Published in final edited form as:

J Neurosci Res. 2007 January ; 85(1): 1–18. doi:10.1002/jnr.21093.

5'UTR of the Neurogenic bHLH *Nex1/MATH-2/NeuroD6* Gene Is Regulated by Two Distinct Promoters Through CRE and C/EBP Binding Sites

Martine Uittenbogaard¹, Debra L. Martinka¹, Peter F. Johnson³, Charles Vinson⁴, and Anne Chiaramello^{1,2,*}

¹Department of Anatomy and Cell Biology, George Washington University Medical Center, Washington, DC ²Program of Neuroscience, George Washington University Medical Center, Washington, DC ³Laboratory of Protein Dynamics and Signaling, Center for Cancer Research National Cancer Institute-Frederick, Frederick, Maryland ⁴Laboratory of Metabolism, National Cancer Institute, National Institute of Health, Bethesda, Maryland

Abstract

Expression of the bHLH transcription factor *Nex1/MATH-2/NeuroD6*, a member of the *NeuroD* subfamily, parallels overt neuronal differentiation and synaptogenesis during brain development. Our previous studies have shown that *Nex1* is a critical effector of the NGF pathway and promotes neuronal differentiation and survival of PC12 cells in the absence of growth factors. In this study, we investigated the transcriptional regulation of the *Nex1* gene during NGF-induced neuronal differentiation. We found that *Nex1* expression is under the control of two conserved promoters, *Nex1-P1* and *Nex1-P2*, located in two distinct non-coding exons. Both promoters are TATA-less with multiple transcription start sites, and are activated on NGF or cAMP exposure. Luciferase-reporter assays showed that the *Nex1-P2* promoter activity is stronger than the *Nex1-P1* promoter activity, which supports the previously reported differential expression levels of *Nex1* transcripts throughout brain development. Using a combination of DNaseI footprinting, EMSA assays, and site-directed mutagenesis, we identified the essential regulatory elements within the first 2 kb of the *Nex1* 5'UTR. The *Nex1-P1* promoter is mainly regulated by a conserved CRE element, whereas the *Nex1-P2* promoter is under the control of a conserved C/EBP binding site. Overexpression of wild-type C/EBP β resulted in increased *Nex1-P2* promoter activity in NGF-differentiated PC12 cells. The fact that *Nex1* is a target gene of C/EBP β provides new insight into the C/EBP transcriptional cascade known to promote neurogenesis, while repressing gliogenesis.

Keywords

neuronal differentiation; bHLH transcription factor; C/EBP; CREB; *NeuroD* family

The execution of the neurogenic program requires the action of key intrinsic regulators, such as the basic helix-loop-helix (bHLH) transcription factors, to integrate positional information

from extrinsic signals (Bertrand et al., 2002; Ross et al., 2003). To decipher this dynamic interplay, we focused our attention on the neuronal-specific transcription factor Nex1/MATH-2/NeuroD6, as Nex1 is critical to the execution of the nerve growth factor (NGF)-induced differentiation pathway (Uittenbogaard and Chiaramello, 2002). Nex1 behaves as a differentiation factor and belongs to the NeuroD family, which includes NeuroD, NeuroD2/NDRF, and MATH-3/NeuroM. These differentiation factors essentially execute specific neuronal differentiation programs in neuronal-restricted progenitor cells, resulting in cell cycle arrest and acquisition of specific neural identities (Lee et al., 1997).

NeuroD members are expressed in immature and differentiated neurons at different stages of embryonic and postnatal development, with NeuroD and NeuroD2 expression appearing at the early embryonic stage E11, and Nex1 expression being detected only by E14 (Schwab et al., 1998). In the developing neocortex, Nex1 expression is detected in a defined type of progenitor cells located within the subventricular zone, which differentiate into glutamatergic pyramidal neurons associated with upper cortical layers (Wu et al., 2005). Levels of Nex1 expression peak during the first postnatal week, when neurite outgrowth and synaptogenesis are highly active (Bartholoma and Nave, 1994; Shimizu et al., 1995; Schwab et al., 1998). In the adult brain, its expression is linked with higher brain functions, such as learning and memory formation. It is sustained in mature neocortical and hippocampal pyramidal neurons, as well as in mature neurons of the entorhinal cortex, subicular cortex, cingulate gyrus, amygdala, and cerebellum (Schwab et al., 2000). However, beyond its pattern of expression, our knowledge of Nex1 functions has remained limited, as Nex1-null mice studies failed to show any distinct phenotype, due to functional redundancy (Schwab et al., 1998).

Our functional studies showed that constitutive expression of Nex1 induces spontaneous neuritogenesis and neurite regeneration in the absence of trophic factors, a process that is dramatically enhanced in conjunction with cAMP treatment (Uittenbogaard and Chiaramello, 2002, 2004). Our genomic and proteomic analyses indicated that Nex1 endows PC12-Nex1 cells with such properties by favoring a distinct combination of gene products, many of them being key downstream regulators of the NGF pathway and critical building blocks for neuritogenesis and regeneration (Uittenbogaard and Chiaramello, 2003). Most recently, our studies showed that Nex1 displays survival-inducing properties on withdrawal of trophic factors, by modulating the expression of key anti-apoptotic regulators and G1 phase cyclin-dependent kinase inhibitors (Uittenbogaard and Chiaramello, 2005).

Relatively little is known, however, about the genomic structure of the rat *Nex1* gene, except that the Nex1 coding sequence is located in the single coding exon 2, and the minimal Nex1 promoter maps in the non-coding exon 1a (Bartholoma and Nave, 1994). Thus, the main objectives of this study are: 1) to elucidate the Nex1 promoter region; and 2) to carry out a functional analysis of the Nex1 5' untranslated region (UTR) to identify key regulatory elements responsible for transcriptional regulation of the *Nex1* gene during neuronal differentiation. We used the PC12 cell line, as it is a well-established system to tease apart transcriptional mechanisms contributing to neuronal differentiation on NGF signaling. In this study, we show that Nex1 expression is under the control of two discrete TATA-less promoters, Nex1-P1 and Nex1-P2, located in two separate non-coding exons. We found that on NGF or cAMP extrinsic signaling, the CRE and C/EBP binding sites are key regulatory mediators for Nex1-P1 and Nex1-P2 promoters, respectively. We further showed that C/EBP β directly regulates the Nex1-P2 promoter activity during NGF-mediated differentiation.

MATERIALS AND METHODS

Recombinant Plasmids

The eukaryotic expression plasmids pCMV-CREB and pCMV-KCREB were obtained from Clontech (Palo Alto, CA). The eukaryotic expression constructs LIP-C/EBP β and A-C/EBP have been described previously (Krylov et al., 1997; Baer and Johnson, 2000; Greenwel et al., 2000). All the Nex1 footprinting probes (FP1-8) were cloned by PCR using rat genomic DNA (Clontech) as template and gene-specific primers (Suppl. Table I), which were designed based on the available rat genomic sequence from the GenBank contig accession number NW_043752. All forward primers were preceded by a sequence including an EcoRI site, except for the FP-6 forward primer, which included a PstI site. All the reverse primers were designed to include a HindIII site for cloning purposes. The PCR reactions were carried out using either High Fidelity Master Mix (Roche Molecular Biochemicals, Nutley, NJ) or AccuPrime SuperMix II (Invitrogen, Carlsbad, CA). The amplified DNA fragments were unidirectionally cloned into the pBluescript-SK vector (Stratagene, La Jolla, CA), and the resulting clones were named pBS/Nex1FP1-8. Nucleotide sequences of the cloned DNA fragments were confirmed by sequencing (Veritas Inc., Rockville, MD).

For promoter reporter assay, a series of Nex1 fragments were cloned into the pGL3-basic vector (Promega Inc., Madison, WI) by PCR using the AccuPrime SuperMix II (Invitrogen). The Nex1 genomic fragment containing both Nex1-P2 and Nex1-P1 promoters was amplified using rat genomic DNA as a template, the MluI-containing forward primer pGL3-ANex1P2ForMluI, and the XhoI-containing reverse primer pGL3-Nex1P1minRevXhoI (Suppl. Table I). The 2.027 kb-long Nex1 P2-P1 PCR fragment was unidirectionally cloned into the MluI and XhoI restriction sites of pGL3-basic to generate the pGL3/Nex1 P2-P1 construct. The pGL3/Nex1 HincII-P1 construct, which contains the first 553 bp of the Nex1-P1 promoter region, was obtained by cloning the 553 bp-long HincII/XhoI fragment into the SmaI and XhoI sites of pGL3-basic. Sequences of the generated constructs were verified (Veritas Inc.).

The Nex1-P2 promoter A-D region was cloned by PCR using AccuPrime SuperMix II (Invitrogen), rat genomic DNA as a template, and the set of forward (pGL3-ANex1P2ForMluI) and reverse (pGL3-DNex1P2RevXhoI) primers (Suppl. Table I). The 770 bp-long Nex1-P2 (A-D) fragment was cloned into the MluI and XhoI sites of pGL3-basic to generate the pGL3/Nex1-P2(A-D) construct. Progressive 5' and 3' Nex1 P2 deletion mutants were made by PCR using either pGL3/Nex1-P2(A-D) plasmid or rat genomic DNA as template and specific sets of MluI-containing forward and XhoI-containing reverse primers (Suppl. Table I). The resulting constructs were named according to the generated deletion, such as P2(A2-D), P2(A3-D), P2(A-C), P2(A-C2), P2(A-C3), P2(B-D), P2(B2-D), and P2(B-C) with the A, B, C, and D positions indicated on Figure 8. These deletions were confirmed by sequencing (Veritas, Inc.).

Site-Directed Mutagenesis

PCR-based site-directed mutagenesis was carried out using the GeneTailor system (Invitrogen), according to the manufacturer's recommendations. The CRE, E4, and E1 E-box regulatory elements were mutated by using 125 ng of methylated pGL3/Nex1-P1min construct as template, specific sets of forward (mutagenic) and reverse (complementary) primers (Suppl. Table I), and the Platinum *Taq* DNA Polymerase High Fidelity enzyme (Invitrogen). The cycling parameters were as follows: 94°C denaturation for 2 min (1 cycle); 94°C denaturation for 30 sec, 55°C annealing for 30 sec, and 68°C extension for 6 min (20 cycles); 68°C final extension for 10 min (1 cycle). All mutations were confirmed by sequencing (Veritas, Inc.).

Cell Culture and Luciferase Assay

The rat pheochromocytoma PC12 cells (ATCC, Manassas, VA) were grown on collagen I-coated plates (Becton Dickinson Labware, San Jose, CA) and cultured as described (Uittenbogaard and Chiaramello, 2004). Differentiation was carried out in the presence of NGF (2.5S murine, Roche Molecular Biochemicals) or dibutyryl cAMP (dbcAMP; Roche Molecular Biochemicals) as indicated in the figure legends. For luciferase assays, PC12 cells were plated at 70–80% confluency, and then washed the following day with phosphate-buffered saline (PBS) before being resuspended in F12K medium containing 20% FBS. Cells were transfected by electroporation (BTX, Harvard Apparatus, Holliston, MA) with specific promoter-reporter firefly luciferase pGL3 constructs (5 µg) and eukaryotic expression vectors (8 µg), as indicated in the figure legends. As an internal control for transfection normalization, cells were transfected with 500 ng of the thymidine kinase (TK) promoter driven Renilla luciferase construct, pRL-TK (Promega). The total amount of transfected DNA was kept constant by the addition of carrier DNA. Transfected cells were either untreated or treated with NGF (50 ng/ml) or dbcAMP (1 mM) for 3 days, as indicated in the figure legends, and then lysed to carry out a dual Firefly-Renilla luciferase assay (Promega). Triplicate samples were measured for each construct, and each transfection experiment was independently repeated at least three times. Luminescence was measured with an LMax luminometer (Molecular Devices, Sunnyvale, CA) and analyzed with SOFTmax Pro software (Molecular Devices). The relative luciferase activity was defined as the ratio of the light units of Firefly luciferase vs. Renilla luciferase.

RNA Isolation and Primer Extension

DNA-free total RNA was isolated from untreated and NGF-treated PC12 cells using the RNAqueous kit (Ambion, Austin, TX). Poly(A) RNA was prepared using the Poly(A) Purist MAG kit (Ambion) according to the manufacturer's recommendations. Commercially available poly(A) RNAs from mouse E16.5 embryo, mouse adult whole brain, and adult human cerebellum (Ambion) were also used for primer extension analysis. Transcription start sites were mapped by primer extension assay using an oligonucleotide primer complementary to the previously determined human, mouse, and rat genomic sequences (GenBank contig accession numbers NT_007819, NT_039343, and NW_047693, respectively). Oligonucleotides were labeled by kinase treatment using [γ^{32} P] ATP (3,000 ci/mmol; Amersham Biosciences, Piscataway, NJ) and T4 kinase (Invitrogen). Primers (200 fmol) and poly(A) mRNA (1 µg) were denatured and annealed at 55°C for 30 min. First strand cDNA synthesis was carried out using Superscript II reverse transcriptase (Invitrogen) for 50 min at 42°C. The reaction products were analyzed next to a sequencing ladder from the corresponding region of the rat *Nex1* gene obtained with the same primer used for the primer extension procedure (Table I). Reactions were run on a denaturing 6% polyacrylamide gel containing 1× Tris/boric acid/EDTA (45 mM boric acid, and 1 mM EDTA). Gels were dried and analyzed using the PhosphorImager Storm device (Amersham Biosciences).

5'-Rapid Amplification cDNA Ends

Transcription start sites were mapped by 5'-rapid amplification cDNA ends (5'RACE) following the manufacturer's recommendations (Invitrogen). First strand cDNA synthesis was carried out at 42°C for 50 min using 500 ng of poly(A) RNA, Superscript II Reverse Transcriptase (Invitrogen), and the gene-specific primer (GSP) GSP1Nex1P1 (2.5 pmol) as listed in Supplemental Table I. The original mRNA template was degraded using a mixture of RNase H and RNase T1. A homo-polymeric tail was then added to the 3' end of the cDNA by incubating at 37°C for 10 min with the terminal deoxynucleotidyl transferase (TdT) enzyme and dCTP. The dC-tailed cDNA was amplified by PCR using the provided Abridged Anchor primer, the nested GSP2Nex1P1 primer (Suppl. Table I) and Platinum *Taq* Polymerase

(Invitrogen). The cycling parameters were as follows: initial denaturation at 94°C for 2 min; 94°C denaturation for 30 sec, 55°C annealing for 30 sec, and 72°C extension for 2 min (35 cycles); 72°C final extension for 10 min (1 cycle). Reactions were analyzed by 1.5% agarose gel electrophoresis, purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). Purified 5'RACE fragments were cloned using a TOPO-TA strategy into the pCR4-TOPO vector (Invitrogen), and sequenced (Veritas, Inc.).

Nuclear Extract Preparation

Nuclear extracts from untreated and NGF-treated PC12 cells were prepared essentially as described in Chiaramello, et al., 1996. The nuclear lysate was centrifuged at $17,000 \times g$ for 30 min. The supernatant was precipitated with 0.33 g/ml ammonium sulfate, and then dialyzed against 0.1 M buffer containing 50 mM Tris-HCl (pH 7.9), 100 mM KCl, 1 mM EDTA, 12.5 mM MgCl₂, 1 mM dithiothreitol, 20% glycerol, and protease inhibitors.

DNaseI Footprinting Protection Assay

Plasmids from the pBS/FP series containing different regions of the Nex1 promoter region were digested with the BamHI and HindIII restriction enzymes (Roche Molecular Biochemicals), and the purified fragments were end-labeled by kinase treatment using [γ^{32} P] ATP (3,000 ci/mmol; Amersham Biosciences) and T4 kinase (Invitrogen). Once labeled, all the footprinting probes (FP) were digested with the EcoRI enzyme, except the FP6 probe, which was digested with the PstI enzyme, to obtain single end-labeled FP probes. DNaseI protection assays were essentially carried out as described by Nyborg and Dynan (1990). The binding reactions contained 30 μ g of nuclear extracts, 120 fmol FP probe, 2 μ g poly (dI.dC), 25 mM Tris-HCl (pH 7.9), 6.25 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 80 mM KCl, and 10% glycerol in a 50- μ l reaction. Samples were incubated on ice for 10 min, treated with deoxyribonuclease I (DNaseI) enzyme (Invitrogen), purified, and electrophoresed next to the corresponding sequencing reactions on a 6.5% denaturing polyacrylamide gel. Gels were dried and analyzed using a PhosphorImager Storm device.

Electrophoretic Mobility Shift Assay

The sequences of the top strand of oligonucleotides (Sigma-Genosys, The Woodlands, TX) used as probes, specific or mutated competitors are listed in Supplemental Table I. The complementary strand of each oligonucleotide was annealed and the double-stranded probes were labeled by kinase treatment using [γ^{32} P] ATP (3,000 ci/mmol; Amersham Biosciences) and T4 kinase (Invitrogen). EMSA analyses were done as described in (Chiaramello et al., 1996). DNA binding reactions consisted of 7 μ g of nuclear extracts incubated with 40 fmol of labeled oligonucleotide and 1.2 μ g of poly (dI.dC) (Sigma, St. Louis, MO) for 30 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 MgCl₂, and 5% glycerol). For competition experiments, nuclear extracts were incubated with the appropriate probe in the presence of various amounts of excess unlabeled specific and mutated competitors as indicated on the figure. For supershift, the anti-C/EBP β polyclonal antibody (SC-150X; Santa-Cruz Biotechnology Inc., Santa-Cruz, CA) was pre-incubated with NGF-treated PC12 nuclear extracts for 45 min at room temperature before addition of the labeled oligonucleotide. DNA binding reactions were separated on a 5% native gel containing 0.5 $\times$ Tris/boric acid/EDTA and 0.05% NP-40. Gels were dried and analyzed using a PhosphorImager Storm device (Amersham Biosciences).

RESULTS

Structural Features of the 5' and 3' Flanking Regions of the Rat *Nex1* gene

The rat *Nex1* gene is localized on chromosome 4q24 (GenBank no. NW_047693). Preliminary work on regulation of the rat *Nex1* gene identified a minimal promoter region (167 bp) located in the non-coding exon 1a, separated from the translation initiation site by the 1.3 kb-long intron 1a (Bartholoma and Nave, 1994). The entire *Nex1* coding sequence (337 amino acids) is encoded in exon 2 (Fig. 1). The 3' UTR of *Nex1* gene is relatively short (0.8 kb), and contains two conserved closely spaced polyadenylation signals, located 14 bp and 155 bp beyond the stop codon (Fig. 1). Two alternative mRNA transcripts of 2.4 kb and 1.7 kb were reported, suggesting the existence of alternative 5' or 3' UTR processing (Bartholoma and Nave, 1994; Shimizu et al., 1995; Schwab et al., 1998). However, 3'UTR processing can be excluded because the two polyadenylation sites are separated by only 141 bp and therefore cannot generate two transcripts differing by 700 bp (Bartholoma and Nave, 1994). Moreover, the fact that only a single band was detected by PCR using primers mapping within the *Nex1* coding region, infers splicing of non-coding exons located in the 5'UTR (Shimizu et al., 1995).

To test the possibility of alternative promoter usage in the *Nex1* 5'UTR, we carried out computer analyses of the sequenced genomic region, using the GrailEXPv.3.3 (<http://www.compbio.ornl.gov>) and PromH(W) (<http://www.softberry.com/berry>) algorithms to predict eukaryotic promoter structures. They both independently predicted the presence of a novel additional promoter region, located 3.1 kb upstream of the ATG site in the rat, mouse (NT_039343) and human (NT_007819) sequences and 1.6 kb upstream from the already established *Nex1*-P1 promoter (Fig. 1). This putative upstream promoter region is referred to as *Nex1*-P2 promoter. The fact that the two *Nex1* transcripts differ in size by only 800 bp and that the two potential *Nex1* promoter regions are separated by 1.6 kb suggest that the *Nex1*-P2 promoter may not be located on the same non-coding exon as the *Nex1*-P1 promoter. Thus, we searched for potential donor splice sites in the 5'UTR of the rat *Nex1* gene using the NetGene2 software (Brunak et al., 1991). In addition to the already identified donor splice site of intron 1a, we identified a novel donor splice site mapping at the -2172 position, generating the novel intron 1b and non-coding exon 1b, the latter containing the putative *Nex1*-P2 promoter region (Fig. 1). Thus, the predicted *Nex1*-P2 transcript would splice out a 2.15 kb intron, resulting in a transcript approximately 0.7 kb larger than the *Nex1*-P1 transcript, which supports the Northern blot analyses reported previously (Bartholoma and Nave, 1994; Shimizu et al., 1995; Schwab et al., 1998).

To delineate potential regulatory regions associated with both predicted promoter regions, we carried out a sequence alignment of genomic DNA from the rat, mouse and human 5' flanking sequence extending 7 kb upstream of the *Nex1* coding sequence using ClustalW. Within this sequence, we identified a 0.3 kb-long region of extremely high homology (98%) encompassing the previously defined minimal *Nex1*-P1 promoter and a 0.2 kb-long region of high homology (70%) located about 3.1 kb upstream of the translation start site, at the site of the predicted novel *Nex1*-P2 promoter (Fig. 1). Finally, genomic sequence alignment using ClustalW showed that the new additional donor splice site of intron 1b is also conserved in the three species.

Nex1 5'UTR Contains Two Distinct TATA-Less Promoter Regions, *Nex1*-P1 and *Nex1*-P2

To map the transcription start sites in the mouse, rat, and human *Nex1* 5'UTR sequences by primer extension, we designed primers (PE) complementary to the sense strand, located less than 200 bp downstream from the putative promoter regions. The PE primers are located at positions -2944 to -2923, and -1363 to -1340 relative to the rat +1 translation start site for the *Nex1*-P2 and *Nex1*-P1 promoters, respectively (Figs. 2,3). Poly(A) RNA from NGF-treated

PC12 cells was isolated as described in Materials and Methods. The commercially available poly(A) mRNA from adult human cerebellum, mouse E16.5 embryo, and adult mouse whole brain (Ambion) were also used as template. The primer extension analysis showed that both Nex1-P1 and Nex1-P2 promoters contain multiple transcription start sites clustered in well-conserved regions (Figs. 2,3). Moreover, both promoters are TATA-less and lack an initiator element and SP1 binding sites, which are usually associated with such promoters (Figs. 2B, 3B).

Primer extension analysis on the Nex1-P2 promoter region using poly(A) mRNA isolated from rat NGF-treated PC12 cells and adult human cerebellum showed multiple transcription start sites, which were relatively well conserved in terms of position, with rat transcripts starting 49, 145, 155, 169, and 179 nucleotides (nt) and human transcripts starting 52, 133, 146, 156, and 175 nt upstream of the PE primer (Fig. 2). In contrast, the mouse Nex1-P2 promoter only displayed three transcription start sites located 114, 136, and 175 nt upstream of the PE primer, with the primary transcript at position 114 (Fig. 2). Notably, all these transcription start sites are tightly clustered within a 135 bp-long region exhibiting 70% homology between the three species, suggesting a similar transcriptional regulatory mechanism at work in the three species (Fig. 2B). Thus, the primer extension results validated the predicted Nex1-P2 promoter in the three species, located 1.63 kb upstream of Nex1-P1 promoter.

Using poly(A) mRNA isolated from rat NGF-differentiated PC12 cells, adult human cerebellum, and adult mouse brain, primer extension showed that the Nex1-P1 promoter region contained a major conserved transcription start site located about 1415 bp upstream of the translation start site (+1) and several minor transcription start sites mapping further upstream in a region displaying 98% homology between the three species (Fig. 3A). Interestingly, using embryonic E16.5 mouse poly(A) mRNA, we detected only minor transcription start sites, which overlapped with minor transcription start sites found in rat and human Nex1-P1 transcripts (Fig. 3A). 5' Rapid Amplification of cDNA ends (5' RACE) confirmed the mapping of these multiple transcription start sites (Fig. 3B), and the donor and acceptor splice sites for intron 1a at positions -1316 and -21, respectively, relative to the rat translation start site. We also observed identical bases at the exon/intron borders in all three species, with the sequence of intron 1a exhibiting high homology between the three species (Fig. 1).

The rat, mouse, and human *Nex1* genes display a conserved genomic structure characterized by two non-coding exons, 1a and 1b containing Nex1-P1 and Nex1-P2 promoters, respectively. Both non-coding exons are separated from the coding exon by either a 1292 bp-long intron 1a or a 2150 bp-long intron 1b (Fig. 1).

DNaseI Footprinting Analysis Shows That the Nex1-P1 and Nex1-P2 Promoters Display Distinct Occupied *cis*-Acting Elements During NGF-Induced Differentiation

Our previous study showed that Nex1 expression is triggered after 5 hr of NGF exposure, and is critical to the execution of the NGF differentiation program (Uittenbogaard and Chiamello, 2002). However, specific *cis*-acting elements responsible for Nex1 expression have not been identified. Thus, we searched for conserved transcriptional binding sites using both the TFSEARCH and TESS algorithms. Phylogenetic analysis of the first 4 kb of the Nex1 5'UTR showed that the Nex1-P1 promoter region includes a cluster of four conserved E-boxes, binding sites for bHLH transcription factors, and a conserved CRE (cAMP response element) recognition site, a binding site for the basic leucine zipper transcription factors of the CREB/ATF family (Fig. 3B). The Nex1-P2 promoter region contains several potential regulatory elements, such as Oct, Pax, HNF, and C/EBP-like binding sites (Fig. 2B).

To identify which binding sites are occupied during neuronal differentiation, we carried out DNaseI footprinting assays on the conserved regions of the Nex1 5'UTR, using nuclear extracts

from undifferentiated and NGF-differentiated PC12 cells. We designed eight sets of primers (Suppl. Table I) to generate footprinting probes, each no more than 450 bp in length and exhibiting conserved regulatory elements within regions of high homology (Fig. 4). Using rat genomic DNA as a template, we amplified four DNA fragments covering the Nex1-P1 promoter region (FP2–FP5), one within intron 1a (FP1), two DNA fragments spanning the distal Nex1-P2 promoter region (FP6, FP7) and one fragment (FP8) located further upstream in the Nex1 5'UTR (Fig. 4). These fragments were singly end-labeled, and incubated with either untreated PC12 nuclear extract, 1-day, 3-day, or 6-day NGF-treated PC12 nuclear extracts. To precisely map the occupied binding sites, the FP probes were sequenced.

We obtained several DNaseI footprinted sites with only three of eight footprinting probes, FP7 and FP-6 specific to Nex1-P2 promoter, and FP3 specific for Nex1-P1 promoter (Fig. 4). No footprint patterns were observed with the FP1 probe specific to the conserved intronic sequences, as well as with the FP8 and FP5 probes, specific to the highly conserved upstream Nex1-P2 and Nex1-P1 promoter regions, respectively (data not shown). A weak footprinting of the central nervous system element (CNE), located near the exon/intron junction, was observed with the FP2 probe (data not shown). Protected binding sites were identified by comparison with a DNA sequencing ladder of the same fragment and the TRANSFAC consensus binding site matrices. A summary of the protein-DNA interactions in the Nex1 5' UTR along with the boundaries of the protected regions is presented in Table I.

DNaseI protection assays on the Nex1-P1 promoter region showed three footprinted binding sites, corresponding to the conserved E1 and E4 E-boxes and the CRE element, which displays a perfect match with the known consensus CRE sequence (TGACGTCA) (Fig. 4). A DNaseI hypersensitive site was also observed in the vicinity of the E1 E-box, which is located downstream of the transcription start sites (Fig. 4). Both the E1 E-box and CRE element share a similar footprinting profile, with protection observed before and after 3 days of NGF-induced differentiation (Fig. 4). Only the CRE element remained lightly protected after 6 days of NGF treatment.

We found six footprinted *cis*-regulatory elements in the Nex1-P2 promoter region (Fig. 4). They all share similar footprinting profiles, throughout NGF treatment, except for the 3'Pax and HNF/Oct-3 binding sites, which are mostly protected before and during the early stages of NGF-induced differentiation (Fig. 4). The most distal octameric binding site, 5'Oct1/2 (TATACAAAAT) displays a sequence similar to the canonical binding site (ATGCAAAT) for the Class II factors Oct-1 and Oct-2, and contains both a Pou specific (Pou-S) half-site (ATGC) and a Pou homeodomain (Pou-HD) half-site (CAAAT) (Figs. 2B,4). The sequence of the most proximal protected octameric binding site 3'Oct1/2 (GCCAGAAAT) diverges from the canonical Pou-S half-site sequence, but shares similarity with the functionally important triple-A sequence of the Pou-HD half-site (Figs. 2B,4). Finally, the third octameric binding site displays a consensus sequence characteristic of the composite element HNF/Oct-3, which contains overlapping binding sites for the neuronal specific POU protein N-Oct-3 and either HNF3 β or HNF6 Forkhead transcription factor (Blaud et al., 2004; Alazard et al., 2005).

Two closely spaced putative Pax binding sites (5'Pax and 3'Pax) were footprinted upstream of the transcription start sites of Nex1-P2 promoter (Figs. 2B,4), and were defined by comparing their sequence with reported Pax2/5/8 binding sites and their degenerate consensus sequences (Czerny et al., 1993; Epstein et al., 1994). The 5'Pax binding site shows a significant degree of similarity with the Pax2 consensus sequence (TCAT/CGCATGAC), whereas the 3'Pax sequence shares similarity with a triple-A motif containing a Pax2 repressor binding site (Håvik et al., 1999).

Figure 4 also shows a large protected region, 170 bp downstream from the Nex1-P2 multiple transcription start sites, which contains a putative CAAT/enhancer-binding protein (C/EBP)-like binding site (TTTGCCT) with a sequence diverging from the established C/EBP consensus palindromic sequence carrying the two 5-bp half sites (TTGCGC/TAAC/T). However, substantial variations of this palindromic consensus sequence are well tolerated, as most identified C/EBP binding sites contain a conserved half-site paired with a more divergent sequence containing at least 2-bp of the consensus (Osada et al., 1996). Notably, a major DNaseI hypersensitive site maps at the 3' end of the C/EBP-like binding site (Fig. 4).

Thus, our DNaseI footprinting results suggest a potential differential regulation of the Nex1-P1 and Nex1-P2 promoter regions through distinct *cis*-acting elements during NGF-induced differentiation. Furthermore, the protection pattern of Nex1-P1 and Nex1-P2 regions was confirmed with nuclear extracts isolated from adult murine brains (data not shown).

Analysis of the Protected Binding Sites of the Nex1 5'UTR by Electrophoretic Mobility Shift Assay

We validated the DNaseI footprinting results by electrophoretic mobility shift assay (EMSA). The sense strand of the wild-type and mutated probes are shown in Supplemental Table I. EMSA assays were carried out using nuclear proteins isolated from NGF-differentiated PC12 cells (3 days), unless noted otherwise. For competitive experiments, an excess of either unlabeled wild-type or mutated competitor oligonucleotide was added.

EMSA assays carried out on the 5'Oct-1/2 binding site produced a sequence-specific retarded complex, which was efficiently competed by an excess of cold, wild-type oligonucleotide (Fig. 5A, lanes 1–5). When both Oct-1/2 half-sites were mutated by replacing the triple-A sequence of the Pou-HD motif and the A–T rich Pou-S sequence with G–C rich sequences, no competition was observed (Fig. 5A, lanes 6–8). EMSA on the 3'Oct-1/2 binding site was carried out using nuclear proteins isolated from undifferentiated PC12 cells, because DNA-binding activity of the specific complexes greatly diminished during NGF-induced differentiation. Both complexes were efficiently competed by an excess of cold, wild-type oligonucleotide, while replacing the triple-A motif of the Pou-HD recognition half site (GAAAT) with a G–C rich sequence (GCCAT) did not affect both complexes, confirming that this octameric element behaves as an Oct-1/2 binding site (Fig. 5B). Finally, two specific complexes were efficiently competed by an excess of cold wild-type HNF/Oct-3 oligonucleotide (Fig. 5C, lanes 1–5). Mutation of the HNF/Oct-3 binding site by replacing the triple-A motif, necessary for both HNF3/6 and N-Oct-3 binding activities, with a G–C rich sequence, and by inserting an additional G residue between the Pou-S and Pou-HD sites did not affect the binding activity of both complexes (Fig. 5C, lanes 6–8).

Protein-DNA interactions with the 5'Pax and 3'Pax binding sites were also confirmed by EMSA. The 5'Pax probe generated two distinct DNA-protein complexes, with the fastest migrating complex being dominant (Fig. 5D). Increasing amounts of cold specific 5'Pax oligonucleotide successfully competed for formation of both complexes (Fig. 5D, lanes 2–5), whereas mutation of the 5'T and the 3'TCA nucleotides, critical for Pax-2 binding activity, resulted in a lack of competition for the binding of both complexes (Fig. 5D, lanes 6–7). EMSA on the 3'Pax binding site showed the presence of a major DNA-protein complex, with very weak binding activity and a faster migrating complex with stronger binding activity (Fig. 5E, lanes 1–5). As expected, mutation of the critical nucleotides for Pax binding completely eliminated the ability of the mutated oligonucleotide to compete for the binding of both complexes (Fig. 5E, lanes 6–8).

Figure 6A shows the EMSA results using the whole footprinted C/EBP-like region as a probe. Because the footprinted C/EBP-like binding site encompassed 27 bp, we designed two short

overlapping oligonucleotides, called 5'half and 3'half, to delineate the nucleotides responsible for complex formation (Fig. 6). Only the 3'half oligonucleotide formed a major complex, whereas the 5'half oligonucleotide produced a negligible shift (Fig. 6B). Finally, complex formation with full-length C/EBP-like oligonucleotide was also inhibited by a 200-fold excess of cold 3'half oligonucleotide (Fig. 6C). To confirm that the 3'half of the footprinted element contained a C/EBP recognition site, we shifted the complex in the presence of excess cold oligonucleotide carrying the well-characterized consensus C/EBP sequence (Williams et al., 1995). We found that the C/EBP consensus oligonucleotide was able to compete for protein binding as efficiently as the full-length C/EBP-like probe itself and the truncated 3'half probe, suggesting that the protected C/EBP-like binding site may behave as a C/EBP *cis*-acting element (Fig. 6D). Furthermore, supershift assay using a polyclonal anti-C/EBP β antibody confirmed the presence of C/EBP β protein in the complex shifted with the full-length C/EBP oligonucleotide (Fig. 6E).

Finally, we confirmed by EMSA the DNA-protein interactions with the footprinted CRE, E1, and E4 binding sites of Nex1-P1. The CRE probe generated a major complex, which was efficiently competed out by an excess of cold wild-type CREB oligonucleotide (Fig. 7A, lane 1–5). Mutation of the CRE binding site from TGACGTCA to TTCATTGA prevented competitive binding to the mutated probe (Fig. 7A, lanes 6–8). Both the E1 and E4 probes produced two specific retarded complexes, whereas mutation of either E-box did not inhibit complex formation (Fig. 7B,C).

Nex1-P2 Promoter Activity Is Stronger Than Nex1-P1 Promoter Activity in NGF-Treated PC12 Cells

To confirm that the newly identified Nex1-P2 region has promoter activity, we amplified a 770 bp-long Nex1-P2 (A–D) fragment using rat genomic DNA as template. The Nex1-P2 (A–D) amplicon was cloned into the promoter-less pGL3-basic vector, as described in Materials and Methods. We cloned the 553 bp-long Nex1-P1 fragment into the pGL3-basic vector to compare the relative strength of each promoter region. Promoter activity was assessed by measuring luciferase activity after transfection into PC12 cells, which were subsequently differentiated with NGF (50 ng/ml) or dbcAMP (1 mM). Promoter activity was expressed as ratio of promoter-driven firefly luciferase versus Renilla firefly luciferase from the internal transfection normalization control vector pRL-TK.

Figure 8 shows that both Nex1-P1 and Nex1-P2 display weak basal promoter activities in undifferentiated PC12 cells, with Nex1-P2 activity stronger than Nex1-P1 activity. On dbcAMP exposure, both promoters exhibited a two-fold increased activity, whereas on NGF treatment, Nex1-P2 promoter activity increased by six-fold when compared to naïve PC12 cells (Fig. 8). In contrast, the Nex1-P1 promoter was poorly stimulated by NGF exposure (Fig. 8). As a control, we cloned 2 kb of the Nex1 5'UTR containing both the distal P2 and proximal P1 regions, and found that the Nex1 P1-P2 construct displayed a promoter activity similar to that of the Nex1-P2 construct in naïve, NGF, or dbcAMP-treated PC12 cells (data not shown). Thus, the luciferase assay results combined with the primer extension analysis indicate that the rat *Nex1* gene is under the control of two distinct promoters, Nex1-P1 and Nex1-P2, with the Nex1-P2 promoter playing a dominant role in regulating the *Nex1* gene during NGF-mediated differentiation.

Nex1-P1 Promoter Is Mainly Regulated by the Conserved CRE Element

The DNase I footprinting and EMSA assays suggest that the conserved CRE element may play a role in regulating Nex1-P1 promoter activity. Because the cAMP response element binding protein (CREB) is known to bind and regulate the activity of the CRE element, we investigated whether overexpression of CREB would stimulate the Nex1-P1 promoter activity in untreated

and differentiated PC12 cells. Naïve PC12 cells were cotransfected with the Nex1-P1 plasmid and CREB eukaryotic expression vector pCMV-CREB. No increased Nex1-P1 promoter activity was observed, as compared to the Nex1-P1 plasmid alone (Fig. 9A). Because CREB is activated via phosphorylation of key serine residues in response to changes in cAMP levels (Montminy, 1997), we treated transfected PC12 cells with the cAMP-elevator dbcAMP (1 mM) for 3 days. Overexpression of CREB resulted in a 6-fold increase of the Nex1-P1 promoter activity in dbcAMP-treated PC12 cells (Fig. 9A). To confirm the regulatory role of CREB in the cAMP-induced pathway, we used the dominant negative construct of CREB, KCREB, which fails to bind to the CRE element due to a mutation in its DNA-binding domain. Overexpression of KCREB in dbcAMP-treated PC12 cells abolished Nex1-P1 promoter activity (Fig. 9A).

Surprisingly, overexpression of CREB led to a decreased NGF-induced Nex1-P1 promoter activity, suggesting that CREB is competing with another member of the CREB/ATF family, which may preferentially modulate NGF response through the conserved CRE element (Fig. 9A). The CREB/ATF family comprises several isoforms coded by at least three highly related genes, *CREB*, *CREM*, and *ATF-1*, which recognize nearly identical binding sites (Hai et al., 1989; Hurst et al., 1991). Likewise, overexpression of KCREB impaired NGF-induced Nex1-P1 promoter activity, suggesting that KCREB may form a non-functional heterodimeric complex with a critical endogenous CREB/ATF member (Fig. 9A).

To evaluate the functional importance of the CRE element in the overall activity of Nex1-P1 promoter, we carried out site-directed mutagenesis of the CRE element to mutate the core CRE recognition site from TGACGTCA to TTCATTG. We observed a collapse of the Nex1-P1 promoter activity in naïve, dbcAMP- or NGF-treated PC12 cells, even on overexpression of CREB (Fig. 9B). Together, these results show that the conserved CRE element is critical for the overall Nex1-P1 promoter activity.

In addition to the CRE element, the Nex1-P1 promoter harbors two conserved and protected E-boxes, E1 and E4. The E1 E-box is located downstream of the multiple transcription start sites, whereas the E4 E-box is located upstream and near the protected CRE. To investigate whether these E-boxes participate in the overall regulation of Nex1-P1 promoter, we mutated E1 and E4 E-boxes, independently. Mutation of the E1 E-box did not alter Nex1-P1 promoter activity in untreated or treated PC12 cells, whereas mutation of the E4 E-box only led to a decreased basal activity of the Nex1-P1 promoter in naïve PC12 cells (Fig. 9C), suggesting that the E4 E-box may be involved in the previously reported autoregulation of the Nex1-P1 promoter in naïve PC12 cells on transient overexpression of Nex1 (Bartholoma and Nave, 1994). Although no endogenous Nex1 is present in undifferentiated PC12 cells (Bartholoma and Nave, 1994; Uittenbogaard and Chiaramello, 2002), it is plausible that the bHLH transcription factor Mash1, known to be expressed in naïve PC12 cells (Johnson et al., 1990), could mediate basal activity of the Nex1-P1 promoter.

Conserved C/EBP Regulatory Element Is Critical for Nex1-P2 Promoter Activity

The DNaseI footprinting and EMSA assays showed six potential regulatory elements, mapping upstream and downstream of Nex1-P2 transcription start sites. To determine which regulatory element(s) is responsible for maximal Nex1-P2 promoter activity, we generated a series of 5' and 3' deletions by PCR (Fig. 10A, left panel). The PCR amplicons were cloned into the promoter-less luciferase pGL3-basic vector. The corresponding constructs were transiently transfected into PC12 cells, which were subsequently treated with NGF for 3 days. The relative luciferase activity was normalized to that of the full-length Nex1-P2 A-D construct.

To assess the functional significance of the C/EBP binding site in regulating the Nex1-P2 promoter activity, we generated three progressive 3' deletions of the C/EBP binding site by

PCR. The corresponding constructs were called Nex1-P2 AC3, Nex1-P2 AC2, and Nex1-P2 AC. The Nex1-P2 AC3 construct bears a deletion of the 3'half of the C/EBP-like site (TTTTTGCCTTT), which generated a shifted complex by EMSA (Fig. 6). The Nex1-P2 AC2 construct bears further deletion of the C/EBP site to eliminate the potentially critical four-A motif. Finally, the Nex1-P2 AC construct no longer carries the C/EBP binding site. Deleting 11 bp of the 3'half site of C/EBP resulted in total loss of the Nex1-P2 promoter activity (Fig. 10A). The collapse of Nex1-P2 promoter activity was confirmed by further deletion and ablation of the C/EBP binding site (Fig. 10A). These observations argue for regulation of the Nex1-P2 promoter by the critical *cis*-acting element C/EBP. Furthermore, the last 11 nucleotides located at the 3' end of the C/EBP binding site are essential for both DNA-binding and transcriptional activities.

To determine whether C/EBP β upregulates Nex1-P2, we transfected PC12 cells with the eukaryotic expression vector C/EBP β . Figure 10B shows that C/EBP β induces a 12-fold increase in Nex1-P2 promoter activity. This transactivation requires the presence of the C/EBP binding site identified by DNase I footprinting and EMSA assays, as its deletion resulted in a lack of regulation of the Nex1-P2 promoter activity (Fig. 10B). To further confirm the regulatory role of C/EBP β , we used two mutants, the LIP-C/EBP β truncated dominant negative form of C/EBP β (Δ C/EBP β), which lacks the trans-activation domain (Baer and Johnson, 2000), and the A-C/EBP, which can no longer bind to DNA due to the replacement of basic amino acids with acidic amino acids (Krylov et al., 1997; Greenwel et al., 2000). We found that overexpression of either mutant led to total repression of Nex1-P2 promoter activity, even below the level observed with the full-length Nex1-P2 AD construct alone (Fig. 10B). This is most likely due to the fact that either mutant dimerizes with endogenous C/EBP, to form non-functional dimeric complex that cannot bind DNA or activate transcription (Vinson et al., 2002).

To address whether the other DNaseI protected binding sites participate in the overall regulation of Nex1-P2 promoter, we generated a series of 5' deletion of the Nex1-P2 5'UTR. Deletion of the 5'Oct-1/2 element resulted in a 1.5-fold increase of the Nex1-P2 promoter activity, implying that the 5'Oct-1/2 behaves as a mild repressor (Fig. 10A). Deletion of the 5' Pax site abolished the Nex1-P2 promoter activity, whereas deletion of the 3'Pax site restored the promoter activity level similar to that observed with the Nex1-P2-A2D construct lacking the repressive 5'Oct-1/2 element (Fig. 10A). Thus, these deletion results indicate that the 3'Pax binding site acts as a negative regulator in the absence of the neighboring 5'Pax site, which is consistent the triple-A motif associated with Pax-2 and Pax-5 repressive activity (Håvik et al., 1999). Finally, deletion of the HNF/Oct-3 element had no effect on Nex1-P2 promoter activity (Fig. 10).

DISCUSSION

The main objective of this study is to elucidate the transcriptional regulation of the *Nex1* gene during NGF-induced differentiation, as Nex1 is a critical effector of the NGF pathway (Uittenbogaard and Chiaramello, 2002). This study shows that the *Nex1* gene displays a conserved genomic structure between the human, mouse, and rat species. It also provides the first comprehensive functional analysis of the Nex1 5'UTR, from which several conclusions can be drawn.

First, Nex1 expression is under the control of two distinct promoters, Nex1-P1 and Nex1-P2, located in two non-coding exons, exon 1a and exon 1b, respectively. Corresponding introns, 1a and 1b, are spliced out linking the non-coding exons to the single coding exon 2 containing the entire Nex1 coding sequence. Thus, the two Nex1 transcripts reported previously are most likely the direct products of alternative Nex1-P1 or Nex1-P2 promoter usage (Bartholoma and

Nave, 1994; Shimizu et al., 1995; Schwab et al., 1998). Moreover, the Nex1-P2 promoter is much stronger than the Nex1-P1 promoter in differentiated PC12 cells, which may explain the differential expression levels of Nex1 transcripts at specific stages of brain development (Bartholoma and Nave, 1994; Shimizu et al., 1995; Schwab et al., 1998). From the onset of Nex1 expression (E14) until gliogenesis occurs (E16), the presumed Nex1-P2 transcripts are more abundant than the Nex1-P1 transcripts. A similar ratio is also detected during the first postnatal week and in mature brain. However, these studies did not address whether promoter usage was specific to a neuronal subpopulation, because total RNA was isolated from either whole embryos or whole postnatal brains. Differential promoter activity may have functional relevance not only in neurogenesis, but also in synaptic plasticity associated with learning and memory formation.

Second, DNaseI footprinting protection and EMSA assays show that the two Nex1 promoters display distinct *cis*-acting elements. Interestingly, the occupied binding sites display a similar footprinting pattern before and during the early phase of NGF treatment. This may be due to the fact that the implicated transcription factors are expressed in undifferentiated as well as differentiated PC12 cells, and require a signaling event, such as phosphorylation, to trigger their transcriptional activities. In fact, the pre-NGF occupancy of these binding sites may be advantageous to trigger Nex1 expression within 5 hours of NGF-treatment (Uittenbogaard and Chiamello, 2002). There are numerous examples of transcription factors, such as MyoD and NeuroD, whose expression in progenitor cells precedes their functional execution on onset of terminal differentiation. Alternatively, repressors associated with undifferentiated PC12 cells may also bind to specific footprinted elements.

Third, luciferase assays show that the Nex1-P1 promoter is predominantly regulated by a conserved CRE element in NGF or dbcAMP-treated PC12 cells. Furthermore, our results from site-directed mutagenesis of the E4 E-box infer that this regulatory element moderately modulates Nex1-P1 promoter activity in the absence of NGF, which corroborate the reported Nex1 autoregulation through the minimal Nex1-P1 promoter in undifferentiated PC12 cells (Bartholoma and Nave, 1994). However, our results indicate a lack of E-box-mediated regulation during NGF-induced differentiation. The most plausible explanation may be that its effect could be masked by the strong transcriptional activity of the CREB/ATF members through the CRE element, on signaling, or that the bHLH-mediated regulation could be transient on the early stages of NGF exposure, and therefore difficult to capture by luciferase assays. Most bHLH transcription factors are known to be involved in auto- or cross-regulation (Chae et al., 2004). Similarly, pro neuronal bHLH proteins, such as neurogenin1 and Mash1, are expressed in undifferentiated PC12 cells and are known to regulate the expression of NeuroD members, which are downstream in the neurogenic cascade (Sun et al., 2001).

Fourth, the Nex1-P2 promoter is essentially regulated through the conserved non-canonical C/EBP binding site, located downstream of the transcription start sites. DNaseI footprinting analysis and EMSA imply that additional *cis*-acting elements, such as Oct-1/2, Pax, and HNF-/Oct-3 may also intervene in the regulation of Nex1-P2 promoter. However, results from luciferase assays suggest that they most likely play a secondary role during NGF-induced differentiation of PC12 cells. Nevertheless, their functional relevance may vary during brain development and at specific temporal and spatial stages, as specific external cues modulate different combinatorial interactions between transcription factors.

The influence of signaling cues on Nex1 promoter activity is further illustrated by the regulation of the Nex1-P1 CRE element, which is more responsive to the combined cAMP and CREB effect, than the NGF treatment alone or the combined NGF and CREB effect. These observations suggest that NGF signaling may be mediated through another endogenous CREB/ATF member, because the exogenous CREB protein competes in a negative manner.

Of interest is the functional impact of the non-canonical C/EBP binding site on Nex1-P2 promoter activity during neuronal differentiation. C/EBP transcription factors are known to play pivotal roles in coupling growth factor signaling to differentiation in a wide array of cell types, such as adipocytes, hepatocytes, keratinocytes, and neurons (Ramji and Foka, 2002; Johnson, 2005). Among the six members of the C/EBP family (C/EBP α -C/EBP ζ), C/EBP isoforms, α , β , and γ are expressed in cortical progenitor cells as well as mature neurons of the mammalian CNS (Ménard et al., 2002). In fact, several studies have shown C/EBP β to be a key regulatory factor of neuronal differentiation. Overexpression of C/EBP β protein induces neuronal differentiation of neuroblastoma cells (Cortes-Canteli et al., 2002). During cortical development, activation of C/EBP β protein by the MEK/ERK pathway promotes neurogenesis of cortical progenitor cells while inhibiting gliogenesis (Ménard et al., 2002; Paquin et al., 2005). Finally, C/EBP β is a downstream target gene of the NGF pathway in PC12 cells, and its transcriptional activity of the C/EBP β protein, is increased on NGF exposure, due to phosphorylation through the MAPK and PI3K signaling pathways (Sterneck and Johnson, 1998).

Identification of the *Nex1* gene as a downstream target of the C/EBP pathway provides additional clues for understanding the transcriptional mechanisms of growth factor-mediated neurogenesis. Independent studies on either the neurogenic bHLH or C/EBP transcription factors have shown that each family by itself promotes neurogenesis, while repressing gliogenesis (Nieto et al., 2001; Sun et al., 2001; Vetter, 2001). Recent studies from the Miller's group led to the hypothesis that C/EBP functions as a growth factor-regulating differentiation signal that cooperates with the appropriate bHLH transcription factor (Paquin et al., 2005). It was further suggested that C/EBP and neurogenic bHLH regulators might cooperate to transcriptionally activate a neuron-specific gene expression program by binding to separate consensus sites within the same genes (Paquin et al., 2005). Thus, our findings not only further support their hypothesis, but also bring new light on the molecular mechanism by which C/EBP may promote neurogenesis. Our study provides the first evidence of C/EBP directly regulating the expression of a neurogenic bHLH protein.

Another interesting aspect of the regulation of the *Nex1* gene is the dual involvement of CREB and C/EBP β , as both basic leucine zipper transcription factors promote long-term memory consolidation on cAMP signaling in hippocampal neurons (Yukawa et al., 1998). Previous studies on Nex1 pattern of expression have suggested a potential role of Nex1 in synaptic plasticity associated with long-term memory, as Nex1 mRNA and protein are found in adult pyramidal neurons of the hippocampal CA1-4 fields and mature neurons of the entorhinal cortex and neocortex (Bartholoma and Nave, 1994; Schwab et al., 1998). Our findings of the essential regulatory roles of CRE and C/EBP elements further lend credence to the notion that Nex1 may be a key component of the C/EBP β /CREB-initiated transcriptional cascade critical for stabilization of long-term memory (Yukawa et al., 1998).

In summary, our findings suggest that Nex1 is a downstream target gene of C/EBP and CREB/ATF regulators, and that C/EBP and Nex1 may share a transcriptional network to execute neurogenesis, while repressing gliogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by NIH grant R01-NS 041391 to A.C. and by P30HD40677.

REFERENCES

- Alazard R, Blaud M, Elbaz S, Vossen C, Icre G, Joseph G, Nieto L, Erard M. Identification of the 'NORE' (N-Oct-3 responsive element), a novel structural motif and composite element. *Nucleic Acids Res* 2005;33:1513–1523. [PubMed: 15767276]
- Baer M, Johnson PF. Generation of truncated C/EBP β isoforms by in vitro proteolysis. *J Biol Chem* 2000;275:26582–26590. [PubMed: 10856306]
- Bartholoma A, Nave K-A. NEX1: a novel brain-specific helix-loop-helix protein with autoregulation and sustained expression in mature cortical neurons. *Mech Dev* 1994;48:217–228. [PubMed: 7545978]
- Bertrand N, Castro DS, Guillemot F. Proneural genes and the specification of neural cell types. *Nat Rev Neurosci* 2002;3:517–530. [PubMed: 12094208]
- Blaud M, Vossen C, Joseph G, Alazard R, Erard M, Nieto L. Characteristic patterns of N Oct-3 binding to a set of neuronal promoters. *J Mol Biol* 2004;339:1049–1058. [PubMed: 15178247]
- Brunak S, Engelbrecht J, Knudsen S. Prediction of human mRNA donor and acceptor sites from the DNA sequence. *J Mol Biol* 1991;220:49–65. [PubMed: 2067018]
- Chae JH, Stein GH, Lee JE. NeuroD: the predicted and the surprising. *Mol Cells* 2004;18:271–288. [PubMed: 15650322]
- Chiamello A, Neuman T, Peavy DR, Zuber MX. The GAP-43 gene is a direct downstream target of the basic Helix-Loop-Helix transcription factors. *J Biol Chem* 1996;271:22035–22043. [PubMed: 8703010]
- Cortés-Canteli M, Pignatelli M, Santos A, Pérez-Castillo A. CCAAT/enhancer-binding protein beta plays a regulatory role in differentiation and apoptosis of neuroblastoma cells. *J Biol Chem* 2002;277:5460–5467. [PubMed: 11733516]
- Czerny T, Schaffner G, Busslinger M. DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site. *Genes Dev* 1993;10:2048–2061. [PubMed: 8406007]
- Epstein JA, Glaser T, Cai J, Jepeal L, Walton DS, Maas RL. Two independent and interactive DNA-binding subdomains of Pax6 paired domain are regulated by alternative splicing. *Genes Dev* 1994;8:2022–2034. [PubMed: 7958875]
- Greenwel P, Tanak S, Penkov D, Zhang W, Olive M, Moll J, Vinson C, Di Liberto M, Ramirez F. Tumor necrosis factor alpha inhibits type I collagen synthesis through repressive CCAAT/enhancer-binding proteins. *Mol Cell Biol* 2000;20:912–918. [PubMed: 10629048]
- Hai TW, Liu F, Coukos WJ, Green MR. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev* 1989;12B:2083–2090. [PubMed: 2516827]
- Håvik B, Ragnhildstveit E, Lorens JB, Saelemyr K, Fauske O, Knudsen LK, Fjose A. A novel paired domain DNA recognition motif can mediate repression of gene transcription. *Biochem Biophys Res Commun* 1999;266:532–541. [PubMed: 10600536]
- Hurst HC, Totty NF, Jones NC. Identification and functional characterization of the cellular activating transcription factor 43 (ATF-43) protein. *Nucleic Acids Res* 1991;19:4601–4609. [PubMed: 1653949]
- Johnson JE, Birren SJ, Anderson DJ. Two rat homologues of *Drosophila* achaete-scute specifically expressed in neuronal precursors. *Nature* 1990;346:858–861. [PubMed: 2392153]
- Johnson PF. Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. *J Cell Sci* 2005;118:2545–2555. [PubMed: 15944395]
- Krylov D, Kasai K, Echlin DR, Taparowsky EJ, Arnheiter H, Vincon C. A general method to design dominant negative bHLHZip proteins that abolish DNA binding. *Proc Natl Acad Sci U S A* 1997;94:12274–12279. [PubMed: 9356439]
- Lee JE. Basic helix-loop-helix genes in neural development. *Curr Opin Neurobiol* 1997;7:13–20. [PubMed: 9039799]
- Ménard C, Hein P, Paquin A, Savelson A, Yang XM, Lederfein D, Barnabé-Heider F, Mir AA, Sterneck E, Peterson AC, Johnson PF, Vinson C, Miller F. An essential role for a MEK-C/EBP pathway during growth factor-regulated cortical neurogenesis. *Neuron* 2002;36:597–610. [PubMed: 12441050]
- Montminy M. Transcriptional regulation by cyclic AMP. *Annu Rev Biochem* 1997;66:807–822. [PubMed: 9242925]

- Nieto M, Schuurmans C, Britz O, Guillemot F. Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. *Neuron* 2001;29:401–413. [PubMed: 11239431]
- Nyborg JK, Dynan WS. Interaction of cellular proteins with the human T-cell leukemia virus type I transcriptional control region. Purification of cellular proteins that bind the 21-base pair repeat elements. *J Biol Chem* 1990;265:8230–8236. [PubMed: 2335523]
- Osada S, Yamamoto H, Nishihara T, Imagawa M. DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. *J Biol Chem* 1996;271:3891–3896. [PubMed: 8632009]
- Paquin A, Barnabé-Heider F, Kageyama R, Miller F. CCAAT/enhancer-binding protein phosphorylation biases cortical precursors to generate neurons rather than astrocytes in vivo. *J Neurosci* 2005;25:10747–10758. [PubMed: 16291948]
- Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J* 2002;365:551–575.
- Ross SE, Greenberg ME, Stiles CD. Basic helix-loop-helix factors in cortical development. *Neuron* 2003;39:13–25. [PubMed: 12848929]
- Schwab MH, Druffel-Augustin S, Gass P, Jung M, Klugmann M, Bartholoma A, Rossner MJ, Nave K-A. Neuronal basic helix-loop-helix proteins (NEX, NeuroD, NDRF): spatio-temporal expression and targeted disruption of the NEX gene in transgenic mice. *J Neurosci* 1998;18:1408–1418. [PubMed: 9454850]
- Schwab MH, Bartholoma A, Heimrich B, Feldmeyer D, Druffel-Augustin S, Goebbels S, Naya FJ, Zhao S, Frotscher M, Tsai M-J, Nave K-A. Neuronal basic helix-loop-helix proteins (NEX, and BETA2/NeuroD) regulate terminal granule cell differentiation in the hippocampus. *J Neurosci* 2000;20:3714–3724. [PubMed: 10804213]
- Shimizu C, Akazawa C, Nakanishi S, Kageyama H. MATH-2, a mammalian helix-loop-helix factor structurally related to the product of *Drosophila* proneural gene, *atonal*, is specifically expressed in the nervous system. *Eur J Biochem* 1995;229:239–248. [PubMed: 7744035]
- Sterneck E, Johnson PF. CCAAT/Enhancer binding protein beta is a neuronal transcriptional regulator activated by nerve growth factor receptor signaling. *J Neurochem* 1998;70:2424–2433. [PubMed: 9603207]
- Sun Y, Nadal-Vicens M, Misono S, Lin MZ, Zubiaga A, Hus X, Fan G, Greenberg ME. Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* 2001;104:365–376. [PubMed: 11239394]
- Uittenbogaard M, Chiaramello A. Constitutive expression of the basic Helix-Loop-Helix Nex1/MATH-2 transcription factor promotes neuronal differentiation of PC12 cells and neurite regeneration. *J Neurosci Res* 2002;67:235–245. [PubMed: 11782967]
- Uittenbogaard M, Chiaramello A. Expression profiling upon Nex1/MATH-2-mediated neuritogenesis in PC12 cells and its implication in regeneration. *J Neurochem* 2004;91:1332–1343. [PubMed: 15584910]
- Uittenbogaard M, Martinka DL, Chiaramello A. The basic Helix-Loop-Helix differentiation factor Nex1/MATH-2 functions as a key activator of the GAP-43 gene. *J Neurochem* 2003;84:678–688. [PubMed: 12562512]
- Uittenbogaard M, Chiaramello A. The basic Helix-Loop-Helix transcription factor Nex1/Math-2 promotes neuronal survival of PC12 cells by modulating the dynamic expression of anti-apoptotic and cell cycle regulators. *J Neurochem* 2005;92:585–596. [PubMed: 15659228]
- Vetter M. A turn of the helix: preventing the glial fate. *Neuron* 2001;29:559–562. [PubMed: 11301015]
- Vinson C, Myakishev M, Acharya A, Mir AA, Moll JR, Bonovich M. Classification of human B-ZIP proteins based on dimerization properties. *Mol Cell Biol* 2002;18:6321–6335. [PubMed: 12192032]
- Williams SC, Baer M, Dillner AJ, Johnson PF. CRP2 (C/EBP β) contains a bipartite regulatory domain that controls transcriptional activation, DNA binding and cell specificity. *EMBO J* 1995;14:3170–3183. [PubMed: 7621830]
- Wu S-X, Goebbels S, Nakamura K, Nakamura K, Kometani K, Minato N, Kaneko T, Nave K-A, Tamamaki N. Pyramidal neurons of upper cortical layers generated by NEX-positive progenitor cells in the subventricular zone. *Proc Natl Acad Sci U S A* 2005;102:17172–17177. [PubMed: 16284248]

Yukawa K, Tanaka T, Tsuji S, Akira S. Expressions of CCAAT/Enhancer-binding proteins beta and delta and their activities are intensified by cAMP signaling as well as Ca²⁺/calmodulin kinases activation in hippocampal neurons. J Biol Chem 1998;273:31345–31351. [PubMed: 9813043]

Fig. 1.

Genomic structure of the rat *Nex1* gene. The schematic representation of the first 4,388 bp of the rat *Nex1* 5'UTR shows blocks of homology between human, mouse, and rat species, as identified by ClustalW. Corresponding percentages are also indicated. The arrows represent multiple transcription start sites associated with the *Nex1*-P1 and *Nex1*-P2 promoters. Numbers indicate the position of each promoter relative to the translation start site (+1). The two introns, 1a and 1b, are indicated along with their sizes. Double arrows denote the non-coding exons, 1a and 1b, as well as the single coding exon 2. Specific structural features of the *Nex1* protein are schematically represented. The two transactivation domains, TAD1 and TAD2, are indicated by hatched boxes, and the nuclear localization domain (NL) is represented by a black box. The bHLH motif is shown by a checkered box. The polyadenylation sites located in the 3'UTR are denoted by thin vertical lines and arrows.

Fig. 2.

The novel Nex1-P2 promoter is TATA-less and displays multiple transcription start sites clustered within a conserved region. **A**: Mapping of the transcription start sites using human, mouse, and rat brain mRNA. Primer extension was carried out as described in the Materials and Methods. The location of the primer is shown in **(B)**. The left panel shows the primer extension analysis using 1 µg of poly(A) RNA from NGF-treated PC12 cells (lane 5, 2 days; lane 6, 6 days), whereas the right panel represent the results obtained using poly(A) mRNA from mouse adult brain (lane 5), mouse embryo E16.5 (lane 6), and adult human cerebellum (lane 7). The extension products were analyzed next to a DNA sequence ladder (lanes 1–4) on a 6% denaturing polyacrylamide gel. Arrows denote major transcription start sites. **B**: Sequence

alignment of the mouse, rat, and human Nex1-P2 promoter regions. The primer used for primer extension analysis is indicated by PE and the corresponding nucleotides are underlined. The forward and reverse primers used for cloning the rat Nex1-P2 promoter into the promoter-less pGL3-basic vector are indicated by the letters A and D, respectively. PCR primers used for 5' and 3' deletions of the Nex1-P2 promoter are also indicated by letters followed by a number indicative of the progressive 5' or 3' deletion. The transcription start sites from each species are shown by arrows above the sequence. The numbers on the right are the nucleotide positions relative to the translation initiation start site ATG. Pertinent putative consensus binding sites are bolded with their names indicated below the sequence. The stars indicate the homology between the three species.

Fig. 3.

The multiple transcription start sites of the TATA-less Nex1-P1 promoter map within a region of a very high degree of homology. **A:** The Nex1-P1 promoter is characterized by multiple transcription start sites. Primer extension was carried out as described in Materials and Methods. The location of the primer is shown in **(B)**. The left panel shows the primer extension analysis using 1 µg of poly(A) mRNA from NGF-treated PC12 cells (lane 5, 2 days; lane 6, 6 days), whereas the right panel represents the results obtained using poly(A) mRNA from mouse adult brain (lane 5), mouse embryo E16.5 (lane 6), and adult human cerebellum (lane 7). The extension products were analyzed next to a DNA sequence ladder (lanes 1–4) on a 6% denaturing polyacrylamide gel. Arrows indicate major transcription start sites, whereas stars denote minor transcription start sites. **B:** Sequence alignment of the mouse, rat, and human Nex1-P1 promoter regions. The primer used for primer extension analysis is indicated by PE and the corresponding nucleotides are underlined. The forward and reverse PCR primers used for cloning the Nex1-P1 promoter into pGL3-basic are indicated by P1 For and P1 Rev, respectively. The transcription start sites identified by primer extension and 5'RACE analyses from each species are shown by arrows above the sequence. The numbers on the right are the nucleotide positions relative to the translation initiation start site ATG. The intron located between non-coding exon 1a and coding exon 2 is illustrated by a vertical line, and therefore its sequence (1,294 bp) is not shown. Pertinent putative consensus binding sites are bold with their names indicated below the sequence. The stars indicate the homology between the three species.

Fig. 4.

DNase I footprinting analysis of the rat Nex1-P1 and Nex1-P2 promoter regions. The top figure shows a graphic representation of gene alignment analysis of the rat Nex1 5'UTR using ClustalW. Degree of homology is indicated, and the multiple transcription start sites are denoted by arrows. The binding sites protected from DNaseI digestion are also shown for each promoter region. Finally, the footprinting probes (FP) used for this analysis are shown below, and their numbers are indicative of their position relative to the translation start site. Results of the DNaseI footprinting protection assays are shown below the schematic representation of Nex1 5'UTR. Oligonucleotides used to generate FP probes are listed in Supplemental Table I, and the FP probes were cloned and single end-labeled as described in Materials and Methods. Nuclear extracts were prepared before (–) and during NGF treatment (1d, 3d, and 6d), as indicated at the top of each gel. DNaseI protection assays were carried out in the absence (C) or presence of 30 µg of nuclear extracts as described in the Materials and Methods. DNaseI digestion products along with corresponding sequencing reactions were resolved on a 6.5% denaturing polyacrylamide gel. Protected binding sites are indicated by a vertical bar along with the corresponding enhancer element. Arrows denote DNaseI hypersensitive sites.

Fig. 5.

EMSA analysis of putative regulatory elements found in the Nex1-P2 promoter region. **A–E:** Radiolabeled oligonucleotide probes were incubated with 7 μ g of nuclear extracts isolated from undifferentiated or NGF-differentiated PC12 cells (3 days), as indicated at the top of each figure. Binding reactions were carried out as described in Materials and Methods, and the products were separated on a 5% native gel. Lane 1 of each panel shows the corresponding probe alone as a control, whereas lanes 2–5 show the results of specific competition in the presence of increasing amounts of unlabeled specific wild-type competitors. Lanes 6–8 of each panel show the results of nonspecific competition in the presence of increasing amounts of cold mutated oligonucleotides. Free probes are indicated at the bottom of the gel. Arrows denote

specific DNA-protein complex. Sequences of wild-type and mutated oligonucleotides used for EMSA are indicated below the figure of each panel. A: EMSA analysis with the radiolabeled 5'Oct-1/2 containing probe. B: EMSA analysis with the radiolabeled 3'Oct-1/2 probe. C: EMSA analysis with the radiolabeled HNF/Oct-3 probe. D: EMSA analysis with the radiolabeled 5'Pax probe. E: EMSA analysis with the radiolabeled 3'Pax probe.

Fig. 6.

EMSA of the C/EBP-like binding site mapping downstream of Nex1-P2 transcription start sites. **A–E:** Radiolabeled oligonucleotides probes were incubated with 7 μ g of nuclear extracts isolated from NGF-differentiated PC12 cells (3 days). Binding reactions were carried out as described in Materials and Methods, and the products were separated on a 5% native gel. Free probes are indicated at the bottom of the gel. Arrows denote specific DNA-protein complex. Sequences of wild-type and truncated oligonucleotides are indicated below the figures. Lane 1 of each panel and lane 3 of (B) show the corresponding probe alone, as a control. **A:** The full-length C/EBP-like probe produces two specific complexes. Lanes 2–6 show the results of specific competition in the presence of increasing amounts of unlabeled specific full-length C/EBP-like oligonucleotide, whereas lanes 7–10 show the results of competition in the presence of nonspecific oligonucleotide. **B:** The 3'half of the C/EBP-like binding site is necessary for complex formation. **C:** The 3'half of the C/EBP-like site behaves as a specific competitor. Lanes 2–6 show the results of specific competition in the presence of the radiolabeled full-length C/EBP probe and increasing amounts of cold oligonucleotide carrying the 3'half site of the C/EBP-like binding site. **D:** The consensus C/EBP binding site competes with the full-length C/EBP-like binding site of Nex1-P2 promoter. Lanes 2–5 show the results of specific competition in the presence of radiolabeled full-length C/EBP-like probe and increasing amounts of cold oligonucleotides carrying the consensus C/EBP binding site described in Williams et al. (1995). **E:** C/EBP β protein is present in the shifted complex. NGF-differentiated PC12 nuclear extracts were incubated with either pre-immune antibody (lane 2) or anti-C/EBP β polyclonal antibody (lane 3), in the presence of full-length C/EBP-like probe, as described in Materials and Methods. The black arrows denote the major DNA-protein complexes, whereas the open arrow denotes the supershifted complex.

Fig. 7.

EMSA analysis of the putative regulatory elements associated with the Nex1-P1 promoter. **A–C:** Radiolabeled oligonucleotide probes were incubated with 7 μ g of nuclear extracts isolated from NGF-differentiated PC12 cells (3 days). Binding reactions were carried out as described in the Materials and Methods and the products were separated on a 5% native gel. Free probes are indicated at the bottom of the gel. Arrows denote specific DNA-protein complexes.

Sequences of wild-type and truncated oligonucleotides are indicated below the figures. Lane 1 of each panel shows the corresponding probe alone, as a control. Lanes 2–5 of each panel show the results of specific competition in the presence of increasing amounts of unlabeled, specific, wild-type competitors. Lanes 6–8 of each panel show the results of nonspecific competition in the presence of increasing amounts of cold mutated oligonucleotides. **A:** The CRE-containing oligonucleotide produces a single specific DNA-protein complex with nuclear extracts from NGF-differentiated PC12 cells. **B:** The conserved downstream E1 E-box produces two specific DNA-protein complexes with nuclear extracts from NGF-differentiated PC12 cells. **C:** The conserved upstream E4 E-box produces two specific DNA-protein complexes with nuclear extracts from NGF-differentiated PC12 cells.

Fig. 8.

The Nex1-P2 promoter is stronger than the Nex1-P1 promoter in NGF or dbcAMP treated PC12 cells. A schematic representation of the Nex1-P1 and Nex1-P2 luciferase reporter constructs is shown at the top of the figure. The protected binding sites are shown for each promoter, and the introns 1a and 1b are indicated by dashed lines. PC12 cells were transfected with 5 μ g of Nex1-P1 or Nex1-P2 luciferase-reporter constructs and 0.5 μ g of pRL-TK internal control vector, as described in the Materials and Methods. Transfected cells were either untreated (open bars) or treated with NGF (50 ng/ml) (black bars) or dbcAMP (1 mM) (gray bars) for 3 days. The relative luciferase activity is defined as the ratio of the light units of Firefly luciferase vs. Renilla luciferase. Results are expressed as fold increase $\pm$ SD. The experiments were carried out in triplicate and repeated three times independently.

Fig. 9.

The conserved CRE element is critical for Nex1-P1 promoter activity on NGF or dbcAMP signaling. A schematic representation of wild-type or mutated Nex1-P1 luciferase reporter construct is shown on the left side of the graph. The mutated enhancer element is marked by an X. PC12 cells were transfected as described in the Materials and Methods with 0.5 μ g of pRL-TK internal control vector, 5 μ g of Nex1-P1 luciferase-reporter construct, and 8 μ g of CREB or KCREB eukaryotic expression vector, as indicated in the figure. Transfected cells were either untreated (open bars) or treated with NGF (50 ng/ml) (black bars) or dbcAMP (1 mM) (gray bars) for 3 days. The relative luciferase activity is defined as the ratio of the light units of Firefly luciferase vs. Renilla luciferase. Results are expressed as fold increase $\pm$ S.D. The experiments were carried out in triplicate and repeated three times independently. **A:** Synergistic effect of cAMP on CREB-mediated Nex1-P1 promoter activity. **B:** The conserved CRE element is essential for Nex1-P1 promoter activity. **C:** Mutation of E1 or E4 E-boxes does not alter Nex1-P1 promoter activity on NGF and cAMP signaling.

Fig. 10.

The conserved C/EBP binding site is essential for the Nex1-P2 promoter activity in NGF-differentiated PC12 cells. A schematic representation of wild-type or truncated Nex1-P2 luciferase reporter construct is shown on the left side of the graph. PC12 cells were transfected as described in Materials and Methods with 0.5 μ g of pRL-TK internal control vector and 5 μ g of wild-type or truncated Nex1-P2 luciferase-reporter construct, as indicated in the figure. Transfected cells were treated with NGF (50 ng/ml) for 3 days. The relative luciferase activity is defined as the ratio of the light units of Firefly luciferase vs. Renilla luciferase. Results are expressed as fold increase relative to the full-length Nex1-P2-AD construct $\pm$ SD. The experiments were carried out in triplicate and repeated three times independently. **A:** The C/EBP binding site is the only required regulatory element for maximal Nex1-P2 promoter activity. **B:** C/EBP β upregulates the activity of Nex1-P2 promoter in NGF-differentiated PC12 cells. PC12 cells were cotransfected with 5 μ g of either full-length Nex1-P2-AD, 3'truncated Nex1-P2-AC3, or 3'truncated Nex1-P2-AC2 luciferase-reporter and 2.5 μ g of eukaryotic expression construct carrying either wild-type C/EBP β , truncated LIP-C/EBP β , or dominant-negative A-C/EBP mutant. PC12 cells were then differentiated with NGF, and luciferase assays were carried out as described above.

TABLE I

Sequence of DNase I Protected Areas of the P1 and P2 Promoter Regions

Binding Site	DNase I Protected Sequence	Promoter Coordinates
Oct1 distal	5' CTGGTGTTT <u>ATACAAA</u> ATCATATTTA 3'	P2 -3389 to -3363
5' Pax	5' TATATTT <u>AAACACATCA</u> CTAGT 3'	P2 -3334 to -3314
3' Pax	5' CAAATT <u>TCTAAAATG</u> ACTGATGTGA 3'	P2 -3296 to -3273
HNF/Oct3	5' TATTTTAATCTATA <u>AAATGAATAG</u> 3'	P2 -3234 to -3211
3' Oct1	5' CCAGGCTGCC <u>CAGAAAT</u> TAT 3'	P2 -2916 to -2898
C/EBP-like	5' ACTTTTTCAAAAGTTCAC <u>TTTTCGCCT</u> 3'	P2 -2824 to -2800
E4 E-box	5' ACAGGGATGCC <u>CAGATG</u> ATCAGTG 3'	P1 -1572 to -1550
CRE	5' <u>TGTGACGTC</u> ACTAGCAGATG 3'	P1 -1505 to -1486
E1 E-box	5' CTGG <u>CAGTTG</u> GCATCAATGTCA 3'	P1 -1470 to -1449
CNE	5' GGAAAGATCC <u>AAAAT</u> GAAAACCTGATTATTGAATGAAATTAACCTAAGGTAATATAAATAAAGAT 3'	P1 -1363 to -1299

The core consensus binding site is indicated by bold and underlined letters. The promoter coordinates are relative to the translation start site.