The GAP-43 Gene Is a Direct Downstream Target of the Basic Helix-Loop-Helix Transcription Factors*

(Received for publication, November 20, 1995, and in revised form, May 2, 1996)

Anne Chiaramello‡§, Toomas Neuman§, Dena R. Peavy‡§, and Mauricio X. Zuber†§†

From the Departments of §Biochemistry and Molecular Biology and ‡Anatomy and Neurobiology and the §Program of Neuronal Growth and Development, Colorado State University, Fort Collins, Colorado 80523

The GAP-43 promoter region contains seven E-boxes (E1 to E7) that are organized in two clusters, a distal cluster (E3 to E7) and a proximal cluster (E1 and E2). Deletion analysis and site-directed mutagenesis of the GAP-43 promoter region showed that only the most proximal E1 E-box significantly modulates GAP-43 promoter activity. This E-box is conserved between the rat and human GAP-43 promoter sequences in terms of flanking sequence, core sequence (CAGTTG), and position. We found that endogenous E-box-binding proteins present in neuronal N18 cells recognize the E1 E-box and that the E1 E-box is conserved in the N18 promoter. The transcriptional activity of the GAP-43 promoter was repressed not only by the negative regulator Id2 protein, but also by two class A basic helix-loop-helix proteins, E12 and ME1a. In vitro analyses showed that both ME1a and E12 bind to the E1 E-box as homodimers. By Northern analyses, we established an inverse correlation between the level of E12 and ME1a mRNAs and GAP-43 mRNA in various neuronal cell lines as well as in ME1a-overexpressing PC12 cells. Therefore, we have identified a cis-acting element, the E1 E-box, located in the GAP-43 promoter region that modulates either positively or negatively the expression of the GAP-43 gene depending on which E-box-binding proteins occupy this site. Together, these data indicate that basic helix-loop-helix transcription factors regulate the expression of the GAP-43 gene and that the class A ME1a and E12 proteins act as down-regulators of GAP-43 expression.

Elucidation of the molecular mechanisms governing the development and differentiation of neural cells has been greatly expanded by the study of the basic helix-loop-helix (bHLH) transcription factors (1–3). In Drosophila, the proneural bHLH genes achaete-scute and daughterless are involved in the determination of cells to become neuronal precursors (4). The daughterless mutants are characterized by an abnormal cell pattern in the central nervous system and by the absence of neurons in the peripheral nervous system (5). The proneural gene aonal also encodes a bHLH transcription factor that is essential for the commitment of the mother cells of internal sensory (chordotonal) organs and the ommatidia of the compound eye (6). The rat homologue of Drosophila achaete-scute, mash-1, is expressed in the developing nervous system during neurogenesis and is essential for the generation of the olfactory system and the autonomic nervous system (7, 8). Recently, a mammalian bHLH gene, neuroD, has been isolated and characterized (9). It is transiently expressed in postmitotic differentiating neurons during mouse embryonic development and triggers premature neuronal differentiation when ectopically expressed.

Accumulating evidence suggests that tissue-specific genes are likely candidates for timely regulation by bHLH transcription factors. Upon differentiation, postmitotic neuroblasts express the GAP-43 gene at high levels in their growth cones during axonal growth and synapse formation (10–12). While the potential functions of GAP-43 have been extensively investigated, the molecular mechanisms responsible for the spatial and temporal expression of GAP-43 are still poorly understood. As brain development proceeds, the pattern of GAP-43 expression becomes progressively more restricted to areas of the brain associated with synaptic plasticity such as the hippocampus and olfactory bulb. In addition, GAP-43 expression augments during regeneration of peripheral nerves and certain types of central nervous system injury (13, 14). It has been postulated that GAP-43 may play a role in axonal outgrowth or growth cone formation by interacting with the transduction machinery responsive to extracellular as well as intracellular signals (15–18). Recently, it was demonstrated by gene targeting that GAP-43 is involved in axon pathfinding at the optic chiasma during perinatal development (19).

Although the mechanism of GAP-43 regulation remains unclear, recent studies suggest that the most likely mechanism of regulation might occur at the level of transcription initiation, although the GAP-43 mRNA stability may also be affected (10, 20, 21). As a step toward the elucidation of GAP-43 regulation, the rat and human GAP-43 promoter regions have been sequenced and characterized (22, 23). Recently, two distinct promoter regions in the 5′-regulatory region of GAP-43 have been mapped, P1 (located between −750 and −407) and P2 (located within the −233 bp from the translational start codon), which is activated during neuronal differentiation of P19-EC cells and in 8-day-old rat brain (24). Furthermore, it seems that the ratio of P1 and P2 promoter activity varies during neuronal differentiation and at different developmental stages of neurogenesis. Therefore, regulatory elements must exist in these two promoter regions to modulate their activities during neurogenesis.

Our objective has been to determine whether there is any cis-acting element responsible for the neuron-specific expres-
tion of GAP-43. No classical regulatory elements involved in transcriptional regulation, such as TATA and CAAT boxes and SP1-binding sites, were detected in the P2 350-bp promoter region (22). Potential consensus binding sites for AP1, AP2, Myb, and NF-IL6 have been found in the GAP-43 promoter region (24). However, none of these putative responsive elements are core-binding sites for proteins that are cell type-specific and developmentally regulated. Upon examination of the rat gap-43 5′-regulatory region, seven putative cis-acting enhancer E-boxes were found spanning the two promoter regions P1 and P2. These E-box elements, characterized by a core DNA sequence (CANNTG), are binding sites for bHLH transcription factors that are responsible for tissue-specific transcription (25, 26). The BHLL transcription factors are characterized by a conserved basic helix-loop-helix structural motif and bind DNA as dimers to modulate transcription of target genes. The BHLL proteins have been classified into distinct classes based on their structure and pattern of expression (reviewed by Murre et al. (27)). BHLL proteins like Id1, Id2, and Emc lack the basic domain and function as dominant-negative BHLL proteins to form nonfunctional heterodimers with BHLL proteins (28–30). In this study, we addressed the question whether BHLL proteins are involved in the regulation of GAP-43 expression. To understand the molecular mechanisms underlying the transcriptional regulation of GAP-43 expression, we examined the transcriptional activity of the seven putative E-boxes by deletion analysis. We demonstrated that the first proximal E1 E-box is crucial to the modulation of GAP-43 promoter activity. The fact that this E-box is conserved in terms of position and sequence between the rat and human GAP-43 promoter regions strengthens the idea of its potential role to control the specific spatial and temporal expressions of GAP-43 during brain development. We found that the E1 E-box has a dual function in the regulation of GAP-43, depending on which BHLL proteins recognize this site. The E1 E-box has the potential to either enhance or silence GAP-43 promoter activity. We identified two class A BHLL proteins, E12 and ME1a, that suppress GAP-43 promoter activity, indicating that class A BHLL proteins have the ability to function as either positive or negative regulators of transcription. In vivo, we observed a correlation between the level of endogenous GAP-43 expression and the BHLL proteins E12 and ME1a in various neuronal cell lines. EXPERIMENTAL PROCEDURES Recombinant Plasmids—The eukaryotic expression plasmids pRcCMV/ME1a and pRcCMV/Id2 were as described previously (31). The eukaryotic expression plasmid pRcCMV/E2 was a gift from H. Weintraub. All GAP-43 CAT constructs (GHC, GXC, GAC, and GNC) were derivatives of the promoterless CAT plasmid pSP64/CAT. The GHC plasmid contains a kilobase pair of rat gap-43 promoter sequence with the three major transcriptional start sites as well as the seven E-boxes (E1 to E7). The 5′-deletions of the rat gap-43 promoter region were created from the pHGH plasmid using the GEC construct was obtained by Nhe digestion and does not carry any E-box. The GAC construct was obtained by digesting the GHC plasmid with the Accl enzyme and carries the first proximal E1 E-box. The GXG construct was obtained by Xbal digestion and contains the first two proximal E-boxes (E1 and E2). The HindIII-XbaI fragment containing the last four E-boxes (E4 to E7) was subcloned into pminiCAT (30) and is referred as the G4miniCAT reporter plasmid. The first two E-boxes (E1 and E2) were subcloned into the XbaI and BamHI sites of the pminiCAT vector by polymerase chain reaction using two primers producing two different termini, XbaI and BamHI, and the Taq polymerase enzyme (Life Technologies, Inc.). The sequence of each primer is as follows, with the incorporated restriction enzyme site underlined: XbaI primer, 5′-CCGGCTGACAGAGAATGCTATGC-3′; and BamHI primer, 5′-GGTGGATCATCTTCCTTCACACAAC-3′. Site-directed Mutagenesis—Site-directed mutagenesis within the rat gap-43 promoter was performed by the method of Deng and Nickoloff (33).
Transcriptional Regulation of GAP-43 Promoter by bHLH Proteins

amounts of 1d2 were added to the binding reactions. Recombinant ME1a protein was purified by nickel affinity chromatography (31), and E12 protein was purchased from Santa Cruz Biotechnology. DNA binding reactions consisted of 60 ng of either ME1a or E12 protein incubated with 40 fmol of labeled specific oligonucleotides and 100 ng of poly(dI-dC). For competition experiments, a 100-fold excess of specific (E1) and nonspecific (mutated E1) competitors was added to the binding reaction. The products of the DNA binding reactions were separated on a 5% native gel containing 0.5× Tris/boric acid/EDTA (45 mM Tris, 45 mM boric acid, and 1 mM EDTA). Gels were dried and exposed on Hyperfilm MP x-ray film (Amersham Corp.) at −70°C.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated using the acid guanidinium/phenol/chloroform extraction procedure (40). Twenty-five micrograms of total RNA were run in each lane and fractionated on a 1.2% formaldehyde-agarose gel before transfer onto a nylon membrane (Hybond N, Amersham Corp.). The amount and quality of transferred RNA were monitored either by methylene blue staining of the filters before hybridization or by using an internal control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The Spcl-BamHI fragment from ME1a cDNA, the full-length E12 cDNA, the full-length GAPDH cDNA, and the HindIII-PstI fragment from rat gap-43 cDNA were isolated, radiolabeled with [32P]dCTP using the Multiprime DNA labeling system (Amersham Corp.), and used as probes. The blots were washed at high stringency (0.2× SSC, 65°C) and exposed to x-ray film for 1–10 days.

RESULTS

The E1 E-box is Critical for GAP-43 Promoter Activity—Upon analysis of the 1-kilobase pair rat gap-43 promoter region, we detected seven putative E-boxes, which are binding sites for the developmentally regulated and cell type-specific bHLH transcription factors (Fig. 1A). These E-boxes were designated E1 to E7 according to the order of the distance from the translation start site of GAP-43. The E-boxes E3 to E7, located in the P1 promoter region (−705 to −205), are not conserved between rat and human GAP-43 promoter sequences, whereas the E1 and E2 E-boxes, clustered in the P2 promoter region (−233 to −1), are conserved between rat and human GAP-43 sequences. However, only the first E-box E1 is perfectly conserved in terms of position, core sequence (CAGTTG), and flanking sequences (23), suggesting that this regulatory element has a critical function.

To investigate the activity of these E-boxes, we first constructed several GAP-43 5′-deletion mutants that were fused to the promoterless bacterial CAT reporter gene (Fig. 1B). The GNC construct represents a negative control that does not contain any E-box element, but has three major transcription start sites as defined by Grabczky et al. (22). The GAC construct carries a single E-box, the E1 E-box, whereas the GXC construct contains the two clustered E-boxes, E1 and E2. The GHC construct represents the wild-type GAP-43 promoter region (1 kilobase pair) with the seven E-boxes. In all of these constructs, the expression of the CAT gene is driven by the GAP-43 promoter. We examined the transcriptional activity of the various GAP-43 reporter-promoter plasmids by transient CAT assays using the N18 neuroblastoma cell line. This cell line was chosen based on the Northern analysis results, which indicate high expression of endogenous GAP-43 (see Fig. 7). As shown in Fig. 2, the 90-bp fragment containing the E1 E-box was sufficient to induce maximal activation of the GAP-43 promoter (6.4-fold), whereas only basal promoter activity was observed with the GNC construct, which does not contain any E-box. In contrast, the GXC construct, which carries an extra E-box (E2) compared with the GAC construct, did not display stronger transcriptional activity compared with GAC plasmid (6.8-fold). In addition, the transcriptional activity observed with both the GAC and GXC constructs was similar to the activity of the full-length GAP-43 promoter construct GHC. These data suggest that the E1 E-box, located in the P2 promoter region, is a functionally important regulatory cis-acting element for GAP-43 expression. Furthermore, the six distal E-boxes do not seem to contribute to GAP-43 promoter activity. To further characterize these E-box elements, we cloned the two clusters of E-boxes, the proximal E1-E2 cluster and the distal E4-E7 cluster, upstream of the minimal herpes simplex virus thymidine kinase (tk) promoter (G2minCAT and G4minCAT, respectively). Transient CAT assays were performed in N18 neuroblastoma cells (Fig. 3). Only the G2minCAT construct showed transcriptional activity above
background levels, with a 5.4 fold-increase, which is comparable to the activity obtained using the homologous reporter-promoter construct GXC. In contrast, the G4minCAT construct did not display any transcriptional activity, which is in agreement with the transcriptional data obtained by 5'-deletion analysis of the GAP-43 promoter region. Taken together, these data imply that the E1 E-box element may be sufficient to modulate the transcriptional activity of both the GAP-43 promoter and the heterologous tk promoter.

To determine whether the E1 E-box is critical to GAP-43 promoter activity, we introduced mutations into the E1 or E2 E-box of either the GXC or GHC plasmid by site-directed mutagenesis (Fig. 1C). The GXC reporter-promoter plasmid, in which the E1 E-box sequence (CAGTTG) was replaced by a mutated E-box sequence (TAGTTC), showed a 18-fold reduction in transcriptional activity (Fig. 4, lanes 5 and 6) compared with the wild-type reporter-promoter plasmid GXC (lanes 1 and 2). In contrast, the transcriptional activity of another mutant construct, GXC/E2m, in which the E2 E-box sequence (CATATG) was replaced by a mutated E-box sequence (TATATC), showed a 18-fold reduction in transcriptional activity (Fig. 4, lanes 5 and 6) compared with the wild-type reporter-promoter plasmid GXC (lanes 1 and 2). In the absence of the eukaryotic expression plasmid Id2, 8 μg of the parental eukaryotic expression plasmid were used. The CAT activities were quantified by PhosphorImager analysis. The percent acetylated [14C]chloramphenicol, -fold activation, and repression are indicated above the lanes. Acetylated (Cm-3-Ac) and non-acetylated (Cm) forms of chloramphenicol are indicated on the left.

Endogenous E-box-binding Proteins Recognize the E1 E-box—To examine the binding activity of endogenous E-box-binding proteins that interact with the E1 E-box, we performed EMSA using nuclear proteins isolated from N18 neuroblastoma cells and an oligonucleotide containing the E1 E-box sequence. Incubation of the E1 E-box probe with N18 nuclear extracts produced a major retarded band (Fig. 5, lane 2). This shifted band was competed with a 50-fold excess of unlabeled specific oligonucleotide (Fig. 5, lane 5). When an excess of unlabeled competitor containing a mutation in the E1 E-box sequence (nonspecific competitor) was added to the binding...
The transcriptional regulation of GAP-43 promoter by bHLH proteins

Overexpression of Id2 Suppresses GAP-43 Promoter Activity—To examine whether GAP-43 promoter activity would be repressed by the negative transcriptional regulator Id-like proteins, we overexpressed Id2 in N18 neuroblastoma cells and performed CAT assays. As Id2 protein lacks the DNA-binding basic region, it forms nonfunctional heterodimers and consequently negatively regulates the transcriptional activity of some E-box-binding proteins. Fig. 3 shows that Id2 significantly reduced the transcriptional activity of the G2minCAT reporter plasmid, which contains the clustered E1 and E2 E-boxes. In contrast, the last four E-boxes from the G4minCAT construct were not affected by Id2 (Fig. 3). We therefore tested whether the E1 E-box was also a target for Id2 repression in the native context of the GAP-43 promoter by cotransfecting N18 cells with the GAC reporter plasmid and the eukaryotic expression plasmid pRcCMV/Id2. Overexpression of the negative regulator Id2 resulted in a decrease in CAT activity (Fig. 4). Thus, Id2 protein suppresses the binding of endogenous E-box-binding proteins to the E1 E-box element. Eight micrograms of N18 nuclear extracts were incubated with 40 fmol of labeled oligonucleotide that carries the wild-type E1 E-box sequence for 20 min at room temperature (lane 2). When heterodimer formation was studied between Id2 and the endogenous E1 E-box-binding proteins, increasing amounts of purified recombinant protein Id2 were added to the binding reaction. The specific DNA-protein complex was blocked in a dose-dependent manner (Fig. 4, lanes 3–5). As a control, lane 1 shows the probe alone.

ME1a and E12 bHLH Proteins Repress GAP-43 Promoter Activity—If the E1 E-box is involved in the orchestration of the appropriate expression of GAP-43, GAP-43 promoter activity should be regulated by ubiquitously expressed class A bHLH proteins. We examined the effect of the class A bHLH transcription factors E12 and ME1a on GAP-43 expression. ME1a is the murine homologue of the human HEB transcription factor (41). E12 and ME1a are expressed at high level in areas of the brain where rapid cell proliferation occurs and no GAP-43 expression is detected (42, 43). To examine the relationship between the enhancer activity of the E1 E-box and the class A bHLH proteins E12 and ME1a, we cotransfected the

Fig. 5. E-box-binding proteins present in N18 cells bind to the E1 E-box sequence. An oligonucleotide carrying the wild-type sequence of the E1 E-box (underlined; 5'-TAGACCTACAGTTGCT-GCTAAGTGGCCTGAA-3') was end-labeled, and 40 fmol of labeled oligonucleotides were incubated in the presence of 8 µg of N18 nuclear extracts. The electrophoretic mobility shift assay was also performed in the presence of different excesses of unlabeled specific and nonspecific competitors as indicated above the lanes. The nonspecific competitor contains a mutation (boldface) in the E1 E-box sequence (5'-TAGAC-CTTATAGTCTCTGACTGGCCTGA-3'). The DNA-protein complex formed between neuronal nuclear proteins and the E1 E-box is specific since it cannot be competed by nonspecific competitor. As a control, lane 1 shows the probe alone.

Fig. 6. Suppression of GAP-43 promoter activity by overexpression of Id2. N18 neuronal cells were transfected with 2 µg of the reporter plasmid GAC in the presence of 8 µg of either the expression plasmid pRcCMVneo without any DNA sequence or eukaryotic expression plasmid containing Id2 cDNA by a calcium phosphate precipitation technique. Cells were harvested, and CAT activities were determined by PhosphorImager analysis.

Fig. 7. Id2 protein suppresses the binding of endogenous E-box-binding proteins to the E1 E-box element. Eight micrograms of N18 nuclear extracts were incubated with 40 fmol of labeled oligonucleotide that carries the wild-type E1 E-box sequence for 20 min at room temperature (lane 2). When heterodimer formation was studied between Id2 and the endogenous E1 E-box-binding proteins, increasing amounts of purified recombinant protein Id2 were added to the binding reaction. The specific DNA-protein complex was blocked in a dose-dependent manner (Fig. 7, lanes 3–5). As a control, lane 1 shows the probe alone.
reporter-promoter plasmid GAC with a eukaryotic expression plasmid carrying the coding sequence for either E12 or ME1a. As shown in Fig. 8, cotransfection of the E12 expression vector with the GAC reporter-promoter plasmid resulted in a complete repression of GAP-43 promoter activity (lanes 5 and 6). When the activity of the E1 E-box was challenged in the presence of either E12 or ME1a as indicated under the lanes. Cells were harvested, and CAT was activity determined. Percent acetylation and -fold repression in the presence of either E12 or ME1a are shown above the lanes. Acetylated (Cm-3Ac) and non-acetylated (Cm) forms of chloramphenicol are indicated on the left.

**Fig. 8.** Both class A bHLH proteins E12 and ME1a repress GAP-43 promoter activity. N18 neuronal cells were transfected by calcium phosphate with 2 µg of reporter plasmid and 8 µg of eukaryotic expression plasmid containing the coding sequence of either E12 or ME1a as indicated under the lanes. Cells were harvested, and CAT was activity determined. Percent acetylation and -fold repression in the presence of either E12 or ME1a are shown above the lanes. Acetylated (Cm-3Ac) and non-acetylated (Cm) forms of chloramphenicol are indicated on the left.

The Level of the Endogenous Expression of the GAP-43 Gene Is Inversely Correlated to the Endogenous Expression of ME1a and E12. To assess the ME1a- and E12-mediated repression of GAP-43 expression, we examined the correlation between the levels of expression of the E12, ME1, and GAP-43 genes in various neuronal cell lines by Northern blotting. The rat hippocampal cell line HTF4, the rat glioblastoma cell line C6, the neuroblastoma cell line N18, and the mouse neuroblastoma × rat glioma hybrid cell line NG108 were used to evaluate the endogenous expression of GAP-43, ME1, and E12. Northern blot analyses showed that GAP-43 was expressed in all cell lines examined, except in the hippocampal HTF4 cell line (Fig. 10). N18 neuroblastoma cells displayed the highest level of endogenous GAP-43 expression (Fig. 10). In contrast, ME1 and E12 mRNAs were both expressed at high levels exclusively in the hippocampal HTF4 cells, where no GAP-43 expression was detected (Fig. 10). Therefore, we observed an inverse correlation between the endogenous level of ME1 and E12 and the neuron-specific GAP-43 gene, which is in full agreement with the transcriptional data obtained by CAT assays.

To further confirm the functional relationship between the elevated level of ME1a and the lack of GAP-43 expression in HTF4 cells, we generated PC12 cell lines that overexpress ME1a and analyzed the expression of the GAP-43 gene in these cell lines by Northern blotting. The level of ME1a mRNA was barely detectable in control PC12 cells, which carry only the selectable marker, whereas the level of GAP-43 mRNA was high in these cells (Fig. 11, lane 1). NGF treatment of the control PC12 cells resulted in a 2–3-fold increase in GAP-43 mRNA and a slight increase in ME1a mRNA (Fig. 11, lane 2). In contrast, GAP-43 expression decreased significantly, concomitant with the increase of ME1a mRNA in control ME1a-overexpressing PC12 cells (Fig. 11, lane 3). Nerve growth factor treatment of ME1a-overexpressing PC12 cells resulted in stimulation of the endogenous expression of ME1a accompanied by a low level of GAP-43 expression (Fig. 11, lane 4). Despite the induction of GAP-43 expression in ME1a-overexpressing cells after NGF treatment, the level of GAP-43 mRNA remained 10–15 times lower than in control NGF-treated PC12 cells (Fig. 11, lanes 2 and 4). Although Fig. 11 represents the data obtained from one ME1a-overexpressing clone, identical results were obtained with three additional clones as well as a pool of populations of ME1a-overexpressing clones (data not shown).
Transcriptional Regulation of GAP-43 Promoter by bHLH Proteins

**Fig. 10.** The level of endogenous GAP-43 expression is inversely correlated to the level of ME1 and E12 in various neuronal cell lines. Total RNA was isolated from different neuronal cell lines: rat hippocampal HTF4 cells, murine N18 neuroblastoma cells, NG108 mouse neuroblastoma × rat glioma hybrid cells; and rat glioblastoma C6 cells. Twenty-five micrograms of each RNA were electrophoresed on a 1.2% formaldehyde-agarose gel. The RNA was transferred onto a nylon membrane and hybridized with various radiolabeled probes against GAP-43, ME1, and E12. Equal amounts of RNA were run in each lane, as indicated by the intensities of the 18 S and 28 S ribosomal RNA bands revealed by methylene blue staining of the filter before hybridization.

**Fig. 11.** Overexpression of ME1a represses GAP-43 expression in PC12 cells. Total RNA was isolated from untreated and NGF-treated PC12 cells and ME1a-overexpressing PC12 cells. Northern blot analysis was carried out as described under "Experimental Procedures" with two different probes, ME1a and GAP-43. Equal loading of RNA was confirmed by hybridization with the GAPDH probe. The position of each RNA is indicated by an arrow on the right.

These results support the Northern analysis data obtained from various neuronal cell lines. Hence, these in vivo data, in combination with the CAT assay data, indicate that down-regulation of GAP-43 expression by ME1a and E12 proteins could have a significant biological function.

**DISCUSSION**

The central issue addressed in this study is the transcriptional regulation of the neuron-specific GAP-43 gene. Previous experiments showed that the expression of the GAP-43 gene is mainly controlled at the transcriptional level during neuronal development and regeneration (10, 22) and that the first 1.5 kilobase pairs of promoter sequence are sufficient to confer neuron-specific expression (44). Furthermore, it was recently demonstrated that only one of the two promoters, the P2 GAP-43 promoter (−233 to −1), is transcriptionally active during neuronal differentiation of P19-EC cells and in 8-day-old rat brain (24). Since the expression of the GAP-43 gene is first detected in postmitotic neuroblasts, these immature neurons must express specific transcription factors that recognize cis-acting elements within the promoter to modulate transcription of the GAP-43 promoter.

We detected seven putative E-boxes (E1 to E7) in the 5′-flanking sequence of the rat gap-43 gene that are organized in two clusters, a proximal cluster (E1 and E2) and a distal cluster (E3 to E7). In this study, we have demonstrated that the expression of the GAP-43 gene is regulated by bHLH transcription factors. This regulation is mainly mediated through the first proximal E1 E-box, which is located in the neurally active P2 promoter. Furthermore, among the seven E-boxes present in the GAP-43 promoter region, the E1 E-box is the only E-box to be conserved between the rat and human GAP-43 promoter regions in terms of core sequence, flanking sequence, and position. Five lines of evidence provide strong support that the E1 E-box is a critical regulatory element for GAP-43 promoter activity. First, 5′-deletion analysis showed that the 90-bp region containing the E1 E-box is necessary and sufficient to confer activity to the GAP-43 promoter. Second, mutation of the E1 E-box abolishes the transcriptional activity of the GAP-43 promoter. Third, endogenous E-box-binding proteins specifically recognize the E1 E-box sequence. Fourth, the activity of the E1 E-box-binding proteins is suppressed by the negative regulator Id2. Finally, repression of GAP-43 promoter activity is mediated by the two class A bHLH proteins ME1a and E12.

Two distinct approaches were used to study the transcriptional modulation of the GAP-43 promoter, CAT assays and RNA analysis by Northern blotting. To elucidate which cis-acting elements are critical to GAP-43 promoter activity in CAT assays, we used homologous reporter-promoter constructs (GHC, GXC, and GAC) to maintain the original conformation of the GAP-43 promoter region. The GAP-43 promoter region carries unusual structural features such as two long homopurine-homopyrimidine stretches (22). They are both located on the coding strand, one between −238 and −371 containing a perfect 80-bp-long dinucleotide GA repeat and the other GA-rich region between −118 and −168. These homopurine-homopyrimidine regions have the potential to form a triple-stranded structure referred to as H-DNA and also may bind proteins (45, 46). These sequences are often found in the vicinity of promoters and are associated with S1-sensitive sites (47).

Induced triplex formation detected within the c-myc promoter has been found to promote c-myc expression (48, 49). Mutational analysis demonstrated that the E1 E-box is crucial for GAP-43 expression. The importance of the E1 E-box was confirmed by transient CAT assay experiments using the heterologous herpes simplex virus tk promoter containing either the two proximal clustered E-boxes (E1 and E2) or the four distal clustered E-boxes (E4 to E7). Enhancer activity was obtained only through the proximal E-box cluster (E1 and E2) and not through the distal E-box cluster (E3 to E7). However, one cannot exclude that these distal E-boxes may become active in a different developmental or cellular context.

To further support the idea that bHLH transcription factors are involved in GAP-43 gene expression, the transcriptional activity of both clusters (E1-E2 and E3-E7) was challenged by overexpressing the Id2 protein. The Id-like proteins interfere with transcriptional activation mediated by bHLH proteins by forming nonfunctional dimers (28, 30). Only the transcriptional activity of the E1-E2 cluster was suppressed by overexpressing Id2. Similar results were obtained using the GAP-43 reporter-promoter plasmid GAC, which contains only the E1 E-box. Furthermore, E1 E-box binding activity was present in nuclear extracts prepared from N18 neuroblastoma cells and was affected by the negative regulator Id2 protein. The protein complex interacting with the E1 E-box was not competed by the mutant oligonucleotide in which CAGTTG was substituted by
TAGTTC. Thus, these results suggest that endogenous bHLH proteins bind to the E1 E-box and consequently activate GAP-43 promoter activity, an activity that is affected by the action of Id2.

Thus, in this study, we have identified a cis-acting element, the E1 E-box, that modulates the transcriptional activity of the GAP-43 promoter. In previous studies, Starr et al. (44) have suggested that the GA region (~168 to ~118) is necessary for minimal GAP-43 promoter activity, based on results from deletion analysis. Due to the fact that the E1 E-box and the GA region were both deleted in this study, it is impossible to conclude whether the lack of transcriptional activity was due to the absence of either the GA repeat or the E1 E-box or both. However, our present results provide evidence that the E1 E-box is a key element in the regulation of GAP-43 promoter activity. Nevertheless, it is likely that the GA region contributes to the conformation of the GAP-43 promoter region, allowing the binding of regulatory E-box-binding proteins to the E1 E-box. In addition, since the GA sequence contains several Ets-binding sites, it is possible that the Ets proteins facilitate the binding of bHLH proteins on the E1 E-box. It was previously reported that the presence of the Ets-binding sites is crucial to the bHLH-mediated transcriptional activity of the Ig heavy chain enhancer element (50).

When the activity of the E1 E-box was challenged with the expression of two class A bHLH transcription factors, E12 and ME1a, we observed a strong repression of GAP-43 promoter activity. The notion that the GAP-43 promoter acts as a direct downstream target for ME1a- and E12-mediated repression is also supported by the results of our in vivo experiments. We analyzed the correlation between the ME1, E12, and GAP-43 mRNA levels in various cell lines. An inverse correlation between ME1, E12, and GAP-43 mRNA levels has been observed over four different cell lines. The rat hippocampal HTF4 cells, which are characterized by a significant amount of both ME1 and E12 mRNAs, do not express GAP-43 mRNA. In contrast, the N18 neuroblastoma cells, as well as two others cell lines, NG108 and C6, display a significant level of GAP-43 expression accompanied by the undetectable expression of either ME1 or E12. When the level of ME1a mRNA was artificially elevated by engineering ME1a-overexpressing PC12 cell lines, the expression of GAP-43 was also drastically diminished, supporting the notion that in vivo, ME1a regulates the transcriptional activity of the GAP-43 promoter through the E1 E-box. Previous experiments demonstrated that in NGF-treated PC12 cells, GAP-43 expression is regulated at the post-transcriptional level by affecting the stability of GAP-43 mRNA (20, 21). This is in agreement with our data since the level of GAP-43 mRNA in NGF-treated ME1a-overexpressing PC12 cells remains at a detectable level. Therefore, GAP-43 expression is regulated at both the transcriptional and post-transcriptional levels. In summary, the effect of down-regulation of GAP-43 expression seems to be directly correlated with the level of expression of both transcription factors E12 and ME1a. The fact that they suppress GAP-43 promoter activity in CAT assays as well as the expression of the GAP-43 gene indicates that this repression obtained by CAT assays is not a reflection of an artificially created conformation of the GAP-43 promoter region. The negative transcriptional effects of ME1a and E12 stand in contrast to previous studies that demonstrate E12- and ME1a-mediated transcriptional activations from various heterologous promoters (31, 41). They suggest that the activity of ME1a and E12 as either an activator or a repressor may be influenced by the promoter context. Thus, the E1 E-box is a target for both positive and negative regulators and plays a dual role. It can function either as an enhancer or a repressor depending on which bHLH proteins occupy this site. There is precedent for ME1a-mediated negative regulation of promoter activity. The rat and human low affinity nerve growth factor p75LNGFR promoters are characterized by a single conserved E-box located 20 bp upstream from the major transcription start sites (38). The activity of this E-box is also differentially modulated depending on the bHLH proteins binding to the site. In the case of the p75LNGFR promoter, only ME1a mediated repression, whereas E12 did not affect promoter activity. In vivo and in vitro data demonstrated that ME1a protein binds and represses the p75LNGFR promoter and consequently may block transcriptional activation by a lack of interactions with components of the transcriptional machinery (38). Therefore, it is possible that ME1a and E12 may repress GAP-43 promoter activity by a similar mechanism. These two class A bHLH transcription factors would dislodge positive E-box-binding proteins and inactivate GAP-43 promoter activity. Thus, GAP-43 promoter activity would be the result of the outcome of a protein competition occurring at the E1 E-box.

Little is known about the mechanisms of active transcriptional repression. By analogy with the mechanism of transcriptional activation, active repressors may destabilize or inhibit the formation of preinitiation complexes, perhaps by interfering with the interactions between positively acting transcriptional machinery. This suggests that the position of an enhancer element relative to the transcription start site is critical. Consistent with this idea is the fact that the GAP-43 E1 E-box is conserved between the rat and human GAP-43 promoters, not only in terms of sequence, but also in terms of positioning. The p75LNGFR E-box is also conserved in terms of position and sequence between the rat and human promoter sequences (38). In the case of the desmin promoter, it was reported that insertion of a half-turn DNA helix between the E-box and the TATA site significantly affected the transcriptional activity of the desmin promoter, suggesting a stereospecific alignment with the TATA box binding protein complex in order to function (51). To further support this model, it has been shown that the bHLH transcription factor USF interacts cooperatively with the transcription initiator TFI-I, which might result in a more stable preinitiation complex to augment the initiation of transcription by RNA polymerase II (52). The bHLH protein Myc has been shown to interact with TFI-I and TATA box binding protein (53). The role of TFI-I is particularly critical for the assembly of a preinitiation complex on promoters lacking a TATA element, such as the GAP-43 promoter. Therefore, bHLH transcription factors may regulate transcription through physical interactions with transcription components of the polymerase II machinery.

The observation that class A bHLH proteins repress GAP-43 and p75LNGFR promoter activities is intriguing as both promoters are developmentally regulated and do not contain a TATA box. Although class A bHLH proteins are ubiquitously expressed, their level of expression has been shown to be developmentally regulated (42, 54, 55). This raises the question of how class A bHLH proteins repress transcription. Several plausible mechanisms may be outlined. First, class A bHLH proteins may repress transcription as homodimers and consequently transiently maintain specific developmentally regulated genes in a state of repression during stages of development where their expression should be restricted. Although our in vitro data support this hypothesis, it is necessary to design dominant-negative class A bHLH mutants that would only form heterodimers in vivo in order to validate this mechanism. In addition, it has been reported that in a specific cellular context, class A proteins are more likely to form homodimers due to post-translational modifications, such as
phosphorylation and redox events (56, 57). However, the possibility remains that class A bHLH proteins would repress through the formation of heterodimers. E12 or ME1a protein may repress GAP-43 expression by interacting with an exogenous partner and therefore form a repressing heterodimer. On the other hand, one cannot exclude a potential interaction between class A bHLH homodimers and another protein that would be the direct repressor of the GAP-43 promoter. The repression action of class A bHLH proteins represents a novel strategy for the regulation of neuronal target genes. They may assume more diverse functions during development since our studies demonstrate that they can act either as repressors or activators. Negative regulation by bHLH proteins is not unprecedented, as the bHLH protein HES-1 negatively regulates its target genes (58). However, HES-1 protein has not been shown to function as an activator. Therefore, the difference in behavior between HES-1 protein and the class A E12 and ME1a proteins suggests different mechanisms of repression exerted by bHLH proteins.

Acknowledgments—We thank the late Dr. H. Weintraub for gifts of plasmids. We thank Marcy Haire for photographic work.

REFERENCES

1. Jan, Y. N., and Jan, L. Y. (1993) Cell 75, 827–830
2. Weintraub, H. (1993) Cell 75, 1241–1244
3. Campos-Ortega, J. A., and Jan, Y. N. (1991) Annu. Rev. Neurosci. 14, 399–420
4. Caudy, M., Vassin, H., Brand, M., Tuma, R., Jan, L. Y., and Jan, Y. N. (1988) Cell 55, 1061–1067
5. Jarman, A. P., Grau, Y., Jan, L. Y., and Jan, Y. N. (1993) J. Cell Biol. 124, 1241–1244
6. Lee, J. H., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N., and Weintraub, H. (1995) Science 268, 836–844
7. Basi, G. S., Jackson, R. D., Virag, I., Schilling, J., and Skene, J. H. P. (1987) Cell 49, 785–791

The rest of the references are...
The GAP-43 Gene Is a Direct Downstream Target of the Basic Helix-Loop-Helix Transcription Factors
Anne Chiaramello, Toomas Neuman, Dena R. Peavy and Mauricio X. Zuber

J. Biol. Chem. 1996, 271:22035-22043.
doi: 10.1074/jbc.271.36.22035

Access the most updated version of this article at http://www.jbc.org/content/271/36/22035

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 57 references, 20 of which can be accessed free at http://www.jbc.org/content/271/36/22035.full.html#ref-list-1