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The Basic Helix-Loop-Helix Transcription Factor NeuroD1 Facilitates Interaction of Sp1 with the Secretin Gene Enhancer

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Received 14 March 2007/Returned for modification 24 April 2007/Accepted 8 September 2007

The basic helix-loop-helix transcription factor NeuroD1 is required for late events in neuronal differentiation, for maturation of pancreatic β cells, and for terminal differentiation of enteroendocrine cells expressing the hormone secretin. NeuroD1-null mice demonstrated that this protein is essential for expression of the secretin gene in the murine intestine, and yet it is a relatively weak transcriptional activator by itself. The present study shows that Sp1 and NeuroD1 synergistically activate transcription of the secretin gene. NeuroD1, but not its widely expressed dimerization partner E12, physically interacts with the C-terminal 167 amino acids of Sp1, which include its DNA binding zinc fingers. NeuroD1 stabilizes Sp1 DNA binding to an adjacent Sp1 binding site on the promoter to generate a higher-order DNA-protein complex containing both proteins and facilitates Sp1 occupancy of the secretin promoter in vivo. NeuroD1-dependent transcription of the genes encoding the hormones insulin and proopiomelanocortin is potentiated by lineage-specific homeodomain proteins. The stabilization of binding of the widely expressed transcription factor Sp1 to the secretin promoter by NeuroD1 represents a distinct mechanism from other NeuroD target genes for increasing NeuroD-dependent transcription.

The gene encoding the gut hormone secretin is highly restricted in expression to S-type enteroendocrine cells of the small intestine in adult animals. In addition, the secretin gene is expressed transiently during development in pancreatic islets and in serotonergic neurons of the central nervous system (18, 34). A proximal enhancer localized within 200 bp of the transcription initiation site of the secretin gene is required and sufficient for its expression in secretin-expressing cells. The same enhancer is relatively inactive in cell lines that do not express the endogenous secretin gene (34). Mutational analysis revealed that the enhancer consists of four distinct protein binding sites important for transcription. These include two binding sites for Sp1, one sequence motif that binds to the DNA binding protein Finb/RREB1 (28), and an E-box that binds to the basic helix-loop-helix (bHLH) protein NeuroD1 heterodimerized with E12/E47 (20).

NeuroD1 is a member of the tissue-specific class (class B) of bHLH transcription factors. It is expressed in neurons, the anterior pituitary gland, pancreatic islets, and enteroendocrine cells. Thus, NeuroD1 is the only identified protein binding to the secretin enhancer that is expressed in a very limited number of cell types, whereas expression of Sp1 and Finb/RREB1 is widespread.

A number of studies suggest a potentially significant role for NeuroD1 in the terminal differentiation of pancreatic islets (22, 23) and enteroendocrine cells (21, 22) and in the development of various structures in the nervous system (12, 16, 17, 19). Potential target genes that depend on NeuroD1 for expression include the genes for the hormones secretin, insulin, glucagon, and proopiomelanocortin (POMC), as well as the gene encoding the homeodomain protein PDX-1 (4, 21, 23, 26, 30).

Of note, the secretin gene is the only target gene identified thus far that shows an absolute requirement for NeuroD1 for in vivo expression. NeuroD1-null mice fail to develop any secretin-producing enteroendocrine cells. A moderate reduction in the number of glucagon-expressing α cells and insulin-expressing β cells was noted in the endocrine pancreas, although both insulin and glucagon immunoreactivity were readily detected in the remaining cells (22). Corticotroph differentiation was delayed during fetal development in NeuroD1-null mice with no reduction in POMC-expressing cells in older animals, indicating a nonessential role for NeuroD1 (15).

In addition to its direct effects on secretin gene transcription, NeuroD1 may play a role in coordinating expression of secretin with cell cycle exit as secretin cells terminally differentiate. The effects of NeuroD1 on cell proliferation may result from increased p21 expression (21). NeuroD1-dependent transcription is repressed by cyclin D1 by a mechanism independent of cyclin-dependent kinases (27). The presence of cyclin D1 in the proliferating cells of intestinal crypts may serve to prevent relatively immature, proliferating cells in the intestinal crypts from prematurely differentiating. Thus, NeuroD1 has a central role in the regulation of secretin cell differentiation.

Our earlier work suggested that NeuroD1 is a relatively weak yet essential transcriptional activator of the secretin gene (28). The organization of the secretin enhancer bears little similarity to that of the insulin or POMC enhancers, suggesting that the function of NeuroD1 in transcription of the secretin, insulin, and POMC genes in enteroendocrine cells, pancreatic β cells, and pituitary corticotrophs, respectively, may depend on other factors recruited to each enhancer.

Finb/RREB1, a ubiquitously expressed DNA binding protein, potentiates transcriptional activation by NeuroD1 despite
its lack of an intrinsic activation domain. The effect of Finb/ RREB1 on NeuroD1 requires both its binding to the enhancer 5′ to the E-box and direct physical association with NeuroD1 (28). We and others previously identified positive cis regulatory elements in the secretin gene enhancer that bind to Sp1 (13, 28). One of the Sp1 sites identified was immediately contiguous to the 3′ end of the NeuroD1 binding E-box. Because of the close proximity between these two sites, we examined whether NeuroD1 and Sp1 functionally and physically interact to increase the transcription-activating functions of NeuroD1. Our results suggest that Sp1 and NeuroD1 synergistically activate expression of the secretin gene through physical interactions that stabilize binding of Sp1 to DNA.

MATERIALS AND METHODS

Cell culture. The human cervical carcinoma cell lines C33A and HeLa (American Type Culture Collection), the hamster insulin tumor cell line HIT-15 M2.2.2 (hereafter called HIT) and the insect cell line STC-1 (29) were cultured in Dulbecco’s modified Eagle’s medium (4.5 g of glucose/liter) supplemented with 10% heat-inactivated fetal calf serum. Drosophila melanogaster Schneider cells (SL2) were grown at 25°C in Schneider medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum.

Plasmid constructions. A secretin-luciferase reporter containing one copy of the secretin enhancer spanning from −209 to +32 has been described previously (30). Transversion point mutations were introduced by site-directed mutagenesis (9) to generate the different mutant secretin reporter plasmids, mGC1 (−119 to −117); the E-box mutant mE (−130 to −127) (20); a mutant with both Sp1 sites mutated, MutGC (−65 to −63, −119 to −117); and a promoter with mutations in the E-box and both Sp1 binding sites, MutGC/E. The Ins 6 reporter was generated by inserting 6 bp with a BamHI site between the E-box and the adjacent G1 Sp1 binding site by PCR using the wild-type secretin promoter (−209 to +32) as a template. The 10-bp insertion between the E-box and the adjacent Sp1 site was generated by digesting the Ins 6 reporter construct with BamHI followed by end filling and religation. The human telomerase reverse transcriptase (hTERT)-luciferase construct extended to −629 used in the present study has been previously described (2). The mammalian expression plasmids for NeuroD1 (23), NeuroD1 in vitro transcription-translation and glutathione S-transferase (GST) fusion plasmids (21), and Sp1-GST fusion plasmids (8) were previously described (2). The mammalian expression plasmids for NeuroD1 and Sp1 of DNA, consisting of 0.25 μg of DNA, were prepared by phenol/chloroform precipitation with a total of 0.82 μg of DNA following reverse transcription and proteinase K digestion. DNA was purified (QIAquick PCR purification kit) following reversal of cross-links and proteinase K digestion. DNA was amplified with primer pairs described below using semiquantitative PCR and compared to serially diluted input DNA to ensure that the immunoprecipitated DNA was present at a concentration that allowed visualization of different amounts.

The primers used for detecting secretin promoter sequences (forward, 5′-CA GGCTCCGAGGCTTGCG-3′, and reverse, 5′-GGCCCTTATGTGGCGG-C-3′) have been described earlier (13). For detecting dihydrofolate reductase (DHFR) promoter sequences, the following primers were used: forward, 5′-TG CACCTGTGAGGAGCA-3′, and reverse, 5′-AGAAGGCGCGTTCAGT GT-3′. For detection of the transfected secretin promoter, the following primers were used: forward, 5′-GGTACCGACTACCT-3′, and reverse, 5′-T GGCGC TTCCTCATTTACA-3′ (from the coding sequence of the lucerase gene). The primers used for detecting the hTERT promoter sequences (forward, 5′-GGT ACCGACCCCCGCGCGCCCGA-3′, and reverse, 5′-AA GACCGCCGCGCCCGC-3′) were described earlier (35). For detection of the human glyceroldehyde-3-phosphate dehydrogenase (GAPDH) coding region, the following primers were used: forward, 5′-TGAAAGTGGAGTAGCAACGG ATT-3′, and reverse, 5′-GTCACACCCATGACCAACAGT-3′. The PCR products were separated in 2% agarose gels stained with ethidium bromide.

RESULTS

Synergistic interaction between NeuroD1 and Sp1 on the secretin promoter. Previous studies showed that expression of the secretin gene in secretin-expressing cell lines is highly dependent on a NeuroD1 binding E-box. In the rat, mouse, and human secretin genes, an Sp1 binding site is present adjacent to the E-box (Fig. 1A). Mutations in the E-box that prevent binding of NeuroD1 to the enhancer reduced transcriptional activity of secretin reporter genes by ~90% in HIT insulinoma cells and STC-1 enterocarcinoma cells, both of which express secretin and NeuroD (20). Introduction of point mutations into the Sp1 binding site (GC1 box) adjacent to the E-box similarly reduced (~84%) transcriptional activity in both cell lines (Fig. 1B). The importance of these two adjacent sites for full transcriptional activity prompted us to investigate whether NeuroD1 and Sp1 functionally cooperate to regulate transcription of the secretin gene.

To determine whether Sp1 and NeuroD1 functionally interact, we examined the effects of Sp1 on NeuroD1-dependent
transcription of the secretin gene as described previously (28) using transient-expression assays in C33A cells, a cell line that does not express NeuroD1. Expression of a secretin-luciferase reporter gene increased by 4.7-fold above the basal level when coexpressed with NeuroD1, in agreement with our earlier observations that this bHLH protein has only a modest transactivating function (Fig. 1C). Cotransfection of an Sp1 expression plasmid increased reporter gene expression by 4.9-fold. Coexpression of both Sp1 and NeuroD1 increased reporter expression approximately 32.2-fold, far exceeding the sum of the activation from either factor alone.

We confirmed that synergistic transcriptional activation of the secretin gene required the binding sites for both NeuroD1 and Sp1 by expressing both factors with reporter genes containing mutations in either the E-box (20) or GC1 box (28). We observed no synergism between Sp1 and NeuroD1 with either mutant, suggesting that their interaction was dependent on promoter occupancy of their adjacent binding sites (Fig. 1C). The GC1 mutant reporter was weakly activated by Sp1, probably from the binding of Sp1 to a second binding site 48 nucleotides 3′ to the GC1 box. However, the effects of Sp1 and NeuroD1 on the GC1 mutant reporter were additive, highlighting the importance of the GC1 site for synergism (Fig. 1C).

To further assess the function of Sp1 in secretin gene transcription, we examined the activity of the secretin-luciferase reporter with cotransfected NeuroD1 and/or Sp1 plasmids in Schneider SL2 cells, which express neither NeuroD1 nor Sp1. Cotransfection of an Sp1 expression plasmid increased low basal reporter activity by ~6.4-fold. NeuroD1 had no effect by itself on expression of the secretin reporter in SL2 cells (Fig. 1C). However, NeuroD1 and Sp1 together resulted in a greater-than-additive (~21.2-fold) activation of the reporter gene in SL2 cells, suggesting that they may functionally interact (Fig. 1C).

Formation of a ternary DNA-protein complex involves binding cooperativity between NeuroD1 and Sp1. We examined DNA-protein complexes formed by Sp1 and NeuroD1 on the secretin enhancer by electrophoretic mobility shift assays (EMSAs) to determine if the functional interaction between these proteins observed in transfection assays was related to the binding of these factors to the enhancer. It has previously been shown that NeuroD1 binds to E-boxes as a heterodimer with ubiquitously expressed bHLH proteins like E12 or E47 but not as a homodimer (23). Binding of in vitro-translated NeuroD1 and E12 to a probe containing the contiguous E-box and Sp1 binding site generated a DNA-protein complex representing a NeuroD1-E12 heterodimer (Fig. 2A, lane 1), whereas NeuroD1 alone did not form a complex (not shown). A distinct, faster-migrating complex was generated with Sp1 (Fig. 2A, lane 2), suggesting the formation of a ternary complex that contained all three proteins bound to the DNA.

The higher-order complex was not detected if all three proteins were incubated with a probe containing a mutation in either the E-box or the adjacent Sp1 binding site (Fig. 2A, lanes 4 and 7) or with a probe containing 6-bp or 10-bp spacing between the two binding sites (lanes 10 and 11), indicating that both binding sites as well as their close proximity to each other were required for the formation of this complex. Although E12 bound to the wild-type probe as a homodimer to generate a complex, it could not form the more slowly migrating complex with Sp1 (Fig. 2B, lanes 1 and 2), indicating that NeuroD1 was required for ternary complex formation and that E12
homodimers appear to inhibit Sp1 binding to the same probe molecule.

The synergistic transcriptional activation by NeuroD1 and Sp1 could arise from interactions that enhance their binding to DNA. To determine whether the observed ternary complex resulted from cooperative DNA binding by the two proteins to the enhancer, we performed an EMSA and quantitated by phosphorimaging the fraction of probe retained in each DNA–protein complex generated by Sp1 or NeuroD1/E12 alone as well the complexes generated by all three proteins together. Sp1 and NeuroD1/E12 independently bound 15.6% and 6.4% of the total probe, respectively (Fig. 2C). If Sp1 and NeuroD1–E12 bound to DNA independently of each other, the probability of the two proteins simultaneously binding to a single probe molecule should equal the product of their individual probabilities of binding. The two proteins together generated a ternary complex containing approximately 6.7% of the total probe versus the average predicted value of 1.8% if binding occurred independently and randomly. This indicates that generation of the higher-order DNA-protein complex was enhanced by cooperative binding interactions.

Ternary complex formation and transactivation depend on the close proximity of the NeuroD1 and Sp1 binding sites. We examined DNA-protein complexes generated with the secretin enhancer probe and nuclear extracts from HIT cells by EMSA to determine if a similar ternary complex is formed with proteins in crude nuclear extracts as described with purified proteins. A number of DNA-protein complexes were identified, including one that consisted of several complexes that were not well resolved on the gel (complex II) (Fig. 3A, lane 1). A consensus wild-type Sp1 oligonucleotide but not a mutant oligonucleotide completely competed a slowly migrating band

FIG. 2. NeuroD1 and Sp1 bind cooperatively to the secretin gene to form a ternary DNA-protein complex. (A) EMSA showing protein complexes formed by indicated combinations of in vitro-translated NeuroD1, E12, or Sp1 bound to a 32P-labeled probe containing the E-box and GC1 Sp1 site (lanes 1 to 3) or the same probes with mutations in either the E-box (lanes 4 to 6) or GC1 (lanes 7 to 9) or with the insertion of a 6-bp (Ins 6) or a 10-bp (Ins 10) sequence between the two sites (lanes 10 and 11). The arrowhead denotes a slow-migrating complex seen only in the presence of all three proteins (lane 2) but not seen with any of the mutant probes (lanes 4 to 11). (B) Failure to generate the ternary complex with E12 and Sp1 in the absence of NeuroD1 (lane 2). (C) Quantitation of Sp1 and NeuroD1/E12 binding to DNA. The fraction of probe present in complexes formed by NeuroD1 plus E12 alone (panel A, lane 1) or Sp1 alone (panel A, lane 3) or in the ternary complex formed by NeuroD1, E12, and Sp1 together (panel A, lane 2) was measured by phosphorimaging. Results are shown as the means ± standard errors of the means for at least three separate experiments. *, significantly different (P < 0.05) from the fraction predicted for independent binding events measured as described before (24). WT, wild type.

FIG. 3. Presence of NeuroD1 and Sp1 in a higher-order DNA-protein complex formed from nuclear extracts. Shown are results of an EMSA of factors in HIT cell nuclear extract binding to the secretin promoter regions containing the E-box and adjacent Sp1 binding site. (A) Effect of competition with unlabeled oligonucleotides for an Sp1, mutant Sp1 site, or E-box on ternary complex formation. (B) Presence of Sp1 and NeuroD1 in the ternary complex. Antibodies to NeuroD1 (lane 2) or E12 (lane 5) reduced the slow-migrating complex. Sp1 antibody (lane 3) supershifted the ternary complex. (C) EMSA showing that probes with increased spacing between the E-box and Sp1 binding site cannot generate the ternary complex from nuclear proteins extracted from HIT cells (lanes 2 and 3). (D) Effect of spacing (6 or 10 bp) between the E-box and the adjacent Sp1 binding site on secretin gene transcription. Luciferase activity of the insertion mutants is expressed as a percentage of activity of the wild-type reporter gene transfected in HIT (white bars) or STC-1 (black bars) cells. Results are shown as the means ± standard errors of the means for at least four independent experiments. Significant differences from the wild type are shown by an asterisk (P < 0.001). WT, wild type.
(complex I), as well as complex III, and partially competed complex II (Fig. 3A, lane 2 versus lane 3). An Sp1 antibody completely supershifted the slowly migrating complex I and reduced the amount of the broad complex II, suggesting that Sp1 was present in complex I and probably in complex II (Fig. 3B, lane 3). Complex III was minimally altered by the Sp1 antibody, suggesting that it may contain another protein with an affinity for GC-rich sequences.

Similarly, the more slowly migrating complex I was competed by unlabeled E-box sequences indicating the presence of bHLH proteins in this complex with Sp1 (Fig. 3A, lane 4). Antibodies to NeuroD1 (Fig. 3B, lane 2) or E12 (Fig. 3B, lane 5) completely disrupted the formation of complex I, indicating the presence of NeuroD1 and E12 in addition to Sp1. This same complex was not generated by extracts of C33A cells, which do not express NeuroD1 (not shown). Finally insertion of 6 bp or 10 bp between the E-box and the adjacent Sp1 binding site of the probe disrupted the formation of complex I without affecting the other complexes, as was shown earlier with the reconstituted complex I (Fig. 2A). The presence of both Sp1 and NeuroD1 in complex I as well as its dependence on the normal spacing between the Sp1 and NeuroD binding sites indicates that this complex corresponds to the higher-order complex observed in Fig. 2A with recombinant proteins.

To determine if transcriptional activation also was dependent on the proximity of the E-box to the Sp1 binding site, we compared the activity of reporter genes where the spacing between the E-box and the adjacent Sp1 binding site in the secretin promoter was increased by either 6 bp or 10 bp versus the wild-type secretin-reporter in two secretin-expressing cell lines, HIT cells and STC-1 cells. Insertion of either 6 bp or 10 bp significantly reduced the transcriptional activity of the enhancer in both cell lines, indicating that the position of the E-box adjacent to the Sp1 binding site was necessary for maximal transcription of the gene (Fig. 3D). Thus, increasing the spacing between the two sites both reduced transcription and prevented formation of the higher-order complex, suggesting that generation of the ternary complex may be required for maximal transcription.

**Sp1 physically associates with NeuroD1.** The spacing between the E-box and GC1 box required for ternary complex formation and maximal enhancer activity suggested that NeuroD1 and Sp1 might physically interact with each other. We immunoprecipitated NeuroD1 from unfractonated extracts of HIT cells with NeuroD1 antibodies and tested for the presence of Sp1 by immunoblotting the precipitated proteins (Fig. 4A). Sp1 antibodies detected a single band in the NeuroD1 immunoprecipitates (lane 3) but not proteins precipitated by control immunoglobulin G (IgG) (lane 2), indicating that Sp1 and NeuroD1 associate at their native levels. To rule out the possibility that DNA present in extracts led to an artifactual association between NeuroD1 and Sp1, we repeated the immunoprecipitation experiment with the HIT cell extracts following pretreatment with ethidium bromide (50 μg/ml) as described before (10). The interaction of NeuroD1 with Sp1 was not significantly changed by ethidium bromide, indicating that their interaction did not result from the presence of DNA (not shown). We expressed full-length Sp1 as a GST fusion protein and tested its ability to directly bind to NeuroD1 using in vitro binding assays. Labeled in vitro-translated NeuroD1 was retained on an affinity column containing full-length GST-Sp1, indicating that these two proteins directly interact with each other (Fig. 4B).

We expressed three deletion mutants of Sp1 as GST fusion proteins and examined whether they could bind to NeuroD1 to identify the region of Sp1 that associates with NeuroD1. A GST fusion protein containing residues 622 to 788 of Sp1 was sufficient for NeuroD1 binding (Fig. 4B and C). This 167-
residue fragment at the C terminus contained the three zinc fingers of Sp1 that comprise its DNA binding domain. Neither of two N-terminal transactivation domains rich in serine-threonine and glutamate residues appears to physically interact with NeuroD1 (Fig. 4B and C). In contrast, E12, the ubiquitously expressed bHLH dimerization partner of NeuroD1, did not bind to GST-Sp1, indicating that the interaction between Sp1 and NeuroD1 is specific for NeuroD1 as opposed to other bHLH proteins capable of binding to the same E-box (Fig. 4B).

To determine the domain of NeuroD1 necessary for interaction with Sp1, we tested the GST-Sp1 fusion containing the C-terminal 167 amino acids (GST-Sp1_{622–788}) for its ability to bind to different truncation/deletion mutants of NeuroD1. The 158 amino acids at the N terminus of NeuroD1 were sufficient for binding to Sp1 (Fig. 4D and E). The inability of the mutant Δ49/155 to bind Sp1 indicates that the N terminus of NeuroD1, which contains the bHLH domain, is necessary for Sp1 binding, whereas the C-terminal activation domain of NeuroD1 does not appear to be involved. The NeuroD1 deletion mutants Δ49/96 and Δ113/128 retained the ability to bind to Sp1, suggesting that the deleted acidic and basic domains and helix 1 were not required. A final NeuroD1 truncation, N138, which retained 138 residues at the N terminus but lacks helix 2, was unable to bind to Sp1, suggesting that sequences in helix 2 of the bHLH domain of NeuroD1 are essential for its association with Sp1 (Fig. 4D and E).

**Sp1 binding to the secretin enhancer is stabilized in Sp1-NeuroD1 ternary complex.** To determine whether the binding cooperativity between NeuroD1 and Sp1 arises from stabilized DNA binding of either protein in the presence of the other, we compared the relative stability of the ternary complex compared to complexes generated by Sp1 or NeuroD1/E12 alone by EMSA. In order to better resolve the ternary complex from the complexes generated by either Sp1 alone or NeuroD1/E12, we used the truncated GST-Sp1_{622–788} fusion protein that contains both the DNA binding and NeuroD1-interacting domains of Sp1. The truncated Sp1 protein by itself generated a fast-migrating DNA-protein complex (complex iii, Fig. 5A, lane 2) that was easily resolved from the slower-migrating complex produced by NeuroD1/E12 (complex ii, Fig. 5A, lane 1). A more slowly migrating complex appeared with NeuroD1/E12 and GST-Sp1_{622–788} (complex i) (Fig. 5A, lane 3), suggesting that the truncated Sp1 protein could form the ternary complex as well. All three complexes (i, ii, and iii) were competed by an excess of unlabeled competitor containing the contiguous E-box and Sp1 binding site added to the probe prior to the binding reaction, indicating that all complexes resulted from sequence-specific DNA binding (lane 4).

We compared the stabilities of each of the three DNA-protein complexes by measuring their rate of dissociation after equilibrium binding following addition of an excess of competing DNA fragments. For these studies we incubated GST-Sp1_{622–788}, NeuroD1, and E12 with a probe containing the contiguous E-box and Sp1 binding site. Upon completion of the binding reaction, a 500-fold excess of unlabeled probe DNA was added and samples were withdrawn every 4 min for EMSA (Fig. 5B). The amount of probe remaining in each complex was quantitated for each time point by phosphorimaging (Fig. 5C). The results indicated that the dissociation of the Sp1-DNA complex (complex iii) was much faster than that of the Sp1-NeuroD1/E12-DNA complex (complex i). Only 35% of the probe initially present in complex iii remained after 4 minutes, and only 22% remained after 8 minutes. In contrast, the rate of loss of complex i or ii was lower than that of complex iii at all of the time points examined, suggesting that both the NeuroD1/E12 and ternary complexes were more stable than the complex generated by Sp1. The relative stability of the ternary complex (complex i) suggests that Sp1 binding is stabilized in the presence of NeuroD1 bound to the adjacent E-box, potentially accounting for its enhanced formation. To determine whether NeuroD1 stabilizes Sp1 binding to the secretin promoter in vivo, we examined the effects of NeuroD1 on chromatin occupancy of the secretin enhancer by Sp1. As expected ChIP assays revealed no occupancy of the secretin enhancer by NeuroD1 in HeLa cells, which do not
normally express NeuroD1 (Fig. 6A, top panel, lane 5). However, in HeLa cells transfected with a NeuroD1 expression plasmid we readily identified the presence of NeuroD1 at the secretin enhancer in vivo (top panel, lane 10) but not at the DHFR promoter, which lacks a NeuroD1 binding site (bottom panel, lane 10), indicating that transiently expressed NeuroD1 was associated with the endogenous secretin gene.

ChIP assays showed occupancy of the endogenous secretin promoter by Sp1 in NeuroD1-expressing HeLa cells, whereas recruitment of Sp1 was minimal in control HeLa cells, suggesting that NeuroD1 increases recruitment of Sp1 to the secretin promoter (Fig. 6B, left panel). The DHFR promoter contains several Sp1 binding sites. Recruitment of Sp1 to the DHFR promoter appeared identical in both wild-type HeLa cells and HeLa cells expressing NeuroD1, indicating that promoter occupancy by Sp1 is not generally dependent on NeuroD1 (Fig. 6B, middle panel). The dependence on NeuroD1 for Sp1 binding appears to be specific for the secretin gene. Little signal was detected with control IgG or with the GAPDH gene, which lacks Sp1 binding sites, confirming the specificity of protein-DNA complexes immunoprecipitated in ChIP assays for the secretin or the DHFR promoter (Fig. 6B).

We further confirmed the importance of NeuroD1 for occupancy of the secretin promoter by Sp1 in vivo in NeuroD1-expressing STC-1 enteroendocrine cells by knocking down NeuroD1 expression by DNA-based RNA interference using a previously described NeuroD1 shRNA (6). We used transient ChIP assays (11) to examine occupancy of a transiently transfected secretin promoter by endogenous NeuroD1 or Sp1. Both Sp1 and NeuroD1 were recruited to the wild-type promoter but not to a mutant promoter containing mutations in the E-box and both Sp1 sites (MutE/GC) that prevent binding of either factor to DNA (Fig. 7A, lanes 5 and 6). Introduction of mutations into the Sp1 binding sites of secretin promoter slightly reduced NeuroD1 occupancy, suggesting that Sp1 DNA binding is not essential for NeuroD1 recruitment to the promoter (Fig. 7A, lane 6).

We next transfected STC-1 cells with the wild-type secretin reporter plasmid or a control reporter plasmid containing multiple Sp1 binding sites (hTERT-Luc) plus expression plasmids for either a NeuroD1 shRNA or a GFP shRNA, which served as a control. The NeuroD1 shRNA significantly reduced...
NeuroD1 occupancy of the secretin promoter, indicating a significant decrease in the level of NeuroD1 expression (Fig. 7B, left panel). The effectiveness of the NeuroD1 shRNA was further confirmed by an 85% loss of secretin promoter activity (Fig. 7C). We consistently observed a significant reduction in Sp1 promoter occupancy in cells with decreased expression of NeuroD1, whereas Sp1 occupancy of the hTERT promoter was unaffected by depletion of NeuroD1 (Fig. 7B, left versus right panel). These observations suggest that in secretin-expressing cells, NeuroD1 stabilizes Sp1 binding to the secretin enhancer to potentiate secretin gene transcription.

**DISCUSSION**

Mice carrying a targeted deletion of the bHLH protein NeuroD1 fail to develop secretin-expressing enteroendocrine cells, indicating that this protein is essential for secretin gene expression. Paradoxically, in vitro studies suggest that NeuroD1 is a relatively weak activator of the secretin gene transcription by itself. Of the transcription factors that bind to the secretin enhancer, only NeuroD1 is relatively restricted to a limited number of cell types, as opposed to the ubiquitous expression of the others, implying a major role for NeuroD1 in regulating secretin gene expression. In the present work, we show that physical interactions between NeuroD1 and the ubiquitously expressed transcription factor Sp1 on the secretin gene enhancer significantly potentiate transactivation by this tissue-specific class B bHLH protein. We previously identified the protein Finb/RREB1 as a transcription-modifying protein that synergistically potentiates NeuroD1. Thus, the interaction of NeuroD1 with two widely expressed DNA binding proteins is one mechanism for potentiating the relatively weak transactivating function of NeuroD1.

Cell-type-specific gene expression depends on the presence of a combination of transcription factors, some of which are also highly restricted in expression, as well as the organization transcription factor binding sites controlling a gene. The proximal enhancer of the secretin gene is conserved in different mammalian species, maintaining a nearly identical arrangement of elements to potentiate transcription. Besides the secretin gene, relatively few target genes that are directly activated by NeuroD1 have been identified. These include the genes encoding the hormones insulin and POMC. Expression of both of these genes depends on interactions between bHLH proteins with lineage-restricted homeodomain transcription factors bound to nearby cis elements to potentiate the transactivating function of NeuroD1.

Lineage-specific transcription of the POMC gene in the pituitary gland is enhanced by the functional synergy between bHLH factors binding to an E-box and the pituitary gland-specific homeodomain protein Ptx1-1 (25). Ptx1-1 indirectly modifies NeuroD1-dependent transcription by physically interacting with one of its ubiquitously expressed dimerization partners rather than by a direct association with NeuroD1. The bHLH domain of Pan1 (E47) serves as a protein-protein interaction domain with the homeodomain of Pan1 (Ptx1-1). The NeuroD1 binding E-box in the POMC promoter is separated from the Ptx1-1 binding site by 67 bp. This spacing may indicate that the interactions on the POMC promoter involve different surfaces of the bHLH proteins than in the case of the insulin and secretin promoter, where Pdx1 and Sp1 bind to sites immediately adjacent to the E-box.

Insulin-expressing pancreatic β cells arise from the primitive gut endoderm and are developmentally related to enteroendocrine cells, and yet insulin gene expression does not occur in the intestine. The expression of insulin and secretin genes, two NeuroD1-dependent genes, in distinct cell types depends in part on the expression of different sets of transcriptional activators in islets versus enteroendocrine cells and on the organization of transcription factor binding sites on each promoter. The major elements responsible for β-cell-specific transcription of the insulin gene are localized to a relatively small region of the promoter that contains an E-box and two TAAT-rich A elements that bind to the homeodomain protein PDX-1. In developing animals, PDX-1 is expressed throughout the proximal duodenum and the pancreas, but intestinal expression is largely absent in adult animals, whereas expression in islets continues.

The homeodomain of PDX-1 associates with several other proteins including E47, NeuroD1, and high-mobility-group protein Y1 to form a higher-order transcription-activating complex (24). Unlike the specific interaction between NeuroD1 and Sp1 on the secretin gene, PDX-1 interacts with the ubiquitous dimerization partners of NeuroD1 as well as with NeuroD1. In the case of the insulin gene, synergistic transcriptional activation occurs with E47 as well as NeuroD1. In addition, high-mobility-group protein Y1 synergizes with the other members of the insulin gene-transactivating complex. The absence of PDX-1 in adult enteroendocrine cells may
explain in part why insulin is not expressed in the intestine. Thus, NeuroD1-dependent expression of the insulin and secretin genes in pancreatic β cells and S-type enteroendocrine cells, respectively, depends on different sets of transcription factors that interact with NeuroD1 to potentiate its activity.

NeuroD1 plays an important role in regulating terminal differentiation of neurons and hormone-producing cell lineages. Relatively few target genes have been identified for this bHLH protein. Other DNA binding proteins potentiate transactivation of the genes encoding the hormones POMC, insulin, and secretin by NeuroD1. The enhancer of the secretin gene shares similarities with the organization of the insulin or POMC genes. Unlike the POMC and insulin genes, where NeuroD1 synergizes with homeodomain proteins specific for their tissue, NeuroD1 synergizes with the widely expressed proteins Sp1 and RREB1 to increase secretin gene transcription. The specific arrangement of factor binding sites in the secretin enhancer provides the critical context for the interaction with Sp1 and Finb that potentiates the activating function of NeuroD1.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants DK43673 and DK67166 to A.B.L. and the GRASP Digestive Disease Center P30-DK34928.

We thank Hans Rotherneder (University of Vienna, Vienna, Austria) and Yang Shi and Azad Bonni (Harvard Medical School, Boston, MA) for generously providing plasmids as noted in Materials and Methods.

REFERENCES


