IA-1 is a novel zinc finger transcription factor with a restricted tissue distribution in the embryonic nervous system and tumors of neuroendocrine origin. The 1.7-kilobase 5′-upstream DNA sequence of the human IA-1 gene directed transgene expression predominantly in the developing nervous system including forebrain, midbrain, hindbrain, spinal cord, retina, olfactory bulb, and cerebellum, which recapitulated the expression patterns of neuroendocrine tissues and childhood brain tumors. The IA-1 promoter deletion reporter gene constructs revealed that the sequence between −426 and −65 bp containing three putative E-boxes (−361 bp) upstream of the transcription start site was sufficient to confer tissue-specific transcriptional activity. Further mutation analysis revealed that the proximal E-box (E9) closest to the start site is critical to confer transcriptional activity. Electrophoretic mobility shift assay and transient transfection studies demonstrated that the NeuroD1 and E47 heterodimer are the key transcription factors that regulate the proximal E-box of the IA-1 promoter. Therefore, we concluded that the IA-1 gene is developmentally expressed in the nervous system and the NeuroD1/E47 transcription factors up-regulate IA-1 gene expression through the proximal E-box element of the IA-1 promoter.

The diffuse neuroendocrine system includes pancreatic islets, gastrointestinal and respiratory neuroendocrine cells, thyroid C, adrenal medulla, and pituitary cells (1). They share phenotypes with neuronal cells by expressing common neuroendocrine markers and signaling pathways. A number of basic helix-loop-helix (bHLH) transcription factors, the neurogenins, Hes-1, and NeuroD1 (also known as β2), have been shown to play important roles in both pancreatic neuroendocrine and nervous system development (2–5). bHLH transcription factors play a critical role in the cell type-specific expression of a variety of genes in many different tissues (6, 7).

The bHLH proteins are classified into two separate groups based on their DNA binding properties and tissue distribution. In general, the class A members are ubiquitous factors including E47 and E12. The class B members are tissue-specific. However, the class B proteins can dimerize with the class A proteins as heterodimers and bind to DNA with high affinity and confer tissue-specific expression (8–12). NeuroD1 is a bHLH transcription factor that was shown to be associated with late neuronal differentiation in Xenopus laevis (13). Gene targeting experiments revealed that deletion of the NeuroD1 gene resulted in defective pancreatic morphogenesis and abnormal enteroneuroendocrine differentiation, which led to the early development of diabetes (14). Furthermore, NeuroD1 is required for differentiation of the granule cells in the cerebellum and hippocampus (4, 5). NeuroD1 regulates several downstream target genes in the pancreatic islets, the intestine, the pituitary, and the developing neural retina (15–19). Previously, we have identified NeuroD1 as a potential target gene of a novel transcriptional repressor, insulinoma-associated antigen-1 (IA-1) (20). Interestingly, in this study, we show evidence that NeuroD1 also regulates IA-1 gene expression. IA-1 encodes a novel zinc finger DNA-binding protein that was isolated from a human insulinoma subtraction library (21). In vitro induction of the AR42J amphicrine cell line into insulin-producing cells suggested that IA-1 gene expression is closely associated with the expression of islet-specific transcription factors including NeuroD1 (22). Functional studies revealed that IA-1 is a transcriptional repressor that binds to a specific DNA element and can autoregulate itself and the NeuroD1 gene (20). The IA-1 gene has a very restricted expression pattern to tumors of neuroendocrine origin (21, 23), including insulinoma, medulloblastoma, retinoblastoma, pituitary tumor, pheochromocytoma, medullary thyroid carcinoma, and small cell lung carcinoma. This pattern of expression largely overlaps the expression pattern observed for NeuroD1 (24). However, little is known about the transcriptional regulation of the IA-1 gene in neuroendocrine tissues. In this study, we have analyzed the IA-1 promoter activity in vitro by introducing an IA-1 promoter-LacZ transgene and monitoring the expression of LacZ activity in the transgenic mice. In addition, we have identified both the E-box element and the E47-NeuroD1 transcription factors that contribute to the tissue restricted expression of the IA-1 gene. Since IA-1 possesses transcriptional repressor activity against the NeuroD1 gene and their expression patterns largely overlap in the neuroendocrine cells, it suggests that the NeuroD1 and the IA-1 gene may counterregulate their expression levels during nervous system development.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfection—HeLa (human cervical carcinoma), WERI-Rb1 and Y79 (human retinoblastomas), Daoy (human medullo-
blastoMA), U87MG (human glioblastoma), and βTC-1, (mouse isletoma)-cells were maintained in Dulbecco's minimal essential medium with either high or low glucose supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin. Twenty-four hours prior to the transfection, the adherent cell lines were seeded at a density of 0.25–0.3 × 10^6 cells/well in a 6-well culture dish. Alternatively, the suspension cell lines were collected by centrifugation on the day of transfection and seeded at a density of 0.5 × 10^6 cells/ml. A 1:1 ratio of 5 μl of LipofectAMINE 2000 reagent (1 mg/ml; Invitrogen) to 5 μg of DNA was used according to the manufacturer's instructions. The DNA complex was added per well in the absence of serum for 5 h at 37 °C. The medium was removed and replaced with fresh fetal bovine serum containing medium for a total of 48 h. The cells were collected at the designated ages, fixed in solution containing 0.2% glutaraldehyde, 1.5% formaldehyde, 5 mM EGTA, 2 mM MgCl₂, and 100 mM sodium phosphate, pH 8.0, for 30 min to 2 h at room temperature depending upon the embryo size. The embryo and tissue were washed three times with 100 mM sodium phosphate buffer, pH 8.0, β-Galactosidase activity was developed in staining solution consisting of 1 mg/ml X-gal, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, in sodium phosphate buffer, pH 8.0, at room temperature for up to 48 h. Whole mount stained embryo was photographed through a dissecting microscope. X-gal-stained embryos or tissues were further fixed in 10% buffered formalin followed by paraffin embedding for histological analysis.

**DNA Constructs**—The various IA-1 promoter CAT constructs were created by subcloning the human IA-1 −1661/+40 bp promoter fragment into the XhoI site of the pcAT3 basic vector (Promega). The −426/+40 bp construct was created by NheI digestion of the −1661/+40 bp IA-1/CAT3 construct followed by religation to eliminate the −1661 to −426 bp fragment, a partial mouse IA-1 3'-UTR fragment (1.3 kb), and full-length hamster β2 β2 DNA (a kind gift from Dr. Ming Tsai; Baylor College of Medicine) probes were random prime-labeled (Invitrogen) in the presence of [α−³²P]dCTP (3000 Ci/mmol; PerkinElmer Life Sciences) and T₄ polynuucleotide kinase (New England BioLabs). E47 and NeuroD1 were synthesized using the expression plasmids pCR3.1β2 and pcDNA3E47 and the TNT-coupled rabbit reticulocyte lysate kit (Promega). Synthesis of E47 and NeuroD1 was confirmed by Western blot analysis using an anti-E47 rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or an anti-NeuroD1 rabbit polyclonal antibody (Chemicon). The EMSA binding reaction included various amounts of in vitro translated proteins in a binding reaction composed of 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM diithiothreitol, 5% glycerol, 200 ng of poly(dI-dC), and 5 × 10⁶ cpm of probe. For supershift assay, 1 μl of a 1.5 dilution of E47 antibody (Santa Cruz Biotechnology) or a 1.25 dilution of NeuroD1 rabbit polyclonal antibody (Chemicon) was added to the reaction mixture with in vitro translated protein and probe. The mixture was then incubated at room temperature for an additional 20 min. The protein-DNA complexes were resolved on a 4% PAGE (40:1) gel in 0.25× TBE buffer. The gels were dried and exposed to autoradiography.

**Northern Blot Analysis**—Total RNA was extracted from Dauer, D283MED, U87MG, WERI-Rb1, Y79, and βTC-1 cell lines using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Twenty micrograms of total RNA were separated on a 1% agarose/formaldehyde gel in 1× MOPS running buffer. Northern blot analysis was carried out as previously described (21). The full-length human IA-1 cDNA (2.9 kb of EcoRI fragment), a partial mouse IA-1 3'-UTR fragment (169 bp fragment of the IA-1 5'- transcript), and the complementary strand were end-labeled using [γ−³²P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences) and T₄ polynuucleotide kinase (New England BioLabs). E47 and NeuroD1 were synthesized using the expression plasmids pCR3.1β2 and pcDNA3E47 and the TNT-coupled rabbit reticulocyte lysate kit (Promega). Synthesis of E47 and NeuroD1 was confirmed by Western blot analysis using an anti-E47 rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or an anti-NeuroD1 rabbit polyclonal antibody (Chemicon). The EMSA binding reaction included various amounts of in vitro translated proteins in a binding reaction composed of 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 200 ng of poly(dI-dC), and 5 × 10⁶ cpm of probe. For supershift assay, 1 μl of a 1.5 dilution of E47 antibody (Santa Cruz Biotechnology) or a 1.25 dilution of NeuroD1 rabbit polyclonal antibody (Chemicon) was added to the reaction mixture with in vitro translated protein and probe. The mixture was then incubated at room temperature for an additional 20 min. The protein-DNA complexes were resolved on a 4% PAGE (40:1) gel in 0.25× TBE buffer. The gels were dried and exposed to autoradiography.

**RESULTS**

**Generation of IA-1/LacZ Transgenic Mice**—Previous analysis of a −2090/+169 bp fragment of the IA-1 5'-flanking sequence demonstrated that the promoter was active in AtT-20 cells and not detectable in HEla cells, consistent with the IA-1 tissue-restricted mRNA expression pattern (26). In order to...
Female mice were collected for whole mount fixation and stained for β-gal activity driven by the IA-1 promoter sequence (−1661 to +40 bp). Strong staining was detected in the nervous system of the early stage embryo (e11.5–e14.5) in the forebrain, midbrain, hind brain, spinal cord, cerebellum, olfactory bulb, and retina (Fig. 1). No β-gal activity was detected in other tissues. In general, the staining patterns of Tg 2–5 and Tg 2–8 are similar. There are differential intensities between the Tg 2–5 and Tg 2–8 lines. For example, the retina staining in the Tg 2–8 was stronger than in Tg 2–5 (e11.5). Further sectioning of β-gal stained embryos (e12.5 day) revealed strong activities in the olfactory bulb, olfactory epithelium, retinal neuron, lens, spinal cord, and trigeminal ganglion. We also stained the postpartum brains collected from e16.5, e18.5, newborn, 1 week, and adult. Strong staining was observed in the cerebellum and pineal gland at e18.5, whereas the LacZ activity gradually decreased while the brain matured to adulthood.

The −426/+40 bp IA-1 Promoter Sequence Contains the Tissue-specific Activity—Based upon the results of the in vivo IA-1 promoter/LacZ transgenic mice, we demonstrated that the −1661/+40 bp human IA-1 promoter region is sufficient to direct both the proper spatial and temporal expression of the IA-1 gene. In order to determine the minimal region(s) necessary to direct tissue-specific expression, three separate deletion constructs of the IA-1 promoter were linked to the CAT reporter gene. We analyzed the promoter activity in three different cell lines, HeLa, Y79, and βTC-1 (Fig. 3). Comparison of the three deletion constructs revealed that the −426/+40 bp/CAT construct activated the reporter gene to the same level as the −1661/+40 bp construct in both Y79 and βTC-1 cells. However, the overall promoter activity was significantly higher in Y79 (−40-fold increase) as compared with βTC-1 cells (−7-fold increase). As expected, promoter activity was much lower in HeLa cells (−3-fold). Therefore, this result demonstrates that the −426/+40 bp region contains most of the cis elements necessary to direct the proper tissue-specific expression of the IA-1 gene.

E-box 3 Is Critical for IA-1 Promoter Activity—Close inspection of the −426/+40 bp IA-1 promoter region revealed the
significant loss in promoter activity (demonstrated that deletion of E-box 3 (E3) resulted in a significant decrease in promoter activity in cell lines (Fig. 1). This CMV-β-gal vector was used as an internal control to normalize transfection efficiency. The transfections were repeated three times.

The presence of three putative E-box elements in the IA-1 promoter establishes the importance of these elements for tissue-specific IA-1 promoter activity. Deletion analysis of the IA-1 promoter in various cell lines demonstrated that the -426/-40 bp region contained an E3 element that was crucial for 70% of the basal promoter activity (Fig. 2). Deletion of one E-box element at a time did not result in a significant change in overall basal promoter activity. Double deletion of E1 and E2, as expected, did not show any significant change to overall basal promoter activity (Fig. 2). As expected, double deletion of either E1 or E2 alone did not result in a significant change in basal promoter activity. These results demonstrate that deletion of E-box 3 (E3) resulted in a significant loss in promoter activity (~70%) in βTC-1 cells (Fig. 4). Deletion of E1 or E2 alone did not result in a significant increase in the overall basal promoter activity. Double deletion of E1 and E2, as expected, did not show any significant change to overall basal promoter activity (Fig. 4). As expected, double deletion of either E1 or E2 or E3 or triple deletion of E1, E2, and E3 mimicked the single deletion of E3 alone, resulting in a ~75, 60, or 60% loss, respectively, in promoter activity when compared with the wild type promoter. Therefore, this result demonstrates the importance of the E3-box for tissue-specific IA-1 promoter activity.

E47-NeuroD1 Heterodimer Activates the -426/-40 bp IA-1 Promoter—Deletion analysis of the IA-1 promoter in various cell lines demonstrated that the -426/-40 bp region contained the majority of the critical information necessary to result in tissue-specific expression of the IA-1 gene (Fig. 3). Further analysis of this region revealed the presence of three E-box elements in this region. Deletion of the individual E-box elements revealed that E3 was crucial for 70% of the basal promoter activity in βTC-1 cells. The sequence of the E3 element resembles a NeuroD1 binding site found in both the rat insulin and glucagon genes (16). Therefore, we performed transient transfections with the wild type -426/-40 bp IA-1 promoter/CAT construct to determine whether NeuroD1 could activate the IA-1 promoter/CAT construct. Using various concentrations of a NeuroD1 expression vector, a dosage-dependent increase in IA-1 promoter activity was observed in HeLa cells (Fig. 5A). NeuroD1 is a tissue-restricted bHLH protein that binds to a 6-bp E-box element as a heterodimer with a ubiquitous partner E47 (15). Therefore, we then performed transient transfections in HeLa cells using the -426/-40 bp IA-1 promoter/CAT construct along with a fixed amount of E47 and NeuroD1 expression vectors to demonstrate that there was a synergistic effect of co-expression of these two molecules on the IA-1 promoter construct. As shown in Fig. 5B, transfection of an E47 expression vector alone could not stimulate the IA-1 promoter/CAT activity; however, the combination of both E47 and NeuroD1 resulted in a higher increase in CAT activity (~4.5-fold) than transfection of NeuroD1 alone (~2-fold) (Fig. 5B). Therefore, this result demonstrates that the IA-1 promoter is activated by the E47-NeuroD1 heterodimer and is responsible at least in part for the tissue-specific expression of the IA-1 gene.

Activation of the Individual E3-box by E47-NeuroD1 Heterodimer—Deletion analysis of the three individual E-box elements from the IA-1 promoter demonstrated that E3 is critical for tissue-specific IA-1 promoter activity. Co-expression of E47-NeuroD1 could significantly activate the -426/-40 bp IA-1 promoter/CAT construct. To demonstrate whether an individual E-box or a combination of the E-boxes could indeed mediate the activating effect of E47-NeuroD1, we cloned three tandem copies of each E-box into a TATA box-containing CAT reporter (E1TATATCAT3). The individual E-box/CAT constructs and
The CMV-roD1 heterodimer showed a strong activation of the E3-box construct. Each reporter vector was co-transfected with pcDNA, NeuroD1, E47, or each E-box (E1, E2, and E3) were cloned into E1bTATA-CAT vector. This activated the individual E3-box of the IA-1 promoter. The radiolabeled IA-1 promoter (-192/-165 bp) containing the E3-box was incubated with E47 or E47-NeuroD1 with or without antibodies to E47 or NeuroD1. Both the homodimer and the heterodimer were shown in shifted bands. E47 antibody supershifted the homodimer, whereas the NeuroD1 antibody interfered with the heterodimer binding. NS, nonspecific band. B, competitive inhibition of the E47-NeuroD1 complex binding with 3× E3-box. Incubation of E47 and NeuroD1 proteins with labeled 3× E3 oligonucleotide in the presence or absence of competitor (50- or 100-fold molar excess) showed that E47-NeuroD1 binding is specific for the E3-box alone.

E47-NeuroD1 cDNA expression vectors were co-transfected into HeLa cells. Transfection of the E47 expression vector alone resulted in a ∼3-fold increase in 3× E1 or 3× E2 activity and a ∼28-fold activation of E3 (Fig. 6). However, the intact -426/+40 bp IA-1 promoter/CAT construct could not be activated by the addition of E47 cDNA alone (Fig. 5B). The reason for this observed difference between the two experiments may be due to in part the inclusion of a heterologous promoter region (E1bTATA box). Alternatively, the 3× E-box constructs may lack critical flanking sequences required for the binding specificity of the E47-NeuroD1 heterodimer. Co-transfection of E47 and NeuroD1 expression vectors did not result in a significant change in the 3× E1 or the 3× E2-box activities; however, the 3× E3 activity was stimulated by ∼47-fold, indicating that the E47-NeuroD1 heterodimer most likely exerts its stimulatory activity through the E3 element. The activation of the 3× E3 was significantly higher than with the -426/+40 bp IA-1 promoter construct. One reason for this discrepancy could be the presence of three copies of the E3-box that results in higher -fold activation than with the wild type promoter. Additionally, the 3× E3-box is linked to a heterologous minimal promoter region that may behave differently than the intact IA-1 promoter region.

E47-NeuroD1 Heterodimers Bind to the E3-box (−192 to −165 bp Region) in an Electrophoretic Mobility Shift Assay—Transient co-transfection experiments using E47 and NeuroD1 cDNA expression vectors demonstrated a synergistic activation of the wild type (−426/+40 bp) IA-1 promoter or the 3× E3/CAT promoter constructs. We further performed EMSA analysis to confirm binding of NeuroD1 protein to the E3-box in the IA-1 promoter. The cDNAs of E47 and NeuroD1 were transcribed and translated in vitro, and the proteins were incubated either alone or together with a double-stranded oligonucleotide spanning the −192 to −165 bp region in the IA-1 promoter (containing E3). The addition of E47 protein alone resulted in a single complex, different from the control rabbit reticulocyte lysate (Fig. 7A, lanes 1 and 2). Co-translation of E47 and NeuroD1 proteins generated one complex with a faster mobility than the E47 homodimer complex (Fig. 7A, lane 5). The addition of excess E47 protein results in the formation of two complexes, one slower migrating complex that co-migrates with the E47 homodimer complex and a faster migrating complex that represents an E47-NeuroD1 heterodimer (Fig. 7A, lane 8). The identities of the complexes were confirmed by antibody supershift experiments. The addition of an E47 antibody results in the supershift of the slower migrating complex or an elimination of the slower and faster migrating complexes, demonstrating that E47 is present in both complexes (Fig. 7A, lanes 3 and 6). NeuroD1 protein alone does not form a complex (data not shown), whereas NeuroD1 antibody does not supershift but partially blocks the E47-NeuroD1 complex (Fig. 7A, lane 7). This result clearly demonstrates that E47-NeuroD1 heterodimers bind to the E3-box in the IA-1 promoter. Additionally, we performed competition experiments using the 3× E3 oligonucleotide along with 50- and 100-fold molar excesses of cold 3× E3 (self), 3× E1, and 3× E2 (Fig. 7B). Incubation of E47 and NeuroD1 proteins with radiolabeled 3× E3 oligonucleotide in the absence of competitor resulted in the formation
Northern Analysis of Various Neuroendocrine Cell Lines—

Northern blot analysis for endogenous IA-1 expression. This observation is consistent with the IA-1 promoter region directing both the appropriate spatial and temporal expression of the IA-1 gene and further supports a role of IA-1 during early embryonic brain development.

Interestingly, homology searches of other genomes have revealed that IA-1 belongs to a highly conserved group of zinc finger-containing proteins that show the highest homology in the first two zinc fingers (30). Members of this group include two novel proteins, Nerfin-1 and Nerfin-2 (for nervous finger-1 and -2) from Drosophila and a C. elegans protein Egl-46 that are expressed in the developing nervous system (30, 31). Nerfin-1 expression was detected during early central nervous system development in neuroblasts prior to lineage formation and was not expressed in neurons and glia. In contrast, Nerfin-2 is expressed in only a subset of brain neurons. Egl-46 represses transcription of touch cell characteristics in FLP cells. Egl-46 mutants show a serotonin-sensitive, impainre-resistant egg-laying defect, and the mutant animals have multiple hermaphrodite-specific neuron defects, including abnormalities in cell migration, axonal outgrowth, and serotonin production (32, 33). Based upon the neuronal restricted expression of these genes, it is likely that they may have conserved function. The highest degree of homology (90%) in these four proteins resides in zinc finger 2 (31). Interestingly, we have shown that zinc fingers 2 and 3 of the IA-1 protein are critical for the DNA binding activity of IA-1, and the NeuroD1 transcription factor was identified as a potential target gene for IA-1 (20). Since NeuroD1 was originally isolated based upon its ability to induce neuronal differentiation when ectopically expressed in Xenopus oocytes (13), it further supports a functional role of the IA-1 gene in neuronal differentiation.

The expression profile seen in the transgenic mouse established that the 1.7-kb region of the IA-1 promoter directs the formation (28, 29). We isolated and identified a novel zinc finger transcriptional repressor, IA-1, from a human insulinoma subtraction library, which is restricted to a limited number of tissues during early embryonic development and is also activated in tumors of neuroendocrine origin (20, 21).

Based upon the tissue-restricted expression pattern observed for the IA-1 mRNA, we sought to identify regions within the IA-1 gene responsible for directing its proper tissue-specific expression. Using in vivo generation of the IA-1 promoter-driven LacZ transgenic mouse line, we convincingly demonstrated that the information required for the tissue-specific expression of the IA-1 gene is present in the 5′-upstream regulatory region. Staining of the early stage whole mouse embryos showed prominent staining in regions of the central nervous system such as the forebrain, midbrain, hind brain, cerebellum, spinal cord, retina, and olfactory bulb. Analysis of brains from e16.5, e18.5, newborn, 1-week-old, and adult mice established a strong expression of the IA-1 gene during early brain development that was rapidly lost as the mice matured. Our results showed the earliest time point for transgene expression was e9.5. Northern blot analysis using whole mouse embryo total RNA showed that the first detectable IA-1 message is at e10.5 (27). This discrepancy is most likely due to the increased sensitivity of detection of LacZ expression due to the high copy number of the transgene construct. Endogenous IA-1 expression was shown to peak around E11.5 and declined by E18.5 in the whole mouse embryo (27). Northern blot analysis on postpartum mouse brain RNA revealed strong expression of IA-1 in e17.5 brain that persisted until 2 weeks postpartum and was completely undetectable by 4 weeks postpartum (27). Our results show a similar temporal expression pattern for the IA-1 transgene construct as compared with the Northern blot analysis for endogenous IA-1 expression. This observation is consistent with the IA-1 promoter region directing both the appropriate spatial and temporal expression of the IA-1 gene and further supports a role of IA-1 during early embryonic brain development.
appropriate spatial and temporal expression of the IA-1 gene in brain. Using transient transfection studies, we demonstrated that a region between -426 and -65 bp exhibited strong promoter activity among a series of reporter constructs made from 5'-end deletions of the 1.7-kb promoter in β-TC-1 and Y79 cells. Although the relative promoter activity was significantly higher in the Y79 cells versus the β-TC-1, it is likely that human IA-1 promoter is more active in human Y79 than in mouse β-TC-1 cells. Northern blot analysis revealed that IA-1 message is stronger in β-TC-1 than in Y79 cells (Fig. 8). Computer analysis of the -426/+40 bp region revealed the presence of three E-box motifs. E-box elements are bound by members of the bHLH transcription factor family. Belonging to this diverse family of transcription factors, there are both tissue-restricted and ubiquitously expressed members. A growing number of neurogenic bHLH factors have been detected in neural tissues and shown to be critical in their formation. The expression of two of these factors, neurogenin 3 (Ng3n) and NeuroD1, has also been shown in the developing endocrine pancreas and nervous system (14, 34). NeuroD1 expression lies downstream of Ngn3 expression due to the lack of NeuroD1 expression in Ngn3 knockout mice and the identification of two Ngn3 binding sites in the NeuroD1 promoter responsible for activation of the NeuroD1 gene (35). Close analysis of the three E-box motifs in the IA-1 promoter showed that E3 was homologous to the NeuroD1-responsive E-box motif in the insulin, glucagon, and secretin gene promoters (10, 15–17). Individual deletion of the three E-box elements revealed that E3 was critical for ~65% of basal IA-1 promoter activity in βTC-1 cells. The E3-box resembles a NeuroD1 binding site and was activated by ~40-fold with the addition of both E47 and NeuroD1 expression plasmids. Finally, we showed binding of in vitro translated NeuroD1 protein to the E3-box in the IA-1 promoter using EMSA analysis. Therefore, we have demonstrated that the neuroendocrine-specific bHLH transcription factor NeuroD1 is involved in the regulation of the IA-1 gene. This has functional significance in tissues that co-express both IA-1 and NeuroD1 in the development of the neuronal tissues.

Northern blot analysis on various neuroendocrine tumor cell lines revealed the expression of IA-1 and NeuroD1 overlaps in all the cell types tested. βTC-1, D283MED, WERI-Rb-1, and Y79 expressed detectable levels of both IA-1 and NeuroD1 message, whereas Daoy and U87MG cells were negative for their expression. This strong correlation between the expression of IA-1 and NeuroD1 in neuroendocrine cells supports a role for NeuroD1 in the regulation of IA-1 expression.

In conclusion, we report that the IA-1 promoter directs the appropriate spatial and temporal expression of the IA-1 gene and that one of the major cis-acting regulatory elements is bound by the E47-NeuroD1 heterodimer. This establishes a dynamic relationship between the tissue-restricted bHLH transcription factor NeuroD1 and the neuroendocrine-specific transcriptional repressor protein, IA-1. The fact that IA-1 is a target gene for NeuroD1 regulation and vice versa suggests that the IA-1 gene may play a pivotal role during early neuronal development by modulating NeuroD1 gene expression.

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