1 expression plasmid (pSV40Nurr1) or empty plasmid (pSV40). The average values of six independent samples are presented as induction fold. The molar ratio of effector plasmid to reporter plasmid used for transfection was 0.5 in each experiment. Mutation of these sites (NL1-3) did not have any effect on the basal promoter activities of the *TH* gene in either cell line (see text). These experiments were repeated twice more using independently prepared DNA samples, resulting in similar results. (c) Nucleotide sequences and locations of functional *cis*-regulatory elements which reside in proximity to NL1. The TATA box partially overlaps with NL1, while the CRE (Kim *et al*. 1993a) and basal element (Patankar *et al*. 1997) reside in the immediate 5′ and 3′ positions, respectively.

(termed NL1 and 2 in this paper) reside within 800 bp proximal region; one at − 35 to − 28 bp and the other at − 357 to − 347 bp. In addition, a potential sequence motif including an exact match for the NBRE, here termed NL3, resides at the − 878 to − 866 bp location (Table 1; Fig. 3a).

NL1 is critical for transactivation of the TH promoter activity by Nurr1

Based on our deletional analysis indicating that the upstream 800 bp region retains most of the responsiveness to Nurr1 transactivation (Fig. 2), we hypothesized that NL1 and/or NL2 may be critical *cis*-regulatory elements for transactivation of the TH promoter activity by Nurr1. In addition, NL3 may also be important because it is the only sequence motif residing in the TH promoter, which perfectly matches with the consensus NBRE motif (Table 1). To test these hypotheses, we mutagenized all or each of these three putative *cis*-elements in the context of the 5.6 kb upstream sequences. As shown in Fig. 3(b), mutation of all three sites (i.e. NL1-3) almost completely abolished transactivation of the TH promoter by Nurr1 in SK-N-BE(2)C and HeLa cell lines. This result thus strongly supports the idea that Nurr1 directly transactivates TH transcription by interacting with some or all of these *cis*-elements. When the NL3 or NL2 site was

mutated, the TH promoter was transactivated as efficiently by Nurr1 as the wild-type promoter. In contrast, mutation of the NL1 site almost completely abolished transactivation of the TH promoter by Nurr1 in SK-N-BE(2)C and HeLa cell lines.

The NL1 site is in proximity of the cAMP response element (CRE), TATA box and basal element, and partially overlaps with them (Fig. 3c), which are known to be important for the basal promoter function of the *TH* gene (Kim *et al*. 1993a; Patankar *et al*. 1997). Therefore, one possible explanation is that mutation of the NL1 site may have fundamentally impaired the basal and general function of the TH promoter. To address this possibility, we tested whether cotransfection of a known transcription activator of the TH promoter, the catalytic subunit of cAMP protein kinase (Kim *et al*. 1993b), can up-regulate the mutant TH promoter. We found that the promoter activity of the mutant TH promoter was up-regulated by forced expression of the catalytic subunit of cAMP protein kinase as robustly (> 50-fold) as the wild-type TH promoter (data not shown). Furthermore, the basal promoter activity of the NL1-mutated construct was as high as the wild-type construct in both SK-N-BE(2)C and HeLa cell lines (data not shown), indicating that the basal promoter function is intact in this mutant construct. We conclude that mutation of NL1 specifically changed the reponsiveness of the TH promoter to transactivation by Nurr1, but neither the basal activity nor the reponsiveness to the other transcriptional activator was affected.

NL3, but not NL1 or NL2, forms prominent complex with Nurr1 as examined by EMSA and DNase I footprinting analyses

To correlate the above functional data with Nurr1-binding properties, we next analyzed and compared the binding affinities of these putative *cis*-regulatory motifs to the Nurr1 protein. We therefore synthesized the full-length Nurr1 protein using coupled *in vitro* transcription and translation using a wheat germ lysate system (see Experimental procedures). In EMSA, Nurr1 generated two major DNA-protein complexes when the NBRE oligonucleotide was used as a probe (C1 and C2; Fig. 4a). Both bands appear to be specific complexes because they are not formed when the protein source was generated using an empty vector in *in vitro* translation (compare lane 2 and 3, Fig. 4a). In addition, formation of both bands was specifically inhibited by molar excess of unlabeled oligonucleotide (Fig. 5). Strikingly, DNA–protein complexes were not clearly detected when

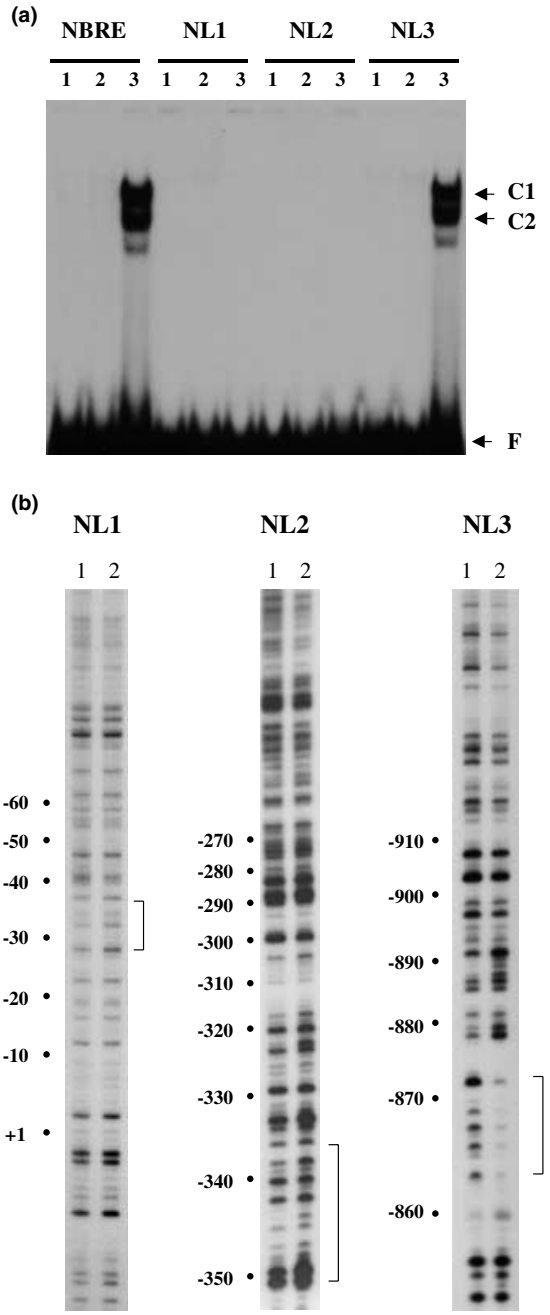

Fig. 4 Specific DNA/protein interaction between putative Nurr1-binding motifs identified on the TH upstream region and the *in vitro* translated Nurr1 protein. (a) For EMSA analysis, 3 μL out of the 50 μL from the *in vitro*-transcription/translation reaction was used for the binding reaction with 40 000 cpm of labeled NBRE, NL1, NL2, or NL3 oligonucleotide (approximately, 0.05–0.1 ng): lane 1, labeled probe only; lane 2, *in vitro* translated product using an empty vector; lane 3, *in vitro* translated Nurr1. Two DNA/protein complexes (C1 and C2) were evidently formed only when NBRE and NL3 were used as the probe. An identical DNA binding pattern was observed in additional EMSA experiments using different buffers such as those lacking EDTA (data not shown). Free probes are indicated by an arrow and F. (b) DNase I footprint analysis of the 5′-*TH* gene promoter using the *in vitro* translated Nurr1 protein (lane 1: control incubation without Nurr1, lane 2: incubation with Nurr1). The coding strands of different upstream areas were radiolabeled and used as probes. This analysis demonstrates that only NL3, but not NL1 or NL2, prominently interacts with the *in vitro* translated Nurr1.

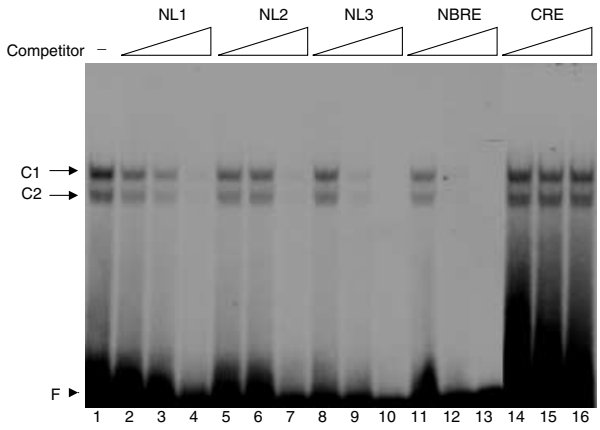

Fig. 5 Competition assay using the *in vitro*-translated Nurr1 and radiolabeled NBRE oligonucleotide. The radiolabeled NBRE oligonucleotide (40 000 cpm) was incubated with 2 μL of the *in vitro*-translated Nurr1 along with a molar excess of cold NL1 (lanes 2–4), NL2 (lanes 5–7), NL3 (lanes 8–10), NBRE (lanes 11–13), and CRE (lanes 14–16). Tenfold (lanes 2, 5, 8, 11, and 14), 100-fold (lanes 3, 6, 9, 12, and 15) and 1000-fold (lanes 4, 7, 10, 13, and 16) of cold oligonucleotides were used. Two DNA–protein complexes (C1 and C2) are indicated by arrows.

either NL1 or NL2 oligonucleotide was used as the probe (Fig. 4a). In contrast, the NL3 probe generated both C1 and C2 as robustly as the NBRE probe did (Fig. 4a). The same pattern of results was obtained using the reticulocyte-generated Nurr1 protein except that only one major DNA-protein complex, with the same mobility as the C1, was formed with the NBRE or NL3 probe (data not shown). Although this result is consistent with the fact that only NL3 contains the consensus NBRE motif, it is unexpected because NL1 was most critical for transactivation of the TH promoter by Nurr1 (see Fig. 3). Therefore, we compared the ability of each site to interact with Nurr1 in the intact TH promoter, using DNase I footprinting analysis. As shown in Fig. 4(b), only the NL3 site, but not NL1 or NL2 site, was protected from DNase I digestion when incubated with the same amount of Nurr1 protein. Taken together, such analyses of DNA–protein interactions indicate that Nurr1 protein has a high affinity to NL3 and NBRE, but not to NL1 or NL2.

This surprising observation, that Nurr1 apparently does not form complexes with NL1, would suggest that this motif may not be a Nurr1-binding site. Therefore, to address if NL1 retains any affinity to Nurr1, we performed competition assays using increasing amounts of unlabeled NL1, NL2, NL3 and a non-related *cis*-element, the CRE of the *TH* gene (Kim *et al*. 1993a). While 100-fold excess of unlabeled NBRE or NL3 almost completely inhibited formation of DNA-protein complexes (lanes 9 and 12, Fig. 5), 1000-fold excess of NL1 or NL2 was required for the same level of interference (lanes 4 and 7). This result indicates that while NL3 and NBRE have similar binding affinities to Nurr1, these affinities are approximately 10-fold higher than that of NL1 or NL2. This analysis also demonstrates that NL1 and NL2 sequences retain some binding affinity because the unrelated CRE was unable to inhibit formation of DNA-protein complexes at all even in the presence of 1000-fold molar excess (lanes 14–16; Fig. 5).

Taken together, our data suggest two possibilities: (i) NL1 is a weak binding site for Nurr1, or (ii) it is a binding site for an as-yet-unidentified protein other than Nurr1. If the latter is true, we speculated that mutation of NL1 to a consensus NBRE site may not improve or even diminish its responsiveness to Nurr1 function. To test this possibility, we generated a mutant reporter construct in which the NL1 site is changed to a consensus NBRE motif. When cotransfected with Nurr1-expression plasmid to HeLa cells, transactivation of the reporter gene expression by Nurr1 was significantly stimulated in the mutant construct than the wild-type one (from nine- to 19-fold; Fig. 6). This result thus does not support the idea that NL1 is a binding site for an unknown factor. Consistent with this idea, DNase I footprinting analyses of the TH promoter using nuclear extracts from SK-N-BE(2)C or HeLa cells did not reveal a footprint at the NL1 site (data not shown; also see Yang *et al*. 1998a).

NL1, NL2 and NL3 can mediate transactivation by Nurr1, when located in an immediately proximal promoter position

Our analysis of DNA/protein interaction between Nurr1 and the TH promoter demonstrates that the NL3 is a high-affinity binding site for Nurr1. However, mutation of NL1 but not NL3 diminished transactivation of the TH promoter by Nurr1 (Fig. 4), indicating that NL3 perhaps does not contribute to TH promoter regulation by Nurr1. One possible explanation for this discrepancy is that a Nurr1-binding motif may be functional only when located in an immediately proximal position relative to the transcription start site and TATA box, as examined in the transient transfection assay. To test this possibility, we subcloned one copy of the NL1 and NL3 motif in front of the minimal promoter (Fig. 7). This minimal promoter contains only the transcription start site and the TATA box, and exhibits promoter activity no greater than the promoter-less plasmid pBLCAT3-1 (Yang *et al*. 1998a). Because the orientation of NL1 is different from that of NL3, we subcloned them in both orientations to examine a potential spatial effect. As shown in Fig. 7, forced expression of Nurr1 increased reporter gene expression driven by pNL1(F)-CAT and pNL1(R)-CAT approximately three- and six-fold, respectively. Remarkably, forced expression of Nurr1 increased reporter expression by pNL3(F)-CAT and pNL3(R)-CAT 17-fold and 10-fold, respectively. Thus, NL3 was able to mediate more robust transactivation by Nurr1 than NL1, when located in an identical proximal position. In addition, both NL1 and NL3 appear to mediate transactivation by Nurr1 in either orientation with a similar efficiency.

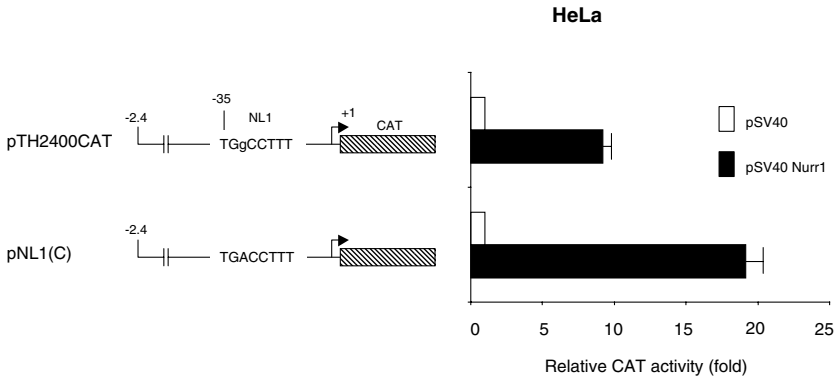

Fig. 6 Responsiveness to Nurr1 is increased when NL1 site is converted to a consensus NBRE motif. In the mutant construct, pNL1(C), G within NL1 of 2.4 kb TH promoter was changed to A to generate consensus NBRE sequence. HeLa cells were transiently cotransfected with reporter plasmids and pSV40 control vector or pSV40-Nurr1 with a molar ratio of 0.5. CAT activity was determined and normalized to the activity of the β-galactosidase, and CAT activity was determined and normalized to the activity of the β-galactosidase, and the means of relative CAT activity (induction fold) ± SEM are presented as the average value from six independent samples. The basal CAT activity driven by pNL1(C) was the same as that by pTH2400CAT.

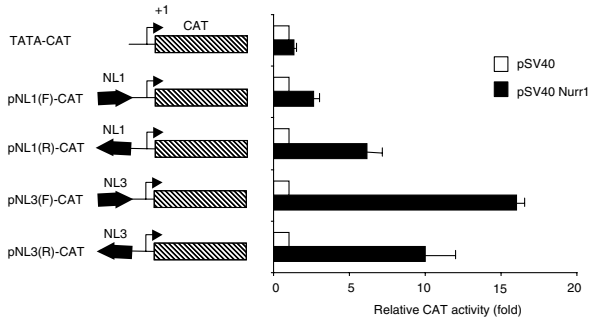

Fig. 7 NL1, NL2, and NL3 are all functional in transcriptional activation when located in an immediately proximal promoter position. One copy each of NL1 and NL3 was inserted upstream of TATA box of a minimal promoter, TATA-CAT (Yang *et al.* 1998b) in both orientations. Each reporter plasmid was cotransfected either with pSV40 or pSV40-Nurr1 into HeLa cells with a molar ratio of 0.5. CAT activity was determined and normalized to the activity of the β-galactosidase, and the means ± SEM of four independent samples are presented as induction fold.

These data strongly suggest that NL3 within the *TH* gene promoter did not show a clear functional role in the transient cotransfection assay, due to its distal position in relation to the transcription start site (Fig. 4). In addition, NL2 was also able to mediate transactivation by Nurr1 when located in an identical proximal position (data not shown). We conclude that NL1, NL2 and NL3 are able to mediate transactivation by Nurr1 in a position-dependent manner, as examined by transient cotransfection assays.

Discussion

As the first and rate-limiting step in the biosynthesis of CA neurotransmitters (Nagatsu 1964), *TH* gene expression is essential for neurotransmitter phenotype specification in all CA neurons including the midbrain DA neurons. Despite extensive investigation during the last two decades, control mechanisms underlying the cell type-specific expression of the *TH* gene remains unclear (reviewed in Kumer and Vrana 1996). It is thus of great interest that inactivation of the Nurr1 gene results in a specific agenesis of midbrain DA neurons although it is expressed in a wide range of brain areas (Zetterstrom *et al.* 1997; Castillo *et al.* 1998; Saucedo-Cardenas *et al.* 1998). Furthermore, recent gain-of-function studies using either neuronal progenitor cells (Sakurada *et al.* 1999; Wagner *et al.* 1999) or embryonic stem cells (Chung *et al.* 2002; Kim *et al.* 2002) indicated that Nurr1 can, directly or indirectly, activate *TH* gene expression. The present study aimed to address control mechanisms how Nurr1 may regulate the transcriptional activity of the *TH* gene.

The *TH* gene may be an immediate downstream target of Nurr1

Because DBH converts DA to NA, differential expression of TH and DBH is crucial for subspecification of DA and NA neurons. To address whether Nurr1 is able to differentially regulate TH and DBH promoter function, we cotransfected the TH-CAT or DBH-CAT reporter construct along with Nurr1-expression vector to different cell lines. In both SK-N-BE(2)C (TH/ DBH-expressing) and HeLa (nonexpressing) cell lines, forced expression of Nurr1 robustly (approximately 10-fold) transactivated the TH promoter activity. In contrast, the DBH promoter was not at all affected by Nurr1 in any cell lines tested (Fig. 1), demonstrating that Nurr1 indeed differentially regulates the TH, but not the DBH, promoter. These data thus clearly explain our recent study which showed that exogenous expression of Nurr1 in embryonic stem cells lead to robust induction of TH, but

not DBH, gene expression after *in vitro* differentiation (Chung *et al*. 2002). Remarkably, Nurr1 barely increased the TH promoter activity in other cell lines tested, including COS-7, SL2, C6 and 293T cell lines (Fig. 1 and data not shown). Thus, it appears that Nurr1 transactivates the TH promoter function in a cell context-dependent manner. In support of this notion, in Nurr1$^{-/-}$ mice, TH expression was abolished only in the midbrain area, but not in other Nurr1-expressing areas such as the olfactory bulb (Zetterstrom *et al*. 1997; Castillo *et al*. 1998; Saucedo-Cardenas *et al*. 1998). It is worthwhile to note that Nurr1 belongs to an orphan nuclear receptor and its ligand is not described. Thus, one possible explanation is that Nurr1 may function only when its ligand is expressed. In line with this possibility, Wagner *et al*. (1999) recently showed that Nurr1-overexpressing neural stem cell lines show TH^{+} phenotypes only when co-cultured with ventral mesencephalic type 1 astrocytes. Based on this result, they proposed that the unidentified ligand for Nurr1 may be produced by local astrocytes. Alternatively, Nurr1 may require a cofactor for its transcriptional regulatory function. Further investigation is warranted to elucidate these possible mechanisms.

Consistent with the differential transactivation of the TH (but not the DBH) promoter activity by Nurr1, our sequence search revealed that only the TH promoter encompasses multiple sites that can potentially interact with Nurr1 (Table 1). Further evidence supporting the direct activation of the *TH* gene transcription by Nurr1 was obtained by site-directed mutational analysis of putative Nurr1-binding sites. Based on promoter deletion analysis showing that the 1.0 kb upstream region retains almost full responsiveness to Nurr1 activation, we mutated three putative Nurr1-binding sites residing within the 1.0 kb proximal region in the context of the 5.6 kb upstream TH promoter. This mutation completely abolished the transactivation of the TH promoter in response to forced expression of Nurr1 (Fig. 3). Taken together, we conclude that the *TH* gene is an immediate target of Nurr1, but its induction may require an as-yet-unidentified cellular factor for its function.

The mechanisms of action of Nurr1 in transcriptional activation of the *TH* gene

Several laboratories have investigated the possible *TH* gene regulation by Nurr1. In the Nurr1-overexpressing neural stem cells derived from the embryonic cerebellum, Nurr1 appeared to be inactive during the later differentiation stage although its earlier transient expression may have conferred upon the clones long-lasting competence to become TH^{+}, followed by co-culture with ventral mesencephalic astrocytes (Wagner *et al*. 1999). Thus, in this experimental system, it does not appear that Nurr1 directly regulates *TH* gene transcription. In another study using adult rat-derived hippocampal progenitor cells, Nurr1 was shown to activate the *TH* gene by interacting with a NBRE-like motif at − 873 to − 866 bp (same as NL3 in the present study), suggesting that TH could be a direct target of Nurr1 (Sakurada *et al*. 1999). In the latter study, however, the authors proposed that Nurr1 does not directly transactivate the TH promoter but act as a derepressor by blocking the function of a negative regulatory element (Sakurada *et al*. 1999). In contrast to the above studies using neural precursor cells, our transient cotransfection assays indicate that Nurr1 directly transactivates the TH promoter activity in certain cellular context. In agreement with our results, a recent study reported that Nurr1 can directly activate the TH promoter activity in a cell-dependent manner and that a proximal *cis*-element (same as NL1 in the present study) is important for activation (Iwawaki *et al*. 2000). However, these results differ from ours in that (i) the functional importance of NL1 was tested in the context of a short (167 bp) promoter region and (ii) NL1 was shown to bind to Nurr1 as efficient as the consensus NBRE motif (Iwawaki *et al*. 2000). At present, it is unclear why NL1 motif showed such different affinity in these two studies. In addition, Jensen and O'Malley (2001) recently reported that Nurr1 can directly activate the TH promoter in a cell-dependent manner via a sequence motif located 1500 bp upstream of the transcription start site. Collectively, all these studies generally support the idea that Nurr1 may regulate *TH* gene expression in a cell type-dependent manner but the precise mechanisms proposed are significantly different.

In agreement with the report by Gage and colleagues (Sakurada *et al*. 1999), our DNase I footprinting and EMSA demonstrated that Nurr1 can prominently interact with the NL3 motif at − 873 to − 866 bp (Fig. 4). However, mutation of this element did not have any detectable effect on the responsiveness of the TH promoter to Nurr1 (Fig. 3). Instead, more proximal motif (NL1), residing at − 35 to − 28 bp, was shown to be critical for transactivation of the 5.6 kb TH upstream promoter by Nurr1. Because NL1 is in close proximity to the transcription start site and directly overlaps with the TATA box, one explanation is that mutation of NL1 may have significantly affected the general promoter function of the *TH* gene, including transactivation by Nurr1. However, our results do not support this possibility because (i) the basal promoter activity of the TH promoter was intact when NL1 was mutated, both in SK-BE(2)C and HeLa cell lines and (ii) cotransfection with another transcriptional activator, the catalytic subunit of protein kinase A, robustly increased the promoter activity of the mutant construct as efficient as the wild-type promoter. We conclude that mutation of NL1 specifically affected the responsiveness of the TH promoter to Nurr1-mediated transactivation.

Despite its critical role for the transactivation of the TH promoter by Nurr1, its binding to the NL1 site was barely detected in both EMSA and DNase I footprinting assays (Fig. 4). One possible explanation for this discrepancy is that the NL1 site binds to an unknown protein that can activate the TH promoter only when it interacts with Nurr1.

Alternatively, NL1 could be a weak Nurr1-binding site, but still mediate its responsiveness to Nurr1 by direct interaction with Nurr1. Several lines of evidence support the second possibility. First, competition assays show that NL1 retains a low but still significant affinity to Nurr1 (approximately one-tenth of the NBRE or NL3; Fig. 5). Second, mutation of NL1 to a consensus NBRE motif increased, instead of decreasing, the responsiveness to Nurr1 (Fig. 6), thus not supporting the idea that it works by interacting with another factor. In this context, one interesting possibility is that interaction of Nurr1 with the as-yet-unidentified ligand or cofactor may facilitate the affinity and/or specificity of its binding to NL1 *in vivo*. This may also explain why Nurr1 can transactivate the TH promoter activity in a cell context-specific manner. A similar, but mechanistically distinct, possibility is that the *in vivo* interaction of Nurr1 with adjacent factor(s) may stabilize its binding to NL1.

Another unresolved question is why NL3 apparently does not contribute to Nurr1-mediated transactivation of the TH promoter regardless of its prominent binding affinity. Previously, we found that the CRE, a critical *cis*-regulatory element of the *TH* gene (Kim *et al*. 1993a), can be functional only when located within a certain proximity (< 350 bp) from the transcription start site (Tinti *et al*. 1997). Based on this finding, we hypothesized that NL3 can likewise be functional if located in a proximal position, as examined in the transient transfection assays. To address this hypothesis, we placed NL1 or NL3 in an immediate upstream of a minimal promoter and compared the promoter activity in the presence of Nurr1. In this proximal position, NL3 was indeed able to transactivate the reporter expression > 10-fold in response to Nurr1, while NL1 was able to transactivate with a lower efficiency (Fig. 7). We also found that these Nurr1-binding motifs can mediate transactivation in either orientation. In addition, another NBRE-like motif, NL2, was also able to mediate transactivation by Nurr1 when located in the immediate proximal position as efficient as NL1. Given that the nucleotide sequences of NL1 and NL2 are not related other than the NBRE-like motif, these results furthermore support the model that NL1 may participate in transactivation of the TH promoter by Nurr1 through direct contact with Nurr1, rather than with an unknown factor. Furthermore, it is tempting to speculate that multiple NBRE-like motifs residing in the TH promoter (Table 1) may co-operate together *in vivo* for transactivation of the *TH* gene by Nurr1.

Role of Nurr1 in phenotypic specification and maintenance of DA neurons

Several gene inactivation studies have established that Nurr1 is essential for development of midbrain DA neurons (Zetterstrom *et al*. 1997; Castillo *et al*. 1998; Saucedo-Cardenas *et al*. 1998). These and subsequent analyses of Nurr$^{-/-}$ mice demonstrated that Nurr1 is required for later stages of DA neuron differentiation instead of earlier neurogenesis (Wallen *et al*. 1999; Witta *et al*. 2000). Importantly, TH has never been induced in the ventral midbrain of Nurr$^{-/-}$ mice, which is in agreement with our present study suggesting that TH is a direct downstream target of Nurr1. Several other markers of midbrain DA neurons, e.g. aldehyde dehydrogenase 2, the homeobox transcription factors engrailed and Ptx3, were shown to be significantly down-regulated in the mutant mice, suggesting that Nurr1 may regulate the expression of multiple target genes. In support of this, exogenous expression of Nurr1 in embryonic stem cells resulted in up-regulation of known DA-specific marker genes including TH, AADC and dopamine transporter (DAT) (Chung *et al*. 2002). Precise mechanisms underlying regulation of these potential target genes by Nurr1 require further investigation. One of these DA-specific genes, DAT, is responsible for termination of DA neurotransmission by rapid re-uptake to presynaptic nerve terminals (Giros and Caron 1993). Recent cloning and characterization of the DAT gene promoter showed that DAT may be a target of Nurr1 (Sacchetti *et al*. 1999, 2001), suggesting the possibility that Nurr1 controls both DA-synthesizing and reuptake genes. In GABA-expressing neurons, a homeobox protein NC-30 is known to control both the glutamic acid decarboxylase and the GABA transporter genes by directly interacting with their promoters (Jin *et al*. 1994; Eastman *et al*. 1999). However, Nurr1 does not appear to require its DNA binding motif for activation of the DAT promoter, indicating that control mechanisms activating the TH and DAT promoters may be distinct (Sacchetti *et al*. 2001).

Acknowledgements

The authors would like to thank Dr Orla M. Conneely at Baylor College of Medicine for kind gifts of a Nurr1-expression plasmid, pSV40Nurr1 as well as an empty vector pSV40. This work was supported by NIH grants MH48866 and (P50)NS39793, and DAMD-17-01-1-0763 (to KSK) and DAMD-17-01-1-0762 (to OI), and NARSAD Independent Award (to KSK).

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