MOLECULAR AND CELLULAR BIOLOGY, Nov. 1995, p. 6036–6044 Vol. 15, No. 11
0270-7306/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Helix-Loop-Helix Transcription Factors Mediate Activation and Repression of the *p75LNFR* Gene

ANNE CHIARAMELLO,[1,2] KÜLLIKI NEUMAN,[3] KAIA PALM,[3,4] MADIS METSIS,[4,5] AND TOOMAS NEUMAN[2,3]*

Department of Biochemistry and Molecular Biology,[1] Department of Anatomy and Neurobiology,[3] and Program of Neuronal Growth and Development,[2] Colorado State University, Fort Collins, Colorado 80523; Laboratory of Molecular Neurobiology, Karolinska Institutet, S-104 01 Stockholm, Sweden[4]; and Institute of Chemical Physics and Biophysics, Tallinn 0026, Estonia[5]

Received 27 June 1995/Returned for modification 25 July 1995/Accepted 4 August 1995

Sequence analysis of rat and human low-affinity nerve growth factor receptor *p75LNGFR* gene promoter regions revealed a single E-box *cis*-acting element, located upstream of the major transcription start sites. Deletion analysis of the E-box sequence demonstrated that it significantly contributes to *p75LNGFR* promoter activity. This E box has a dual function; it mediates either activation or repression of the *p75LNGFR* promoter activity, depending on the interacting transcription factors. We showed that the two isoforms of the class A basic helix-loop-helix (bHLH) transcription factor ME1 (ME1a and ME1b), the murine homolog of the human HEB transcription factor, specifically repress *p75LNGFR* promoter activity. This repression can be released by coexpression of the HLH Id2 transcriptional regulator. In vitro analyses demonstrated that ME1a forms a stable complex with the *p75LNGFR* E box and likely competes with activating E-box-binding proteins. By using ME1a-overexpressing PC12 cells, we showed that the endogenous *p75LNGFR* gene is a target of ME1a repression. Together, these data demonstrate that the *p75LNGFR* E box and the interacting bHLH transcription factors are involved in the regulation of *p75LNGFR* gene expression. These results also show that class A bHLH transcription factors can repress and Id-like negative regulators can stimulate gene expression.

The neurotrophic factors nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5, collectively named neurotrophins, play an important role during neuronal differentiation, regeneration, and survival and also during programmed cell death. Neurotrophins interact with two different types of cell surface receptors, the trk family of tyrosine kinase receptors (2) and the low-affinity neurotrophin receptor, referred to as p75LNGFR (9, 51). Previous studies have suggested several functions for p75LNGFR. p75LNGFR has been shown to increase the affinity of trkA for NGF (24) and to enhance the specificity of the trk family for neurotrophins (5, 30, 32). p75LNGFR is often coexpressed with trkA in the majority of NGF-responsive cells, such as basal forebrain cholinergic neurons and PC12 cells (8, 13, 60). Furthermore, in embryonic spinal cord and brain tissue, p75LNGFR is shown to dimerize with trkA (30) and therefore could be directly involved in regulation of signal transduction by trk receptors. However, it has also been suggested that p75LNGFR may localize NGF to regions where trkA is present and therefore, signal transduction is carried out through the trkA receptor (34). p75LNGFR, which is structurally related to the tumor necrosis factor receptor, can either promote neuronal survival or induce apoptosis when not bound to a ligand (3, 50). This dual function is developmentally regulated: during the phase of neuronal target selection (E13 to E17 in the mouse), p75 LNGFR mediates neuronal survival, whereas in the early postnatal period, it confers cell death (3).

While the potential functions of p75LNGFR have been extensively investigated, the molecular mechanisms responsible for the spatial and temporal expression of p75LNGFR are still poorly understood. p75LNGFR is widely distributed and expressed in a variety of cells, both neuronal and nonneuronal in origin. However, p75LNGFR is differentially expressed and regulated during neurogenesis. During the early stages of central nervous system development, it is transiently expressed in the mantle layer but not in the germinal neuroepithelial layer (7, 26). During postnatal development of the cerebellum, specific spatial and temporal expression of p75LNGFR has been demonstrated in two different cell types, the immature neuroblasts located in the external granular layer and the Purkinje cells (53). p75LNGFR expression in immature neurons is transient and detected early during development, whereas in Purkinje cells it persists throughout adulthood (31, 53). This heterogeneous regulation of p75LNGFR expression suggests a complex interplay of regulatory factors with the *cis*-acting regulatory elements present in the *p75LNGFR* promoter.

Several reports have been dedicated to the identification of critical regulatory sequences, and the structure of the rat *p75 LNGFR* promoter has been investigated in detail. This promoter carries two major start sites and has several characteristics typical of constitutively expressed promoters such as the lack of consensus TATA and CAAT elements and the presence of multiple GC-rich motifs (54). The first 4 kbp of the 5′ regulatory region are sufficient to direct the appropriate mesenchymal expression of p75LNGFR during development and expression in the cerebellum but insufficient to direct the expression of p75LNGFR in other neuronal populations (31); however, the first 390 nucleotides of the promoter region are sufficient for promoter activity. In addition, the distal portion of the *p75LNGFR* promoter carries enhancer elements such as a retinoic acid (RA)-responsive element as well as a silencer element (42, 46). Comparison of the rat and human *p75 LNGFR* promoter sequences revealed strong homology in the proximal promoter region (42).

Since little is known concerning the *cis*-acting elements that

* Corresponding author. Mailing address: Department of Anatomy and Neurobiology, Colorado State University, Fort Collins, CO 80523. Phone: (970) 491-5791. Fax: (970) 491-7907. Electronic mail address: toomas@lamar.colostate.edu.

guide the developmental expression of p75LNGFR during neurogenesis, we analyzed the 5′ flanking sequence of the *p75 LNGFR* gene. We show here that an E box, located 20 bp upstream of the major transcription start sites, is important for both activation and repression of *p75LNGFR* promoter activity in different cell types. This E box is conserved between the rat and human *p75LNGFR* promoters in terms of sequence and position, suggesting a potential role as a regulatory element that allows appropriate expression of p75LNGFR during development. This may be achieved through the binding of various developmentally regulated basic helix-loop-helix (bHLH) transcription factors to the E box. These transcription factors are characterized by a conserved structural motif predicted to form two amphipathic helices separated by an intervening loop (18, 43). The HLH motif is involved in dimerization activity, whereas the basic domain adjacent to the HLH motif is essential for DNA-binding activity (40, 43). A subfamily of HLH proteins, such as Id1, Id2, Id3, Id4, and emc, do not contain a basic domain and function as dominant negative inhibitors by forming nonfunctional heterodimers with bHLH proteins (6, 12, 16, 52, 56). Competition among bHLH proteins to form dimers has a significant role in determining which target genes are regulated and consequently in contributing to the establishment of a particular phenotype for a specific cell.

In this study, we have analyzed the bHLH proteins that recognize the conserved E box located near the major transcription start site of the *p75LNGFR* promoter. We found that this E box has a dual function in the modulation of *p75LNGFR* gene transcription. It can either enhance or repress *p75LNGFR* promoter activity, depending on the bHLH proteins binding to the E box. Both forms of the bHLH protein ME1 (ME1a and ME1b), the murine homolog of the human HEB transcription factor (28), suppress *p75LNGFR* promoter activity, and this suppression can be released by the expression of the negative regulator Id2. ME1a homodimers form stable complexes with the *p75LNGFR* E box and consequently may impair the binding of other bHLH proteins to the site. Our data present evidence that the *p75LNGFR* E box and the bHLH transcription factors interacting with this sequence are involved in the modulation of *p75LNGFR* gene expression. These results also reveal that class A bHLH proteins can repress and Id-like negative regulators can stimulate gene expression.

MATERIALS AND METHODS

Plasmid construction. The rat p75LNGFR-chloramphenicol acetyltransferase (CAT) constructs 0.4 CAT, 1.4 CAT, and 4.0 CAT were made as described previously (42). The human construct 1.2 kb AvaII p75LNGFR-CAT was a gift from M. Chao (54). Full-length cDNAs for bHLH transcription factors ME1a, ME1b, ME2, and Id2 were constructed as described previously (44, 45, 55). The expression plasmids for E12 and human MyoD were a gift from H. Weintraub, the mouse NSCL-1 cDNA was obtained from C. Begley (4), the mouse MASH-1 cDNA was obtained from Franco del Amo (19), and the human AP4 cDNA was obtained from R. Tjian (29). HLH transcription factor cDNAs were cloned into eukaryotic expression vector pRcCMV (Invitrogen). The 5′ deletion mutants of ME1a were created by digesting the full-length ME1a cDNA with the following enzymes: *Eco*RI (Δ-259 construct), *Bam*HI (Δ-488 construct), and *Xmn*I (Δ-561 construct). Each fragment was isolated, blunt ended with Klenow polymerase (Boehringer), and cloned into the blunted *Hin*dIII site of eukaryotic expression vector pRcCMV.

DNA mutagenesis. E-box mutations were created by digesting the rat p75 LNGFR template with the enzyme *Pvu*II whose restriction site is located in the E-box sequence (CAGCTG). This digestion was followed by an exonuclease III digestion. The digested fragments were ligated and cloned into the reporter plasmid pBLCAT3. Each mutation was confirmed by sequencing using the U.S. Biochemical Sequenase version 2.0 kit.

Cell culture. PC12 cells (gift from L. Greene) were maintained and differentiated with NGF as described previously (20). Embryonic carcinoma cell line PCC7 was obtained from S. Pfeiffer and was grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco). PCC7 cells were differentiated into neuronal-like cells with all-*trans* RA (0.5 μM) and dibutyryl cyclic AMP (dBcAMP; 1 mM) treatment. Mouse fibroblast 3T3, rat glioblastoma C6, human osteosarcoma Saos2, and human cervical carcinoma C33A cells were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. ME1a cDNA in expression vector pRcCMV was transfected into PC12 cells, and the cells were then subjected to selection with G418 (400 mg/ml; Gibco) for 18 to 21 days to establish ME1a-overexpressing cell lines.

DNA transfection and CAT assays. Cells were transfected with 15 μg of total plasmid DNA, using the calcium phosphate precipitation method, in 60-mm-diameter dishes (1×10^5 to 3×10^5 cells per dish). If not indicated otherwise, 5 μg of reporter DNA construct and 10 μg of eukaryotic expression construct were used for each transfection. The medium was changed 12 to 18 h after transfection to normal growth medium or differentiation medium with appropriate supplements as indicated for each experiment. Cells were harvested 48 h later, and CAT assays were performed as described previously (10). Quantitation of acetylation ratios was obtained by PhosphorImager (Molecular Dynamics) analysis. To normalize transfection efficiencies, cells were cotransfected with 1 μg of plasmid pRcRSVlacZ. All the reported CAT activities were normalized to total protein and LacZ activity. The CAT assay values represent the means of at least three independent transfections.

Northern (RNA) blot analysis. Total RNA from untreated and NGF-treated control and ME1a-overexpressing PC12 cells was isolated by the acid guanidinium-phenol-chloroform extraction procedure (11). Twenty-five micrograms of total RNA was run in each lane and fractionated on an 1.2% agarose-formaldehyde gel before transfer onto a nylon membrane (Hybond N; Amersham). The amount and quality of transferred RNA were monitored by methylene blue staining of the filters before hybridization. The *Spe*I-*Bam*HI fragment from ME1a cDNA and the *Bst*EII-*Bst*EII fragment from rat p75LNGFR cDNA were isolated and radiolabeled with [α-^{32}P]dCTP, using the Multiprime DNA labeling system (Amersham) and were used as probes. The blots were washed at high stringency (0.2× SSC [2× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 65°C) and exposed to X-ray film for 1 to 10 days.

Preparation of DNA probes for electrophoretic mobility shift assay (EMSA) analysis. The following DNA oligonucleotide probes were synthesized (Macromolecular Resources Facility, Colorado State University). The sequences of the top strands of MEF-1 (10) and rat p75LNGFR oligonucleotides (with the E-box sequence underlined) are as follows: MEF-1,5′-CTAGATCTCCAACACCTGCTGCGGAT-3′; and p75LNGFR,5′-GGCTGCTGCATTGCCTTCACCCAGCTGCTCCCGCCCGCCCGCA-3′. The complementary oligonucleotides were annealed, and the double-stranded probes were labeled at the 3′ recessed ends by using [α-^{32}P]dCTP (ICN) and Klenow polymerase (Boehringer). Unlabeled competitor DNA was prepared by annealing complementary oligonucleotides.

EMSA. Recombinant proteins ME1a, ME2, and Id2 were purified to homogeneity by nickel affinity chromatography (10). DNA binding reaction mixtures consisted of 40 ng of purified protein incubated with 40 fmol of labeled oligonucleotide and 100 ng of poly(dI-dC) (Sigma) for 20 min at room temperature in the presence of binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM $MgCl_2$, 5% glycerol). When heterodimer formation was studied, 40 ng of purified ME1a and various amounts of Id2 were incubated for 20 min at room temperature. The probe was then added, and the reaction mixture was incubated for another 20 min at room temperature. The products of the DNA binding reactions were analyzed as described previously (10). To determine the dissociation rate of the ME1a–*p75LNGFR* E-box complex (off-rate), purified ME1a protein was incubated with 40 fmol of p75LNGFR probe for 20 min, and then 200× unlabeled specific competitor was added. After incubation for various times, an aliquot of the binding reaction was immediately loaded on a 5% native polyacrylamide gel. The gel was dried, and each band was quantified by PhosphorImager (Molecular Dynamics) analysis.

RESULTS

The bHLH transcription factor ME1 represses rat *p75 LNGFR* promoter activity. Sequence analyses of the rat *p75 LNGFR* promoter region revealed an E-box element (CAG CTG) localized 20 bp upstream of the transcription start sites (Fig. 1). This E box is conserved between the rat and human *p75LNGFR* promoters in terms of sequence and position (Fig. 1), suggesting that it plays a potential role in expression of the *p75LNGFR* gene. To assess whether bHLH transcription factors are involved in transcriptional regulation of p75LNGFR expression, we cotransfected expression plasmids of various bHLH transcription factors with a reporter construct containing 396 bp of the rat *p75LNGFR* proximal promoter in front of the CAT gene (0.4 CAT) into PCC7 cells and analyzed CAT activities. Both class A and class B bHLH transcription factors were tested for modulation of *p75LNGFR* promoter activity. Among the class A bHLH transcription factors, only ME1

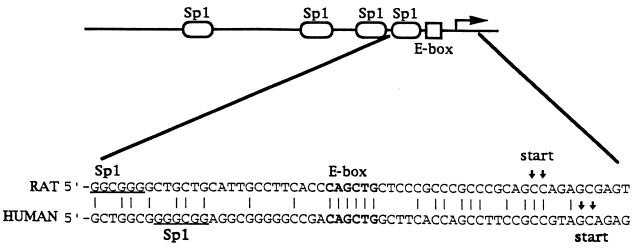

FIG. 1. Schematic representation and nucleotide sequences of the rat and human *p75LNGFR* proximal promoter regions. The binding sites for Sp1 and the E box are represented as an open ellipse and an open box, respectively, in the 396-bp upstream promoter region. The sequences of the rat (42) and human (54) promoter regions are indicated below the map. The E-box core motifs are indicated by boldface letters, and the Sp1 sites are underlined. The transcriptional start sites for each promoter are indicated by arrows.

significantly reduced *p75LNGFR* promoter activity (Fig. 2). Since two different forms of ME1 protein, ME1a and ME1b, are generated by alternative splicing (37, 44, 47), both forms were tested. ME1a is characterized by a 24-amino-acid insertion that might disrupt a leucine heptad repeat which shows a high degree of similarity with ankyrin-like domains (37). As shown in Fig. 2, both forms of ME1 are capable of repressing *p75LNGFR* promoter activity, suggesting that the 24-amino-acid insertion does not interfere with the ability to form dimers and therefore bind to the *p75LNGFR* E box. No effect was observed with other class A bHLH transcription factors such as the ubiquitous E12 and ME2, the murine homolog of human E2-2/ITF-2 (1, 25, 55). Tissue-specific class B bHLH transcription factors NSCL-1 (4) and MASH-1 (21, 36) and the muscle-specific protein MyoD did not affect the activity of the *p75 LNGFR* promoter (Fig. 2). In addition, the ubiquitous class C bHLH factor AP4 did not modulate *p75LNGFR* promoter activity (Fig. 2). Thus, bHLH transcription factor ME1 is a specific repressor of *p75LNGFR* promoter activity.

Since ME1a is the predominant form of ME1 during neuronal differentiation (44), we specifically studied the effect of ME1a overexpression on *p75LNGFR* promoter activity in PCC7 cells. Cotransfection of different amounts of ME1a expression vector with a *p75LNGFR* 0.4 CAT promoter-reporter plasmid resulted in an ME1a concentration-dependent decrease of *p75LNGFR* promoter activity (Fig. 3A). No reduction of *p75LNGFR* promoter activity was obtained in the presence of 0.5 μg of ME1a expression vector, whereas 8 μg of ME1a expression plasmid reduced *p75LNGFR* promoter activity fivefold (Fig. 3A). Next, we examined whether sequences upstream from the 396-bp proximal promoter region could also be involved in the regulation of *p75LNGFR* promoter activity by ME1a. For that purpose, different promoter reporter constructs containing 1.4 and 4 kb of the *p75LNGFR* promoter region (1.4 CAT and 4.0 CAT, respectively) were cotransfected with expression plasmid CMV-ME1a into PCC7 cells. As shown in Fig. 3B, CAT activities expressed by the 1.4 CAT and 4.0 CAT promoter reporter constructs showed similar decreases by ME1a compared with the 0.4 CAT construct. Thus, the E box significantly contributes to the modulation of p75LNGFR expression in the native context of the *p75LNGFR* promoter. Furthermore, treatment of PCC7 cells with all-*trans* RA and dBcAMP induces neuronal differentiation (49) and stimulates seven- to eightfold the activities of all three *p75LNGFR* promoter reporter constructs (Fig. 3B). However, ME1a still represses the RA- and dBcAMP-induced *p75LNGFR* promoter activity in PCC7 cells (Fig. 3B).

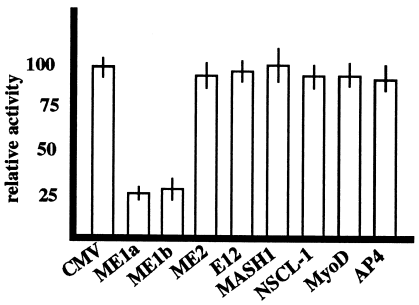

FIG. 2. Effects of various bHLH transcription factors on rat *p75LNGFR* promoter activity. Teratocarcinoma PCC7 cells were cotransfected with the 0.4 CAT reporter plasmid (5 μg) and expression plasmid pRcCMVneo without any cDNA sequence (CMV) or containing either ME1a, ME1b, ME2, E12, MASH-1, NSCL-1, MyoD, or AP4 cDNA (10 μg) by the calcium phosphate precipitation technique. Plasmid pRcRSVlacZ (1 μg) was included in the transfections to normalize the transfection efficiency. CAT activities are expressed relative to the value obtained by transfection of the reporter plasmid and the parental pRcCMV expression plasmid. The data shown are representative of at least three independent experiments.

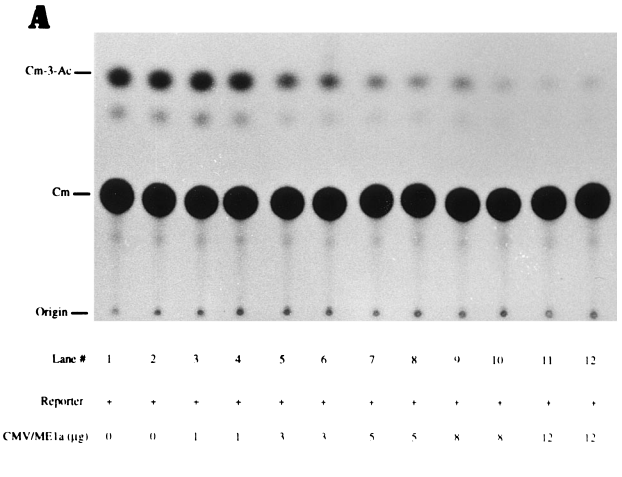

FIG. 3. Effect of ME1a on rat *p75LNGFR* promoter activity. (A) ME1a represses *p75LNGFR* promoter activity in a concentration-dependent manner. Transient cotransfection assays were performed with PCC7 cells, using 5 μg of 0.4 CAT reporter plasmid and various amounts of ME1a expression plasmid as indicated at the bottom. Although the figure represents the results of a single experiment, similar levels of ME1a repression were observed in three independent experiments. Acetylated (Cm-3-Ac) and nonacetylated (Cm) forms of chloramphenicol are indicated on the left. (B) Functional analysis of various constructs of the rat *p75LNGFR* promoter. Transient cotransfection assays were carried out with untreated and RA- and dBcAMP-treated (RA + dBcAMP) PCC7 cells, using 5 μg of different reporter plasmids as indicated and 8 μg of parental plasmid pRcCMV (control) or an ME1a expression plasmid (+ME1a). CAT activities are expressed relative to the value obtained by transfection of the reporter plasmid with the parental plasmid pRcCMV. Values represent the averages of three different experiments. Standard deviations were less than 15% of the average values of all experiments.

The human *p75LNGFR* promoter is also specifically repressed by ME1a. Since the E-box motif is conserved in both the rat and human *p75LNGFR* proximal promoter regions, we investigated the effects of ME1a on human *p75LNGFR* pro-

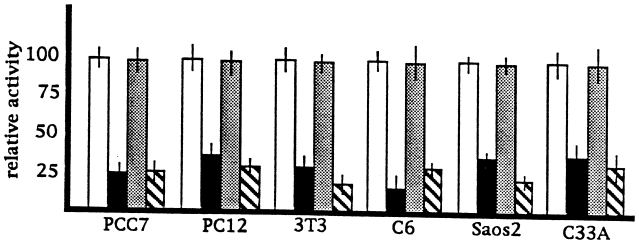

FIG. 4. ME1a suppresses both rat and human *p75LNGFR* promoter activities in different cell lines. Plasmid 0.4 CAT (rat p75LNGFR) or 1.2 kb AvaII p75 LNGFR-CAT (human p75LNGFR) was transfected together with either 8 μg of parental plasmid pRcCMV or 8 μg of an ME1a expression plasmid into mouse teratocarcinoma PCC7, rat pheocromocytoma PC12, mouse fibroblast 3T3, rat glioblastoma C6, human osteosarcoma Saos2, and human cervical carcinoma C33A cells. Rat *p75LNGFR* promoter activity in the absence or presence of ME1a is indicated by open or filled bars, respectively, and human *p75LNGFR* promoter activity in the absence or presence of ME1a is indicated by dotted or hatched bars, respectively. CAT activities are corrected for transfection efficiency and expressed relative to the value obtained by transfection of the rat or human *p75LNGFR* promoter reporter plasmid and expression plasmid pRcCMV. CAT activities are presented as means ± standard deviations and represent results of at least three independent experiments.

moter activity. The human *p75LNGFR* promoter reporter construct contains 1.2 kb of upstream regulatory region and is comparable to the rat 1.4 CAT construct. As shown in Fig. 4, cotransfection of the ME1a expression plasmid with the human p75LNGFR-CAT reporter plasmid resulted in a four- to fivefold decrease in promoter activity. To test whether the repression of *p75LNGFR* promoter activity by ME1a is cell type specific, we performed transient CAT assays in various cell lines, such as rat pheochromocytoma PC12, mouse fibroblast 3T3, rat glioma C6, human osteosarcoma Saos2, and human cervical carcinoma C33A. Similar levels of ME1a-mediated repression of both the human and rat *p75LNGFR* promoters were obtained in all tested cell lines (Fig. 4). This functional similarity between the rat and human *p75LNGFR* promoter E boxes reflects the conserved nature of ME1a–*p75 LNGFR* E-box interactions and suggests a potential regulatory role for the regulation of *p75LNGFR* gene expression.

Functionally dual role of the E box in the *p75LNGFR* promoter. To evaluate the contribution of the E box for overall *p75 LNGFR* promoter activity, we generated several p75LNGFR-CAT reporter constructs in which the E box and its flanking nucleotides were deleted (Fig. 5a). In the mut1 construct, the whole E-box sequence has been deleted; in the mut2 construct, only the 5′ half site of the E box has been deleted; in the mut3 construct, the whole E box as well as the 5′ and 3′ flanking sequences, including the Sp1 binding site located adjacent to the major transcription start site, have been deleted. The CAT activity of each construct was assessed in undifferentiated PCC7 cells and in PCC7 cells treated with RA and dBcAMP in the presence or absence of ME1a (Fig. 5b). Each of the mutations resulted in a significantly lower (about fourfold) *p75 LNGFR* promoter activity compared with the wild type (Fig. 5b). Thus, the E box defines a functionally indispensable part of the *p75LNGFR* promoter that is needed for positive regulation of the *p75LNGFR* gene. Cotransfections with CMV-ME1a did not affect the CAT activity levels expressed by any of the mutants (Fig. 5b), indicating that the integrity of the E-box region is essential for the ME1a-mediated suppression of *p75 LNGFR* promoter activity. Taken together, these data imply that the E box has a dual role, since it can function either as an enhancer or as a silencer, depending on which transcription factors occupy this site.

To identify the domains of ME1a protein necessary for *p75 LNGFR* E-box-mediated repression, we created a set of ME1a amino-terminal deletions that were expressed by using the eukaryotic expression vector pRcCMV (Fig. 6a). Progressive N-terminal deletions showed that removal of either the leucine zipper motif (Δ-259), or the leucine heptad repeat motif (Δ-488) did not affect the degree of inhibition mediated by ME1a (Fig. 6b). Further deletion of the N-terminal region close to the basic domain (Δ-561) did not eliminate the transcriptional repression mediated by ME1a. These results suggest that both the dimerization and DNA-binding functions of ME1a through the bHLH domain appear to be both necessary and sufficient to mediate the repression of *p75LNGFR* promoter activity.

ME1a binds avidly to the *p75LNGFR* proximal promoter E box. To investigate the mechanism underlying the repression of *p75LNGFR* promoter activity by ME1a, we initially determined ME1a DNA-binding characteristics by EMSA, using an oligonucleotide with a *p75LNGFR* E-box sequence. Recombinant ME1a and ME2 proteins were isolated as described elsewhere (10). As depicted in Fig. 7A, ME1a protein produced a sequence-specific retarded complex with the *p75LNGFR* E-box-containing oligonucleotide, whereas no interaction of ME2 was detected with the same oligonucleotide (Fig. 7A). However, both of these proteins form DNA-protein complexes with the MEF-1 E-box-containing oligonucleotide (Fig. 7A), as expected from results of previous studies (10). Hence, this difference in DNA-binding activity between ME1a and ME2 proteins may explain the difference in their abilities to modulate the activity of the *p75LNGFR* promoter.

To evaluate the dissociation rate of the ME1a homodimer–*p75LNGFR* E-box complex, a large-scale binding reaction was

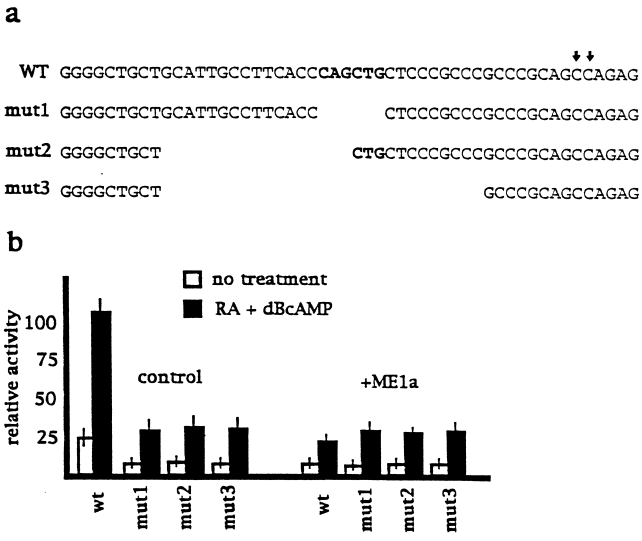

FIG. 5. Effects of E-box deletions on *p75LNGFR* promoter activity. (a) Nucleotide sequences of wild-type (WT) and mutant (mut1, mut2, and mut3) rat *p75LNGFR* proximal promoters. The transcriptional start sites are indicated by arrows, and the E-box motif is in boldface letters. Each mutated *p75LNGFR* promoter region was cloned into a reporter plasmid and designated mut1, mut2, and mut3. (b) Importance of the E box for *p75LNGFR* promoter activity. The wild-type 0.4 CAT construct (wt) or mutated reporter plasmids (mut1, mut2, and mut3) were cotransfected with either the parental plasmid pRcCMV (control) or the ME1a expression plasmid (+ME1a) into PCC7 cells. The medium was changed 12 to 18 h after transfection, and the cells were either untreated (control) or treated with RA (0.5 μM) and dBcAMP (1 mM) as indicated. CAT activities are corrected for transfection efficiency and expressed relative to the value obtained by transfection of the reporter plasmid and parental plasmid pRcCMV. CAT activities are presented as means ± standard deviations and represent results of at least three independent experiments.

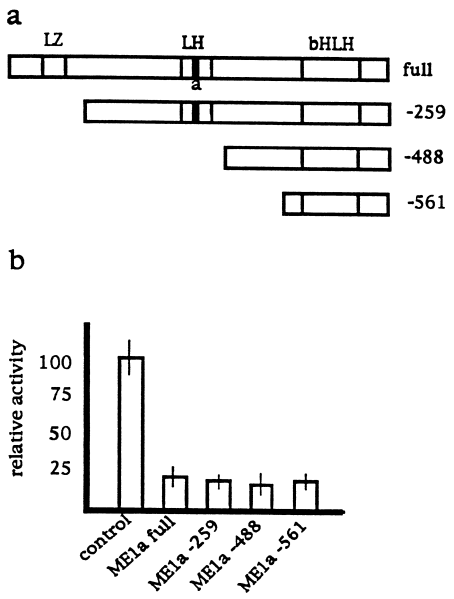

FIG. 6. Effects of ME1a amino-terminal deletion mutants on *p75LNGFR* promoter activity. (a) Schematic representation of the full-length ME1a protein and the different amino-terminal deletion mutants. The major structural-functional regions are indicated: leucine zipper (LZ), loop-helix (LH), and bHLH. The ME1a miniexon is represented by a black box. The designations at the right indicate the number of N-terminal amino acids that have been deleted. (b) Repression by ME1a is mediated through the bHLH motif. The rat 0.4 CAT reporter plasmid was cotransfected with either 8 μg of full-length ME1a expression vector (ME1a full) or 8 μg of each ME1a amino-terminal mutant (ME1a -259, ME1a -488, and ME1a -561) expression plasmid into PCC7 cells. CAT activities are corrected for transfection efficiency and expressed relative to the value obtained by transfection of the *p75LNGFR* promoter reporter plasmid and the parental expression plasmid pRcCMV (control). CAT activities are presented as means ± standard deviations and represent results of at least three independent experiments.

prepared with ME1a protein, labeled oligonucleotide carrying the *p75LNGFR* E box, and a 200-fold excess of competing unlabeled *p75LNGFR* E-box oligonucleotide. A time course of ME1a dissociation was analyzed by EMSA. After 30 min of incubation, approximately 75% of the ME1a–*p75LNGFR* E-box complex remained intact (Fig. 7B), whereas 50% of the ME1a–MEF-1 complex was dissociated after 10 min of incubation and DNA-bound ME1a was not detected after 30 min of incubation (Fig. 7C). Consequently, the dissociation rate of the ME1a–*p75LNGFR* E-box complex is significantly slower than that of the ME1a–MEF-1 complex. Thus, the slow dissociation rate of the ME1a–*p75LNGFR* E-box complex combined with the data from the ME1a amino-terminal deletion analysis suggests that ME1a occupies the *p75LNGFR* E box and may not interact with the basal transcriptional machinery to suppress promoter activity.

Overexpression of Id2 protein releases the repression of *p75 LNGFR* promoter activity mediated by ME1a. *trans*-dominant HLH negative regulators, i.e., Id-like proteins, often modulate the DNA-binding abilities of bHLH proteins by forming nonfunctional heterodimers and therefore regulate their transcriptional activities (6, 56). Previously, we have shown by EMSA analysis that Id2 interacts with ME1a and suppresses its transcriptional activity (10). Therefore, we determined whether the Id2 protein could affect the ability of ME1a to bind to the *p75 LNGFR* E-box element. For these purposes, EMSA analyses were performed with different amounts of recombinant Id2 protein in the presence of a constant amount of ME1a protein (Fig. 8A). The formation of an ME1a–*p75LNGFR* E-box complex was clearly hindered by Id2 protein in a dose-dependent manner. These in vitro data suggest that Id2 may stimulate *p75 LNGFR* promoter activity by forming nonfunctional heterodimers with ME1a protein.

To explore the possibility that Id2 protein releases the ME1a-mediated repression of *p75LNGFR* promoter activity in vivo, increasing amounts (0 to 12 μg) of the expression plasmid pRcCMVId2 were cotransfected with a constant amount (8 μg) of ME1a expression plasmid into PCC7 cells. As shown in Fig. 8B, in the presence of 8 μg of ME1a expression plasmid, 12 μg of the Id2 expression plasmid restored full *p75LNGFR* promoter activity. However, expression of Id2 alone did not affect the level of *p75LNGFR* promoter activity (Fig. 8B). These functional data support the in vitro DNA binding results and clearly demonstrate that Id2 releases the repression mediated by ME1a and consequently positively regulates *p75*

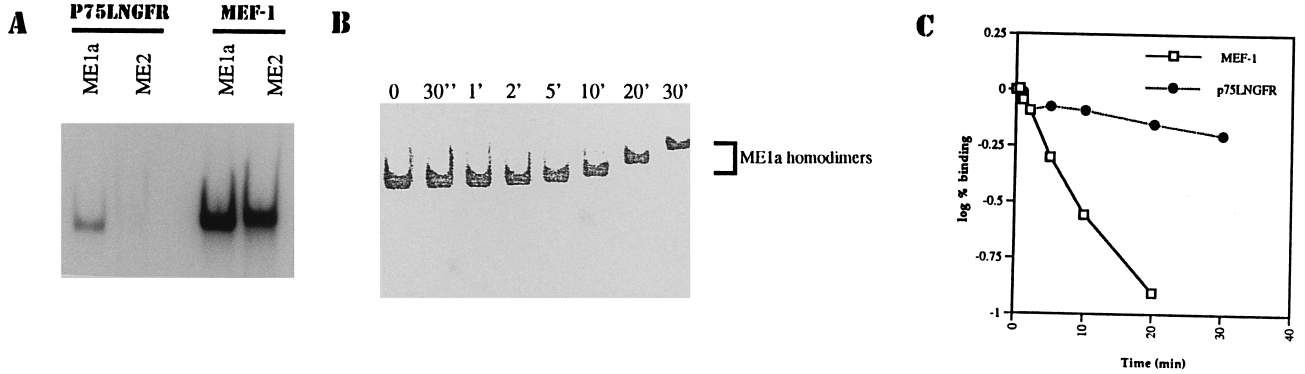

FIG. 7. Binding of ME1a to the *p75LNGFR* E-box region. (A) ME1a binds to the *p75LNGFR* E box. EMSAs were performed with a radiolabeled oligonucleotide which contained either the *p75LNGFR* E box or the MEF-1 site. The radiolabeled DNA (p75LNGFR or MEF-1) was incubated in binding buffer with the purified recombinant ME1a and ME2 proteins as indicated above the lanes. The DNA-protein complexes were resolved by native polyacrylamide gel electrophoresis and detected by autoradiography. (B) Off-rate determination of the ME1a–*p75LNGFR* E-box complex. The recombinant ME1a protein was incubated with 40 fmol of *p75 LNGFR* E-box probe for 20 min, 200× unlabeled specific competitor (*p75LNGFR* E box) was added, and the mixture was incubated for the indicated times and immediately loaded on a 5% native gel. The gel was run on 0.5× Tris-borate-EDTA, dried, and quantified by PhosphorImager analysis. The bound ME1a homodimers are indicated by a bracket on the right. (C) Dissociation rate analysis of ME1a homodimers, using different E boxes, by EMSA. The off-rate of ME1a homodimers was examined with the *p75LNGFR* and MEF-1 E boxes. ME1a was incubated with 40 fmol of either *p75LNGFR* E box or MEF-1 site for 20 min and further incubated in presence of unlabeled specific competitor (*p75LNGFR* E box or MEF-1 site, respectively, for different periods of time (0, 0.5, 1, 2, 5, 10, 20, and 30 min). The DNA-protein complexes were analyzed by native polyacrylamide gel electrophoresis. The data were quantified by PhosphorImager analysis.

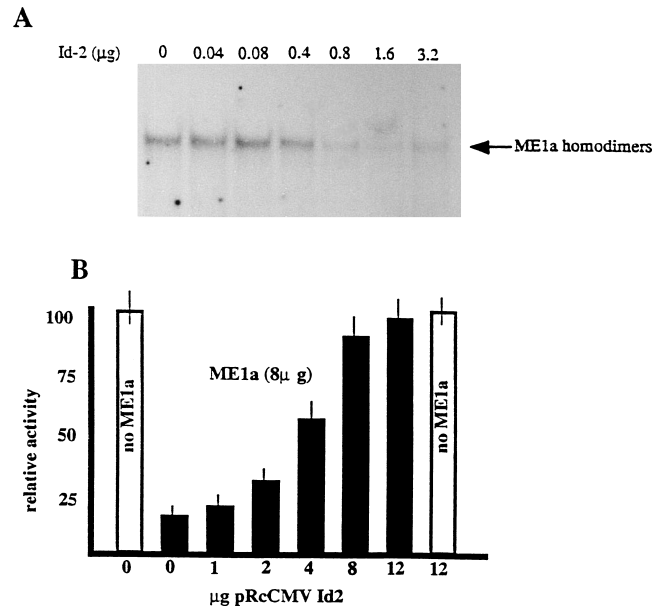

FIG. 8. Id2 impairs the binding of ME1a to the *p75LNGFR* E box and blocks the inhibitory effect of ME1a on *p75LNGFR* promoter activity. (A) ME1a forms non-DNA-binding heterodimers with Id2 protein. The ME1a and Id2 interactions were analyzed by EMSA using recombinant ME1a and Id2 proteins. Forty nanograms of ME1a protein was incubated with different amounts of Id2 as indicated above the lanes. After 15 min of incubation at room temperature, the radiolabeled *p75LNGFR* E box was added, and the mixture was incubated for additional 15 min. The ME1a–*p75LNGFR* E-box complexes were analyzed by 5% native polyacrylamide gel electrophoresis. The bound ME1a homodimers are indicated by an arrow. (B) The transcriptional regulator Id2 releases ME1a-mediated repression but does not affect the level of *p75LNGFR* expression. The rat 0.4 CAT reporter plasmid was cotransfected either with the parental plasmid (no ME1a), with 12 μg of Id2 expression plasmid, or with a constant amount (8 μg) of full-length ME1a expression plasmid (ME1a) and various amounts of Id2 expression plasmid (pRcCMVId2) into PCC7 cells. CAT activities are corrected for transfection efficiency and expressed relative to the value obtained by transfection of the *p75LNGFR* reporter plasmid and the parental expression plasmid pRcCMV (control). Relative CAT activities are presented as means ± standard deviations and represent results of at least three independent experiments.

LNGFR promoter activity. In addition, these data imply that the endogenous E-box-binding proteins responsible for transcriptional activation of the *p75LNGFR* promoter are not affected by the transcriptional regulator Id2.

ME1a affects endogenous expression of the *p75LNGFR* gene. To ascertain that the ME1-mediated repression of *p75 LNGFR* promoter activity is not an artifact of the transient CAT assay, we generated PC12 cell lines that overexpress ME1a and analyzed the expression of p75LNGFR in these cell lines. If high expression of ME1a is necessary to suppress *p75 LNGFR* promoter activity, then artificially elevating the ME1a concentration should alter endogenous expression of the *p75 LNGFR* gene. Northern (RNA) blot analyses revealed that control PC12 cells expressed high levels of *p75LNGFR* mRNA, whereas ME1 mRNA was barely detectable (Fig. 9). In contrast, expression of *p75LNGFR* mRNA was significantly reduced in ME1a-overexpressing PC12 cells (Fig. 9). The level of ME1a mRNA in overexpressing clones was comparable to the ME1 mRNA level in hippocampal cell line HT4 (data not shown), indicating that overexpression of ME1 was at the physiological level. Although Fig. 9 represents the data obtained from one ME1a-overexpressing clone, identical results were obtained with three additional clones as well as a pool population of ME1a-overexpressing clones (data not shown). NGF treatment of PC12 cells resulted in stimulation of *p75LNGFR* mRNA expression in both control and ME1a-overexpressing cells (Fig. 9). Despite the induction of *p75LNGFR* expression in ME1a-overexpressing cells after NGF treatment, the levels of *p75LNGFR* mRNA remain 10 to 15 times lower than in control PC12 cells. These results support the data obtained in transient CAT assays and indicate that endogenous expression of the *p75LNGFR* gene can be controlled by ME1a in an inverse fashion.

DISCUSSION

The bHLH transcription factors have been implicated in the events of determination, differentiation, and morphogenesis of several organ systems, including the nervous system. They act as transcription factors which modulate the expression of neuronal target genes (21, 33, 36, 43). However, most specific target genes remain unknown. On the basis of their expression patterns and interactions with other HLH proteins, bHLH transcription factors have been divided into two classes, A and B. Class A bHLH proteins such as daughterless, E12, E47, ME1, and ME2 form homodimers as well as heterodimers (43) and are expressed in a wide variety of tissues, although their expression becomes more cell type specific as development progresses (23, 44). Class B bHLH transcription factors, which include MyoD, members of the *achaete-scute* complex, MASH-1, MASH-2, NSCL-1, and NSCL-2, generally form heterodimers with class A proteins. They also exhibit a more restricted spatiotemporal expression pattern than class A transcription factors (40).

In this study, we have identified a target gene, *p75LNGFR*, for the HLH transcription factors ME1 and Id2. The *p75 LNGFR* gene has a complex expression pattern during development (7, 26, 31, 53). However, the *cis*-acting elements responsible for its specific spatial and temporal expression have still not been identified. In this study, we have demonstrated that the conserved E boxes of the rat and human *p75LNGFR* promoters significantly contribute to *p75LNGFR* gene expression, since disrupting the E-box element resulted in a 75% decrease in promoter activity. The enhancer activity of the E box is probably due to the binding of specific endogenous bHLH proteins. Furthermore, when the activity of this E box was challenged with the expression of various bHLH transcription factors, we observed a strong repression of *p75LNGFR* promoter activity, which was specific to the bHLH protein ME1, the murine homolog of the human HEB protein, whereas the

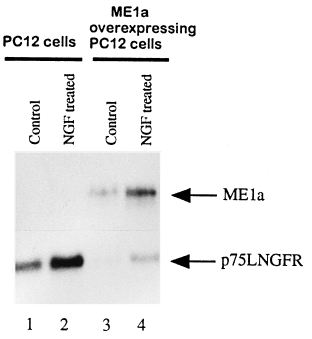

FIG. 9. Overexpression of ME1a downregulates *p75LNGFR* gene expression in PC12 cells. Total RNA was isolated from untreated and NGF-treated PC12 and ME1a-overexpressing PC12 cells. Northern blot analysis was carried out as described in Materials and Methods with two different probes, ME1a and p75 LNGFR. Equal loading of RNA was checked by staining the membrane with methylene blue. Positions of ME1a mRNA and *p75LNGFR* mRNA are indicated by arrows at the right.

related bHLH transcription factors E12 and ME2 (murine homolog of human E2-2) had no effect. This repression is mediated by both forms of ME1 protein, ME1a and ME1b, which differ by the presence of a 72-bp miniexon only in the ME1a form. The negative transcriptional effect of ME1 stands in contrast with previous studies showing that ME1a activates transcription from various heterologous promoters (10, 28). This observation suggests that the promoter context can influence whether ME1 functions as a positive or negative regulator. Interestingly, the repression by ME1 was observed on both the rat and human *p75LNGFR* promoters, suggesting a functional conservation of the E box. The experiments described here indicate that the E box has a dual role in the native context of the *p75LNGFR* promoter: it can function either as an activator or as a repressor, depending on the interacting bHLH proteins. Also, this E box most likely plays a critical role during regulation of rat and human *p75LNGFR* gene expression during development.

Furthermore, in vitro binding assays demonstrate that ME1a protein binds to the *p75LNGFR* E box, whereas the bHLH protein ME2 does not bind or binds significantly more weakly, and affects the promoter activity. Therefore, our results revealed a perfect concordance between the abilities of ME1a to bind to the *p75LNGFR* E box in vitro and to confer repression in vivo. Also, the competition assays indicate a slow dissociation rate of the ME1a–*p75LNGFR* E-box complex, suggesting that when this complex is formed, it remains stable. Therefore, we propose that the activity of the *p75LNGFR* promoter is influenced by the outcome of a protein competition occurring at the E-box site. The bHLH protein ME1a would displace resident bHLH proteins and inactivate the enhancer activity of the *p75LNGFR* E box. Since the amino-terminal region of ME1a is not required for repression of transcription, we suggest that ME1a may repress transcription by binding to DNA as a homodimer and thus blocking transcriptional activation by a lack of interactions with components of the transcriptional machinery. This observation implies that the transcriptional activation domain of ME1a is not likely to form a bridge with TATA-binding protein (TBP) or TBP-associated factors that bind to the *p75LNGFR* promoter region. Thus, it suggests that the *p75LNGFR* E-box-binding proteins have specific regulatory interactions and therefore stimulate *p75LNGFR* promoter activity. Furthermore, the conserved positioning of the E box in the *p75LNGFR* promoter region supports the idea of critical interactions with other transcriptional factors. Consistent with this idea is the fact that other bHLH proteins, such as c-myc and USF, have been shown to interact with key components of the transcriptional machinery (22, 41, 61). Also, in the case of the human immunodeficiency virus type 1 promoter, it is believed that bHLH-ZIP proteins most probably promote the assembly of the TBP–TBP-associated factor complex (48).

The notion that the *p75LNGFR* promoter is a target for ME1-mediated repression is also supported by results of our in vivo experiments. We analyzed the correlation between the ME1 and *p75LNGFR* mRNA levels in control and ME1a-overexpressing PC12 cells. The expression of *p75LNGFR* mRNA is drastically diminished in ME1a-overexpressing PC12 cells, indicating that ME1a acts as a suppressor of *p75LNGFR* gene transcription in vivo. The correlation between increasing ME1a level and decreasing *p75LNGFR* gene expression and the ability of ME1a to strongly bind and repress *p75LNGFR* promoter activity provide evidence that E-box-binding proteins participate in the orchestration of the spatial and temporal expression of the *p75LNGFR* gene during development.

Previous studies demonstrated that the expression of Id-like transcriptional regulators decreases in a variety of cell lines when the cells are induced to differentiate (12, 14, 17). Thus, it has been suggested that Id-like proteins function as negative regulators through their ability to sequester positively acting transcription factors, such as MyoD and MASH-1 (35, 36, 38). To further understand the mechanism of *p75LNGFR* gene expression, we analyzed the effect of Id2 on *p75LNGFR* promoter activity in the presence of ME1a. These experiments demonstrated that Id2 not only alleviated the repression mediated by ME1a in a dose-dependent manner but also completely restored *p75LNGFR* promoter activity. Thus, the results suggest that the endogenous transcription factors interacting with the E box are not affected by the transcriptional regulator Id2. Recently, bHLH transcription factors such as TAL-1 and several other bHLH-ZIP proteins whose activities are not disturbed by Id-like proteins have been identified. The class B bHLH protein TAL-1 forms functionally active heterodimers with E12 in presence of Id-like proteins (27). Class C bHLH proteins TFE3, USF, and AP4, which are characterized by a leucine zipper motif adjacent to the bHLH motif (39, 43), do not form heterodimers with Id-like proteins (56). However, the class C bHLH protein AP4 is not able to modulate the activity of the *p75LNGFR* promoter, as demonstrated by CAT assays. Our results suggest that Id2 can stimulate *p75LNGFR* promoter activity by sequestering a negative bHLH regulator and therefore releasing the repression state. Thus, the Id-like transcriptional regulators can play a dual role: they can function as general inhibitors of the differentiated state by blocking the activity of bHLH proteins required for a specific differentiation program or as positive regulators by sequestering negative regulators. The regulation of *p75 LNGFR* gene expression is an example demonstrating that Id2 positively regulates the expression of a neuronal gene and consequently may promote neuronal differentiation. Consistent with this idea is the fact that some neuronally differentiated cells, like the Purkinje cells in the cerebellum, exhibit a high level of Id2 expression during development and throughout adulthood (45). In addition, it has been shown recently that expression of Id1 is induced during neuronal differentiation of PC12 cells (15). While Id2, ME1a, and p75LNGFR do not have identical expression patterns, there is a striking overlap in their spatial and temporal patterns of expression during neurogenesis (14, 31, 44, 45, 53). For example, in developing cerebellum, p75LNGFR is expressed in external granule cells and Purkinje cells but is absent in internal granule cells (53). ME1 is expressed at a high level in both external and internal granule cells but at a lower level in Purkinje cells (44). Id2 is present in external granule cells as well as in Purkinje cells (45). These expression patterns indicate that p75LNGFR is expressed only in cells in which ME1 is coexpressed with Id2, suggesting that Id2 suppresses the negative transcriptional effect mediated by ME1. In contrast, when ME1 is expressed in the absence of Id2, there is no p75LNGFR expression. Furthermore, during the early stages of the central nervous system development, the expressions of p75LNGFR and ME1 are mutually exclusive, p75LNGFR being expressed in the mantle layer (7, 26) but not in the germinal layer, where ME1 is highly expressed (44). Taken together, these expression patterns further strengthen the conclusion drawn from the studies presented in this report that the level of ME1 is critical for the expression of p75LNGFR and that Id2 releases the ME1-mediated transcriptional suppression of p75LNGFR. Thus, the dual role of HLH transcription factors may be crucial in modulating the cascade of transition events involved in promoting the neural cell fate.

The analysis of *p75LNGFR* gene expression described in this study has provided novel insight into the mechanisms of reg-

ulation of cell-specific genes. The data presented here demonstrate the importance of the conserved E box in the modulation of *p75LNGFR* gene expression as well as the suppressive effect of ME1 on *p75LNGFR* gene expression. There is precedent in other systems for negative regulation of promoter activity by the binding of bHLH proteins. In both the embryonic segmentation and adult peripheral nervous system of *Drosophila* cells, the bHLH protein hairy acts as a negative regulator of the proneural gene *achaete* by directly binding to DNA (59). The mouse bHLH protein HES-1, which is expressed in proliferating neuronal cells and shows structural homology to hairy, acts as a negative regulator by directly binding to DNA (57). However, these bHLH proteins do not function as positive regulators like ME1a. Another paradigm is the negative regulation of the c-*fos* promoter by the myogenic bHLH protein MyoD; in this case, the binding of MyoD interferes with the signal transduction components responsible for c-*fos* activation (58). In contrast, ME1 may interfere with positive endogenous regulators which are likely to belong to the bHLH family. To further understand the regulation of the *p75LNGFR* promoter, it is critical to identify and characterize the proteins which control promoter activity.

ACKNOWLEDGMENTS

We thank H. Weintraub, M. Chao, C. Begley, R. Tjian, and F. Franco del Amo for gifts of plasmids. We thank Marcy Haire for photographic work.

This work was supported by grants from the Spinal Cord Society, NIH (NS33804), Swedish Cancer Society (grants 3473-B94-03XAB and 3474-B95-03XBB), and Swedish Research Council for Engineering Sciences (grant 230, 94-645); by funds from the Karolinska Institute program of collaboration with Baltic countries; and by an International Research Scholar's Award from the Howard Hughes Medical Institute (grant HHMI 75195-541201 to M.M. and K.P.).

REFERENCES

1. **Bain, G., S. Gruenwald, and C. Murre.** 1993. E2A and E-2 are subunits of B-cell specific E2-box DNA-binding proteins. Mol. Cell. Biol. **13:**3522–3529.
2. **Barbacid, M.** 1994. The Trk family of neurotrophin receptors. J. Neurobiol. **25:**1386–1403.
3. **Barrett, G. L., and P. F. Bartlett.** 1994. The p75LNGFR nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. Proc. Natl. Acad. Sci. USA **91:**6501–6505.
4. **Begley, G. C., S. Lipkowitz, K. Göbel, K. A. Mahon, V. Bertness, A. R. Green, N. M. Gouch, and I. Kirsch.** 1992. Molecular characterization of NSCL, a gene encoding a helix-loop-helix protein expressed in the developing nervous system. Proc. Natl. Acad. Sci. USA **89:**38–42.
5. **Benedetti, M., A. Levi, M. Chao.** 1993. Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc. Natl. Acad. Sci. USA **90:**7859–7863.
6. **Benezra, R., D. L. Davis, D. L. Lockson, D. L. Turner, and H. Weintraub.** 1990. The protein Id; a negative regulator of helix-loop-helix proteins. Cell **61:**49–59.
7. **Buck, C. R., H. J. Martinez, I. B. Black, and M. V. Chao.** 1987. Developmentally regulated expression of the nerve growth factor receptor gene in the periphery and brin. Proc. Natl. Acad. Sci. USA **84:**3060–3063.
8. **Cavicchioli, L., T. P. Flanigan, G. Vantini, M. Fusco, P. Polato, G. Toffano, F. S. Walsh, and A. Leon.** 1989. NGF amplifies expression of NGF receptor messenger RNA in forebrain cholinergic neurons of rat. Eur. J. Neurosci. **1:**258–262.
9. **Chao, M.** 1994. The p75 neurotrophin receptor. J. Neurobiol. **25:**1373–1385.
10. **Chiaramello, A., A. Soosaar, T. Neuman, and M. X. Zuber.** 1995. Differential expression and distinct DNA-binding specificity of ME1a and ME2 suggest a unique role during differentiation and neuronal plasticity. Mol. Brain Res. **29:**107–118.
11. **Chomczynski, and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
12. **Christy, B. A., L. K. Sanders, L. F. Lau, N. G. Copeland, N. A. Jenkins, and D. Nathans.** 1991. An Id-related helix-loop-helix protein encoded by a growth factor-inducible gene. Proc. Natl. Acad. Sci. USA **88:**1815–1819.
13. **Doherty, P., P. Seaton, T. P. Flanigan, and F. S. Walsh.** 1988. Factors controlling the expression of the NGF receptor in PC12 cells. Neurosci. Lett. **92:**222–227.
14. **Duncan, M., E. M. DiCicco-Bloom, X. Xhiang, R. Benezra, and K. Chada.** 1992. The gene for the helix-loop-helix protein, Id, is specifically expressed in neuronal precursors. Dev. Biol. **154:**1–10.
15. **Einarson, M. B., and M. V. Chao.** 1995. Regulation of Id1 and its association with basic helix-loop-helix proteins during nerve growth factor-induced differentiation of PC12 cells. Mol. Cell. Biol. **15:**4175–4183.
16. **Ellis, H. M., D. R. Spann, and J. W. Posakony.** 1990. Extramachrochaetae, a negative regulator of sensory organ development in *Drosophila*, defines a new class of helix-loop-helix proteins. Cell **61:**27–38.
17. **Evans, S. M., and T. X. O'Brien.** 1993. Expression of the helix-loop-helix factor Id during mouse embryonic development. Dev. Biol. **159:**485–499.
18. **Ferré-D Amaré, A. R., G. C. Prendergast, E. B. Ziff, and S. K. Burley.** 1993. Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. Nature (London) **356:**38–45.
19. **Franco del Amo, F., M. Gendron-Maguire, P. J. Swiatek, and T. Gridley.** 1993. Cloning, sequencing and expression of the mouse mammalian achaete-scute homolog 1 (MASH1). Biochim. Biophys. Acta **1171:**323–327.
20. **Green, L. A., and A. S. Tischler.** 1976. Establishment of noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA **73:**2424–2428.
21. **Guillemot, F., L.-C., Lo, J. E. Johson, A. Auerbach, D. J. Anderson, and A. L. Joyner.** 1993. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell **75:**463–476.
22. **Hateboer, G., H. T. M. Timmers, A. K. Rustgi, M. Billaud, L. J. van't Veer, and R. Bernards.** 1993. TATA-binding protein and the retinoblastoma gene product bind to overlapping epitopes on c-Myc and adenovirus E1A protein. Proc. Natl. Acad. Sci. USA **90:**8489–8493.
23. **Helms, J. A., S. Kuratini, and G. D. Maxwell.** 1995. Cloning and analysis of a new developmentally regulated member of the basic helix-loop-helix family. Mech. Dev. **48:**93–108.
24. **Hempstead, B. L., D. Martin-Zanca, and D. R. Kaplan.** 1991. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature (London) **350:**678–683.
25. **Henthorn, P., M. Kiledjian, and T. Kadesch.** 1990. Two distinct transcription factors that bind the immunoglobulin enhancer E5/kE2 motif. Science **247:**467–470.
26. **Heuer, J. G., C. S. von Bartheld, Y. Kinoshita, P. C. Evers, and M. Bothwell.** 1990. Alternating phases of FGF receptor and NGF receptor expression in the developing chicken nervous system. Neuron **5:**283–296.
27. **Hsu, H.-L., I. Wadman, J. T. Tsan, and R. Baer.** 1994. Positive and negative transcriptional control by the TAL1 helix-loop-helix protein. Proc. Natl. Acad. Sci. USA **91:**5947–5951.
28. **Hu, J.-S., E. N. Olson, and R. E. Kingston.** 1992. HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. Mol. Cell. Biol. **12:**1031–1042.
29. **Hu, Y.-F., B. Luscher, A. Admon, N. Mermod, and R. Tjian.** 1990. Transcription factor AP-4 contains multiple dimerization domains that regulate dimer specificity. Genes Dev. **4:**1741–1752.
30. **Huber, L. J., and M. Chao.** 1995. A potential interaction of p75LNGFR and trkA NGF receptors revealed by affinity crosslinking and immunoprecipitation. J. Neurosci. Res. **40:**557–563.
31. **Huber, L. J., and M. V. Chao.** 1995. Mesenchymal and neuronal cell expression of the p75LNGFR neurotrophin receptor gene occur by different mechanism. Dev. Biol. **167:**227–238.
32. **Ip, N. Y., T. N. Stitt, P. Tapley, R. Klein, D. J. Glass, J. Fandl, L. A. Greene, M. Barbacid, and G. D. Yancopoulos.** 1993. Similarities and differences in the way neurotrophins interact with the trk receptors in neuronal and non-neuronal cells. Neuron **10:**137–149.
33. **Jan, Y. N., and L. Y. Jan.** 1993. HLH proteins, fly neurogenesis, and vertebrate myogenesis. Cell **75:**827–830.
34. **Jing, S., P. Tapley, and M. Barbacid.** 1992. Nerve growth factor mediates signal transduction through trk homodimers receptors. Neuron **9:**1067–1079.
35. **Johnson, J. E., S. J. Birren, and D. J. Anderson.** 1990. Two rat homologues of Drosophila achate-scute specifically expressed in neuronal precursors. Nature (London) **194:**495–496.
36. **Johnson, J. E., S. J. Birren, T. Saito, and D. J. Anderson.** 1992. DNA binding and transcriptional regulatory activity of mammalian achaete-scute homologous (MASH) proteins revealed by interaction with a muscle-specific enhancer. Dev. Biol. **89:**3596–3600.
37. **Klein, E. S., D. M. Simmons, L. W. Swanson, and M. G. Rosenfeld.** 1993. Tissue-specific RNA splicing generates an ankyrin-like domain that affects the dimerization and DNA-binding properties of a bHLH protein. Genes Dev. **7:**55–71.
38. **Kreider, B. L., R. Benezra, G. Rover, and T. Kadesch.** 1992. Inhibition of myeloid differentiation by the helix-loop-helix protein Id. Science **255:**1700–1702.
39. **Landschultz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240:**1759–1764.
40. **Lassar, A. B., R. L. Davis, W. E. Wright, T. Kadesch, C. Murre, A. Voronova,**

D. Baltimore, and H. Weintraub. 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins *in vivo*. Cell **66:**305–315.
41. **Meisterernst, M., M. Horikoshi, and R. G. Roeder.** 1990. Recombinant yeast TFIID, a general transcription factor, mediates activation by the gene-specific factor USF in a chromatin assembly assay. Proc. Natl. Acad. Sci. USA **87:**9153–9157.
42. **Metsis, M., T. Timmusk, R. Allikmets, M. Saarma, and H. Persson.** 1992. Regulatory elements and transcriptional regulation by testosterone and retinoic acid of the rat nerve growth factor receptor promoter. Gene **121:**247–254.
43. **Murre, C., P. S. McCaw, H. Vassin, M. Caudy, L. Y. Jan, Y. N. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lassar, H. Weintraub, and D. Baltimore.** 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58:**537–544.
44. **Neuman, T., A. Keen, E. Knapick, D. Shain, M. Ross, H. O. Nornes, and M. X. Zuber.** 1993. ME1 and GE1: basic helix-loop-helix transcription factors expressed at high levels in the developing nervous system and in morphogenetically active regions. Eur. J. Neurosci. **5:**311–318.
45. **Neuman, T., A. Keen, M. X. Zuber, G. I. Kristjansson, P. Gruss, and H. O. Nornes.** 1993. Neuronal expression of regulatory helix-loop-helix factor Id2 gene in mouse. Dev. Biol. **160:**186–195.
46. **Neuman, T., M. Metsis, H. Persson, and P. Gruss.** 1993. Cell type-specific negative regulatory element in low-affinity nerve growth factor receptor gene. Mol. Brain Res. **20:**199–208.
47. **Nielsen, A. L., N. Pallisgaard, F. S. Pedersen, and P. Jørgensen.** 1992. Murine helix-loop-helix transcriptional activator proteins binding to the E-box motif of the Akv murine leukemia virus enhancer identified by cDNA cloning. Mol. Cell. Biol. **12:**3449–3459.
48. **Ou, S.-H. I., L. F. García-Martínez, E. J. Paulssen, and R. B. Gaynor.** 1994. Role of the flanking E box motifs in human immunodeficiency virus type 1 TATA element function. J. Virol. **68:**7188–7199.
49. **Pfeiffer, S., H. Jakob, K. Mikoshiba, P. Dubois, J. Guenet, J. Nicolas, J. Gaillard, G. Chevance, and F. Jacob.** 1981. Differentiation of a teratocarcinoma cell line: preferential development of cholinergic neurons. J. Cell Biol. **88:**57–66.
50. **Rabizadeh, S., J. Oh, L.-T. Zhong, J. Yang, C. M. Bitler, L. L. Butcher, and D. E. Bredesen.** 1993. Induction of apoptosis by the low affinity NGF receptor. Science **261:**345–348.
51. **Radeke, M. J., T. P. Misko, C. Hsu, L. A. Herzenberg, and E. M. Shooter.** 1987. Gene transfer and molecular cloning of the rat nerve growth factor. Nature (London) **325:**593–597.
52. **Reichmann, V., I. van Crichten, and F. Sablitzky.** 1994. The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. Nucleic Acids Res. **22:**749–755.
53. **Rocamora, N., F. J. Garcia-Ladona, J. M. Palacios, and G. Mengod.** 1993. Differential expression of brain-derived neurotrophic factor, neurotrophin-3, and low-affinity nerve growth factor receptor during the postnatal development of the rat cerebellar system. Mol. Brain Res. **17:**1–8.
54. **Sehgal, A., N. Patil, and M. Chao.** 1988. A constitutive promoter directs expression of the nerve growth factor receptor gene. Mol. Cell. Biol. **8:**3160–3167.
55. **Soosaar, A., A. Chiaramello, M. X. Zuber, and T. Neuman.** 1994. Expression of basic-helix-loop-helix transcription factor ME2 during brain development and in the regions of neuronal plasticity in the adult brain. Mol. Brain Res. **25:**176–180.
56. **Sun, X.-H., N. G. Copeland, N. A. Jenkins, and D. Baltimore.** 1991. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. Mol. Cell. Biol. **11:**5603–5611.
57. **Takebayashi, K., Y. Sasai, Y. Sakai, T. Watanabe, S. Nakanishi, and R. Kageyama.** 1994. Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. J. Biol. Chem. **269:**5150–5156.
58. **Trouche, D., M. Grigoriev, J.-L. Lenormand, P. Robin, S. A. Leibovitch, P. Sassone-Corsi, and A. Harel-Bellan.** 1993. Repression of c-fos promoter by MyoD on muscle cell differentiation. Nature (London) **363:**79–82.
59. **Van Doren, M., A. M. Bailey, J. Esnayra, K. Ede, and J. W. Posakony.** 1994. Negative regulation of proneural gene activity: hairy is adirect transcriptional repressor of achaete. Genes Dev. **8:**2729–2742.
60. **Verge, V. M. K., J. P. Merlio, J. Grondin, P. Ernfors, H. Persson, R. J. Riopelle, T. Hokfelt, and P. M. Richardson.** 1992. Co-localization of nerve growth factor binding sites, trk mRNA, and low affinity nerve growth factor receptor mRNA in primary sensory neurons: responses to injury, and infusion by nerve growth factor. J. Neurosci. **12:**4011–4022.
61. **Workman, J. L., R. G. Roeder, and R. E. Kingston.** 1990. An upstream transcription factor, USF (MLTF), facilitates the formation of preinitiation complexes during *in vitro* chromatin assembly. EMBO J. **9:**1299–1308.