MOLECULAR AND CELLULAR BIOLOGY, Nov. 1997, p. 6673–6682 Vol. 17, No. 11
0270-7306/97/$04.00+0

NeuroD1/β2 Contributes to Cell-Specific Transcription of the Proopiomelanocortin Gene

GINO POULIN, BENJAMIN TURGEON, AND JACQUES DROUIN*

Laboratoire de Génétique Moléculaire, Institut de Recherches Cliniques de Montréal, Montréal, Québec, Canada H2W 1R7

Received 24 March 1997/Returned for modification 27 May 1997/Accepted 8 August 1997

NeuroD1/β2 is a basic helix-loop-helix (bHLH) factor expressed in the endocrine cells of the pancreas and in a subset of neurons as they undergo terminal differentiation. We now show that NeuroD1 is expressed in corticotroph cells of the pituitary gland and that it is involved in cell-specific transcription of the proopiomelanocortin (POMC) gene. It was previously shown that corticotroph-specific POMC transcription depends in part on the action of cell-restricted bHLH factors that were characterized as the CUTE (corticotroph upstream transcription element) (M. Therrien and J. Drouin, Mol. Cell. Biol. 13:2342–2353, 1993) complexes. We now demonstrate that these complexes contain NeuroD1 in association with various ubiquitous bHLH dimerization partners. The NeuroD1-containing heterodimers specifically recognize and activate transcription from the POMC promoter E box that confers transcriptional specificity. Interestingly, the NeuroD1 heterodimers activate transcription in synergy with Ptx1, a Bicoid-related homeodomain protein, which also contributes to corticotroph specificity of POMC transcription. In the adult pituitary gland, NeuroD1 transcripts are detected in POMC-expressing corticotroph cells. Taken together with the restricted pattern of Ptx1 expression, these results suggest that these two factors establish the basis of a combinatorial code for the program of corticotroph-specific gene expression.

Families of cell-restricted basic helix-loop-helix (bHLH) transcription factors control differentiation in many cell lineages. For example, the myogenic bHLH factors are involved in various steps of myogenesis, and they do so as muscle-specific transcription factors (16, 40, 44, 46, 53, 55). Similarly, Tal-1/SCL is required for differentiation of the hematopoietic lineage (3, 48), and a growing family of neurogenic bHLH factors has been identified in neural tissues. The Mash-1 and HES-1 bHLH factors that were originally cloned by homology to the *Drosophila* genes *achaete-scute*, and *Hairy* and *Enhancer of Split* behave as positive and negative regulators, respectively, of neurogenesis (15, 18, 19). The functions of other neural tissue-specific bHLH factors are not yet known (22, 32, 34). One member of this group, NeuroD1, was recently shown to be associated with late neuronal differentiation in *Xenopus laevis* (30). The same factor was also isolated as β2, a cell-specific transcription factor of the insulin gene (41). Thus, this so-called neural tissue-specific bHLH factor is also expressed in a small subset of endocrine cells of the pancreas. We now report on the expression and transcriptional role of NeuroD1/β2 in specific endocrine cells of the anterior pituitary gland that express the proopiomelanocortin (POMC) gene.

The development of the neuroendocrine system is highly integrated; indeed, pituitary development and hypothalamic development are intimately associated, as disruption of one affects the other (24–26). The pituitary gland develops from a placodal invagination of the stomodeum starting at embryonic day 8.5 (E8.5) in mice (2). The structure formed by this invagination, known as Rathke's pouch, develops in close contact with the neuroepithelium of the ventral diencephalon fated to become the hypothalamus (47). A projection of the hypothalamus (the infudibulum) will develop into the posterior lobe of the pituitary, while Rathke's pouch will become the anterior and the intermediate lobes of the gland. Throughout morphogenesis, specific hormone-producing cells differentiate according to a well-conserved sequence (52). The first cells to reach final differentiation in this sequence (at E12.5) are the POMC-expressing cells of the anterior lobe, the corticotrophs which process POMC into ACTH (adrenocorticotropic hormone). Another POMC-expressing lineage appears later at E14.5: the melanotroph cells of the intermediate pituitary, which process POMC into α-MSH (melanocyte-stimulating hormone) (20).

Previous work to identify mechanisms for corticotroph-specific transcription of the POMC gene indicated that two regulatory elements of the promoter are sufficient to recapitulate this activity. Different transcription factors bind these two elements and exert synergistic effects on transcription (50, 51). Consistent with the apparent tissue specificity of this synergism, cognate DNA-binding proteins for these elements have a restricted distribution. One of these elements (CE3) is the binding site of *Ptx1*, a *bicoid*-related homeogene expressed in the pituitary (27). The activity of this element is synergistically and specifically enhanced in corticotroph cells by a target site for bHLH factors, an E-box element (Fig. 1A). This E box, DE2C, is bound by corticotroph-restricted bHLH proteins that appear as two bands in gel retardation. These two complexes were previously named CUTE (corticotroph upstream transcription element) (51). bHLH transcription factors must form dimers through their HLH domains in order to bind DNA; protein-DNA interactions depend on the basic region (31, 37). The class I (or ubiquitous) bHLH factors can form homodimers, and the class II (or tissue-restricted) bHLH factors are active as heterodimers in association with class I factors (29). Since the CUTE complexes have a restricted distribution, they may very well be heterodimers of class I and II bHLH factors. The DE2C E box of the POMC gene has the same sequence as the cell-specific E box of the insulin promoter that was used as probe to clone β2 (9, 14, 23, 41). Hence, the CUTE

* Corresponding author. Mailing address: Institut de Recherches Cliniques de Montréal, Laboratoire de Génétique Moléculaire, 110 avenue des Pins Ouest, Montréal, QC, Canada H2W 1R7. Phone: (514) 987-5680. Fax: (514) 987-5575. E-mail: drouinj@ircm.umontreal.ca.

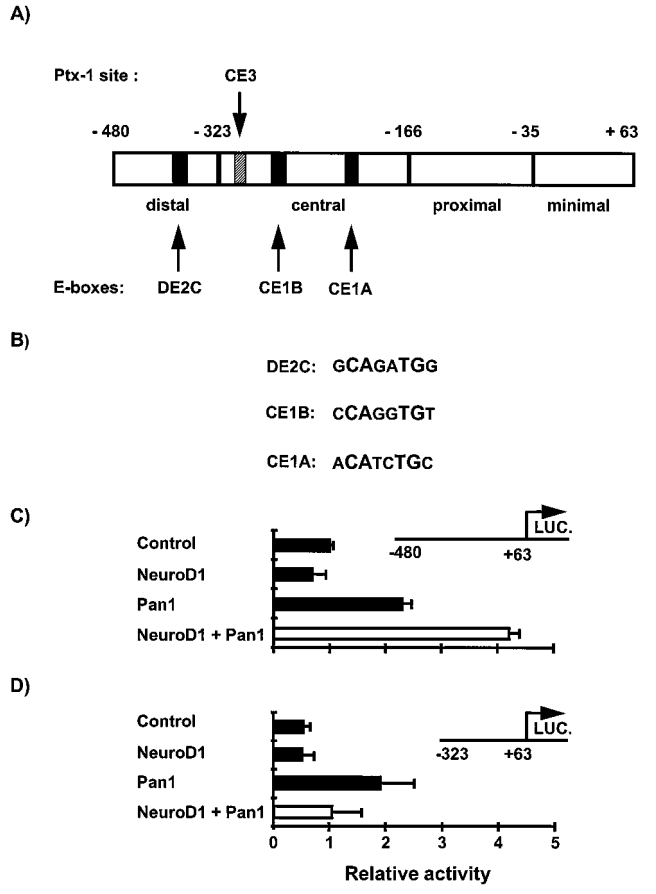

FIG. 1. Schematic representation of the rat POMC promoter (bp −480 to +63). (A) Relative positions of the DE2C, CE1B, and CE1A E boxes and of the Ptx1 binding site (CE3) within the POMC promoter. (B) Nucleotide sequences of the three E boxes of the promoter. Lowercase letters represent variant nucleotides by comparison to the consensus sequence NCANNTGN. (C) Effect of bHLH factor expression on full-length POMC-luciferase reporter (50). Expression vectors for NeuroD1 on/or Pan1 were transfected in CV-1 cells, and the luciferase (LUC.) activity (± standard error of the mean) is shown relative to that of the reporter alone. (D) Same experiment as in C performed with a reporter plasmid containing a POMC promoter fragment deleted to bp −323, which no longer contains the DE2C E box.

complexes may contain NeuroD1 or a related factor of similar DNA-binding specificity.

We now report that NeuroD1/β2-containing heterodimers specifically bind and activate transcription from reporters containing the DE2C E box. Further, we show that these heterodimers activate transcription synergistically with Ptx1 in heterologous cells. We also show that the CUTE complexes contain NeuroD1. In addition, NeuroD1 expression appears to be restricted to a subset of pituitary cells that includes the corticotrophs. These data support the model that NeuroD1 is part of a transcriptional complex which triggers corticotroph-specific transcription and cell differentiation during pituitary ontogeny.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The different reporter plasmids were constructed in the vector pXP1-luciferase as described previously (43). The DE2C, CE1B, CE1A, DE2, and CE3 elements inserted into the reporter plasmids were made from oligonucleotide sequences of the corresponding regions of the rat POMC promoter as described previously (27, 51). The expression vectors of the bHLH factors used in this study were described in previous work: β2/NeuroD1 (41), ME1a and ME1b (7), Pan1 and Pan2 (42), and Id (4).

Cell culture and nuclear extracts. L or CV-1 cells (21) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% newborn calf serum and maintained at 37°C and 5% CO_2. AtT-20 D16v cells (50) were grown under the same conditions, but fetal bovine serum was used, also at a concentration of 10%.

The nuclear extracts were prepared as described previously (51). Briefly the cells were harvested using 0.5 mM EDTA in phosphate-buffered saline (PBS) and gently spun down. The supernatant was removed, and the cells were resuspended in a buffer containing protease inhibitors, 10 mM Tris-HCl (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol. The cells were left on ice for 15 min to swell, and then Nonidet P-40 was added to a final concentration of 0.66%. The cells were then vortexed vigorously for 10 s and centrifuged at 12,000 rpm. The supernatant was subsequently removed, and the pellet of nuclei was resuspended in a buffer similar to that described above but containing 20 mM Tris-HCl (pH 7.9), 400 mM NaCl, and no KCl. The nuclei were then shaken on a shaking platform for 30 min and spun down, and the supernatant was kept at −70°C. The protein concentration was subsequently estimated by the Bradford assay.

Transfection assays. L and CV-1 cells were transfected by the calcium phosphate coprecipitation method. The precipitate containing 3 μg of reporter plasmid, 1 μg of expression vector, 1 μg of plasmid RSV-GH as an internal control, and carrier DNA up to a total of 10 μg was applied to 10^5 cells in a 35-mm petri dish. Control samples contained equivalent amounts of empty expression vector or pSP64. After 16 h, the medium was changed, and the cells were harvested 24 h later, using 0.5 mM EDTA in PBS. In Fig. 8C, 1.5 μg of the reporter plasmid per dish was used; NeuroD1 expression vector was used at 1 μg per dish; Pan1, Ptx1, and Id effectors were used at 0.5 μg. Overexpression of different factors for preparation of nuclear extracts and for use in electrophoretic mobility shift assays (EMSAs) was accomplished by similar transfections using 5 μg of each expression plasmid. AtT-20 cells were transfected by using Lipofectamine (Pharmacia); 0.5×10^6 cells were plated into 35-mm petri dishes. Reporter plasmid (500 ng), Id expression vector (25 ng), and carrier DNA up to a total of 800 ng were used together with 4.5 μl of Lipofectamine (Pharmacia) in a final volume of 200 μl of DMEM without serum. The volume was made up to 1 ml with DMEM after 30 min and left for 4 h on the cells; 1 ml of DMEM containing 10% fetal bovine serum was then added. The cells were harvested 20 h later, using 0.5 mM EDTA in PBS. Data were compared by Student's *t* test.

Northern blot analysis. Total cellular RNA was isolated by the guanidinium thiocyanate-phenol-chloroform method (8). RNA was analyzed by electrophoresis on a 1.2% agarose gel by the RNA-glyoxal method (33). Transfer was performed on a Hybond-N (Amersham) membrane. Probe was made by random priming on an *Apa*I-*Pst*I fragment of unique (i.e., not conserved in NeuroD2 and -3) sequence in the 3′ coding region of NeuroD1. Blots were washed for 30 min at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS). 18S rRNA was revealed by using an 18S RNA-specific oligonucleotide (ACG GTA TCT GAT CGT CTT CGA ACC) labeled with [γ-^{32}P]ATP. Hybridization was performed in 6× SSC–5% Denhardt's solution–1% SDS–0.05% sodium pyrophosphate–salmon sperm DNA (50 μg/ml) at 42°C overnight. Washes were performed in 6× SSC–0.05% sodium pyrophosphate–1% SDS at 55°C for 45 min.

Histochemistry. Immunohistochemistry was performed with an anti-ACTH monoclonal antibody (Cortex Biochemicals) produced against the amino-terminal part of ACTH and anti-thyroid-stimulating hormone beta subunit (TSHβ) antibodies produced in rabbit (AFP4492192 provided by A. F. Parlow, Pituitary Hormones and Antisera Center, Torrance, Calif.). Anti-mouse and anti-rabbit immunoglobulin antibodies (Sigma) coupled to horseradish peroxidase were used to reveal hormone-antibody complexes. Immunohistochemistry and in situ hybridization procedures were as described previously (27, 28). Briefly, adult male pituitaries were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned in slices of 5-mm thickness. The hybridizations were carried out at 52.5°C with a [^{35}S]UTP-labeled probe. The washes were performed in 1× SSC–50% formamide at 62°C. The slides were then treated with RNase A (20 μg/ml) for 30 min at 37°C, followed by another 1× SSC–50% formamide wash at 62°C. Two different cRNA probes were generated for NeuroD1. One probe of about 700 nucleotides contained 3′ untranslated sequence and the C terminus of NeuroD1 including the bHLH-coding domain, whereas another probe of 180 nucleotides excluded the bHLH-coding region. These different probes gave similar results. Sense probes made from corresponding fragments did not give any significant signal.

EMSA. The gel shift assays were performed with 5% polyacrylamide gels (44:1 acrylamide/bisacrylamide) in 40 mM Tris-HCl and 195 mM glycine (pH 8.5) at 4°C. The probe sequences were 5′-GAT CCG GAA GGC AGA TGG ACG CA-3′ (DE2C) and 5′-GAT CCG GGG CCA GGT GTG CGC TA-3′ (CE1B). We used 10,000 cpm per reaction (~15 fmol) of double-stranded oligonucleotide probes, end labeled by fill-in with Klenow enzyme in the presence of [α-^{32}P]dCTP and purified on a Sephadex G-25 column. The reactions were performed in 20 μl containing 25 mM HEPES (pH 7.2), 150 mM KCl, 5 mM dithiothreitol, 10% glycerol, 1 to 2 μg of a 1:1 mixture of poly(dI-dC) and poly(dA-dT), and 5 μg of nuclear extracts. The reaction mixtures were incubated for 30 min on ice prior to loading. The samples were separated by electrophoresis for 3 to 3.5 h. For supershift experiments, the antibodies (from Ming Tsai [Baylor College of Medicine] or Santa Cruz Biotechnology, as indicated) were preincubated with the nuclear extracts for 15 min on ice prior to probe addition.

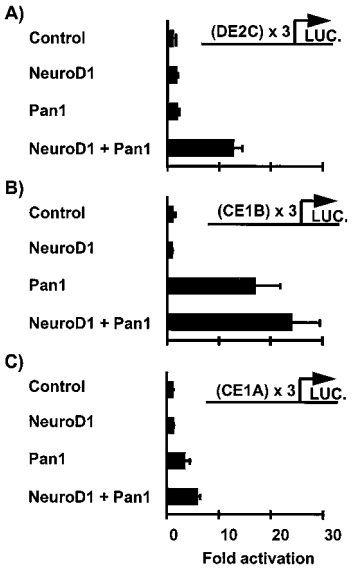

FIG. 2. Specificity of NeuroD1 activation of POMC promoter E boxes. Reporter plasmids containing three copies of E boxes DE2C (A), CE1B (B), and CE1A (C) were tested by cotransfection with expression vectors for NeuroD1 and/or Pan1. Results (± standard error of the mean) are the average of at least three separate experiments, each performed in duplicate. LUC., luciferase.

RESULTS

Transcriptional specificity of NeuroD1. The CUTE complexes were previously characterized as bHLH factors that specifically bind the DE2C E box (51) and not other E boxes of the POMC promoter (35). The POMC promoter contains three E boxes (Fig. 1A and B), but corticotroph-specific transcription can be conferred only by DE2C. Therefore, DE2C plays a unique role by recruiting a corticotroph-restricted bHLH factor(s) to the POMC promoter. Since β2/NeuroD1 was shown to activate transcription from a similar E box in the insulin promoter (41), we tested whether it acts on the POMC promoter E boxes. Expression of NeuroD1 in CV-1 cells did not affect the activity of a bp −480 POMC-luciferase reporter, but cotransfection with the class I bHLH factor Pan1 resulted in significant activation (Fig. 1C). Interestingly, deletion of the distal region of the promoter which contains the DE2C E box prevented this activation (Fig. 1D), suggesting that the other E boxes of the promoter are not targets for the NeuroD1 heterodimers. However, these E boxes are targets for class I bHLH factors (Fig. 1C and D and reference 35).

To define the properties of each POMC promoter E box, three copies of each E box (DE2C, CE1B, and CE1A [Fig. 1A and B]) were cloned upstream of a luciferase reporter containing a minimal POMC promoter (bp −35 to +63 bp) fragment (50). Mammalian expression vectors for NeuroD1 and Pan1 were cotransfected with these reporters into L cells. These cells do not express NeuroD1 (Northern blot analysis [data not shown]), nor do they express significant levels of class I bHLH factors, as they are not detected in Western blots (data not shown) or in EMSA (see Fig. 3 and 4).

NeuroD1 overexpression in L cells did not increase the transcriptional activity of any of the three E-box reporter plasmids (Fig. 2, NeuroD1 compared to control). Overexpression of Pan1 alone did not affect the transcriptional activity of the DE2C reporter (Fig. 2A, Pan1) but enhanced the transcriptional activity of the CE1B and CE1A reporters (Fig. 2B and C, respectively). However, coexpression of NeuroD1 and Pan1

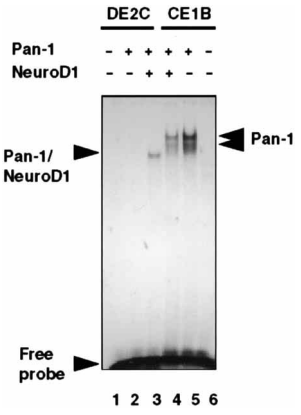

FIG. 3. Specific DE2C binding of NeuroD1/Pan1 heterodimers. EMSA was performed with radiolabeled DE2C and CE1B E boxes as probes and nuclear extracts from L cells transfected with Pan1 and/or NeuroD1 expression vectors as indicated.

led to a significant increase of the DE2C reporter activity (Fig. 2A, NeuroD1/Pan1 compared to NeuroD1 or Pan1) but did not significantly enhance the activity of either the CE1B or CE1A reporter beyond the effect of Pan1 alone (Fig. 2B and C, Pan1 compared to NeuroD1/Pan1). Neither factor affected the activity of reporters devoid of E boxes or containing a mutant E box (reference 51 and data not shown). Further, the few nucleotide differences between the E boxes have marked effects on their activation by NeuroD1 and/or Pan1. These results strongly suggested that NeuroD1 transcriptional activity is dependent on specific recognition of the DE2C E box. Thus, the transcriptional properties of NeuroD1/Pan1 heterodimers are consistent with the properties of the CUTE complexes and of their target, DE2C (51). In contrast, the CE1B and CE1A E boxes of the POMC promoter behave as targets of class I ubiquitous bHLH factors, albeit of different potencies.

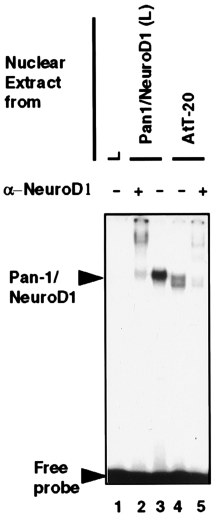

FIG. 4. CUTE complexes contain NeuroD1. EMSA was performed with radiolabeled DE2C E box as probe and nuclear extracts from L cells expressing NeuroD1/Pan1 and from AtT-20 cells to reveal the CUTE complexes. Supershift was produced by addition of 1.5 μl of affinity-purified antibodies against NeuroD1 (α-NeuroD1), provided by M. Tsai, or 2 μl of normal rabbit serum as control (−). The anti-NeuroD1 antibodies did not react with Pan1 (reference 41 and data not shown).

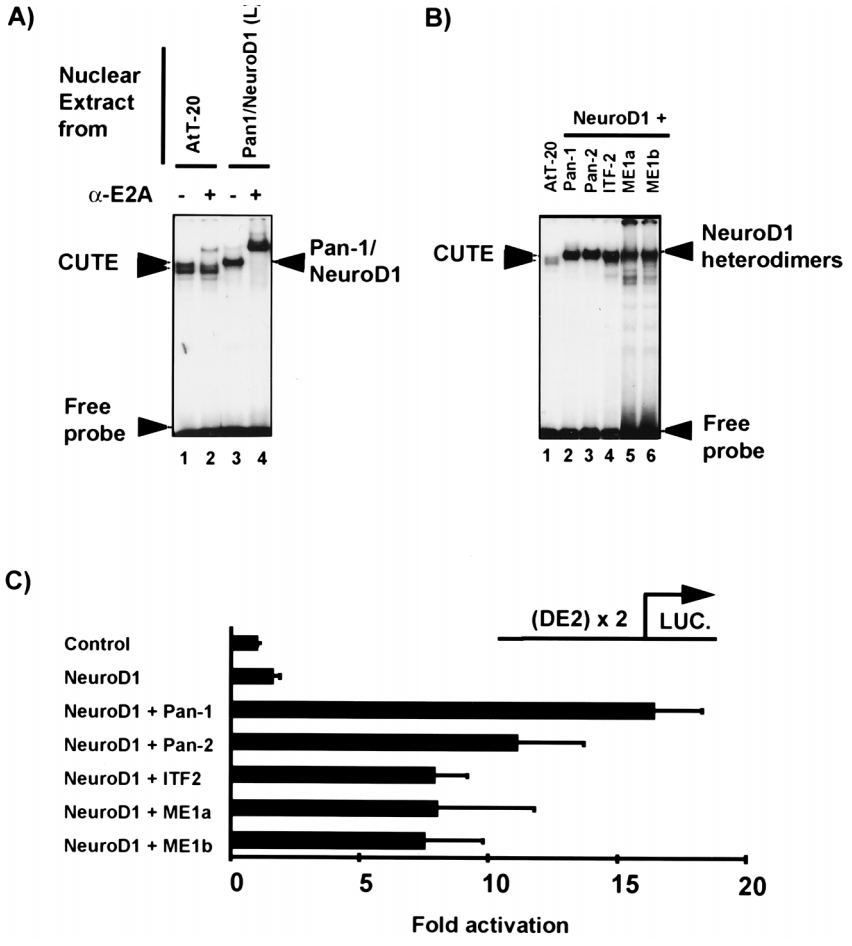

FIG. 5. Pan1, Pan2, ITF2, ME1a, and ME1b can dimerize with NeuroD1. (A) E2A gene products are present with NeuroD1 in the slower-migrating CUTE complexes. Monoclonal antibodies against E2A (α-E2A; 1.5 μg; Santa Cruz) and nuclear extracts from AtT-20 cells were used to demonstrate the presence of E2A products in these heterodimers, as in Fig. 4. These antibodies were shown by the supplier not to cross-react with ITF2 or HEB. (B) NeuroD1 heterodimers formed with Pan1, Pan2, ITF2, ME1a, and ME1b bind the DE2C probe. EMSA was performed as for previous figures, using probe and nuclear extracts from L cells cotransfected with expression vectors for NeuroD1 and indicated class I bHLH factors. (C) Transactivation of the DE2 reporter plasmid by NeuroD1 heterodimers formed with either Pan1, Pan2, ITF2, ME1a, or ME1b. Transfection assays were performed as for Fig. 2. LUC., luciferase.

Specificity of NeuroD1/Pan1 heterodimer exerted at DNA binding. The specificity of E-box recognition by NeuroD1/Pan1 heterodimers was tested by EMSA. The gel shift analyses were performed with the DE2C and CE1B probes and nuclear extracts from L cells expressing Pan1, Pan1 and NeuroD1, or neither. Cells expressing only NeuroD1 did not exhibit binding to either probe (data not shown), in agreement with experiments done with recombinant NeuroD1 (41). NeuroD1 and Pan1 were both required to bind DE2C as a unique band in EMSA (Fig. 3, lane 3). In assays using the same nuclear extracts with the CE1B probe, there was no significant binding of similar migration (Fig. 3, lane 4 compared to lane 3). Instead, all bands observed with the CE1B probe were dependent on Pan1 irrespective of the presence of NeuroD (Fig. 3, lane 5). In agreement with the transfection data showing Pan1 transactivation of the CE1B but not the DE2C reporter (Fig. 2A and B), Pan1 dimer complexes did not bind DE2C (Fig. 3, lane 2). Taken together, these results demonstrate that the specificity of NeuroD1/Pan1 heterodimer action on transcription (Fig. 2) is largely due to specific recognition of the DE2C E box (Fig. 3).

NeuroD1 is part of the CUTE complexes. To test whether NeuroD1 is a component of the CUTE complexes of AtT-20 cells, we used anti-NeuroD1 antibodies (provided by M. Tsai) in supershift EMSAs. As control, we showed that NeuroD1/Pan1 heterodimers expressed in L cells (Fig. 4, lane 3) were supershifted by the anti-NeuroD1 antibodies (Fig. 4, lane 2). The two CUTE complexes (51) of AtT-20 cells (Fig. 4, lane 4) were also supershifted in the presence of the anti-NeuroD1 antibodies (Fig. 4, lane 5). Similar results (Fig. 6 and data not shown) were obtained with another antibody (Santa Cruz) made against a C-terminal NeuroD1 peptide from a region unique to NeuroD1 by comparison to NeuroD2 and NeuroD3 (34). Thus, both CUTE bands of AtT-20 cells contain NeuroD1, and conversely, NeuroD1, rather than other related gene products, appears to be the major class II component of CUTE complexes.

NeuroD1 forms active dimers with various class I bHLH factors. Since NeuroD1 does not bind E boxes on its own and requires a class I dimerization partner for activity (Fig. 2), we tested whether the CUTE complexes contain products of the E2A gene; these include the differential splicing products Pan1 (E47), Pan2 (E12) (42), and ITF1 (17). A commercially available antibody (Santa Cruz) that recognizes an epitope common to these gene products was used in gel shift analyses to demonstrate the presence of E2A gene products in the

CUTE complexes of AtT-20 cells. As control, overexpressed NeuroD1/Pan1 heterodimers (Fig. 5A, lane 3) were supershifted by the anti-E2A antibodies (Fig. 5A, lane 4). However, only the slower-migrating CUTE complex (Fig. 5A, lane 1) was partially supershifted with saturating amounts of the anti-E2A antibody (Fig. 5A, lane 2). This experiment suggested that E2A gene products are present in CUTE complexes. However, they may account for only about 25% of the CUTE complexes. Specific antisera against the other mouse class I bHLH factors are not available to test their presence in CUTE complexes. In an attempt to determine whether other class I factors can act with NeuroD, cotransfection assays and in vitro binding experiments by EMSA were performed. In transfection assays, all ubiquitous bHLH factors tested (Pan1 and -2, ITF2, and HEB [ME1a and ME1b]) activated a DE2 reporter in the presence of NeuroD1 (Fig. 5C) but not when transfected alone (data not shown). In EMSA, all class I bHLH factors formed DE2C-binding heterodimers with NeuroD1 (Fig. 5B). All of these complexes migrated close to the position of the slower-migrating CUTE complexes, suggesting that any of these could be present in CUTE complexes. Thus, ITF2 and/or ME1a/b (HEB) may constitute the other 50% of the slowly migrating CUTE band which is not accounted for by E2A products. Both ITF2 and ME1a/b are expressed in AtT-20 cells (35). The identity of the NeuroD1 dimerization partner of the faster-migrating CUTE complex remains unknown.

NeuroD1 is restricted to corticotroph cells of the pituitary. Since it was previously suggested that the CUTE complexes are restricted to pituitary corticotroph cells (51), and since NeuroD1 and its target DE2C are thought to contribute to POMC cell specificity, we determined whether NeuroD1 expression is restricted to a subset of pituitary cells. First, we used a panel of pituitary-derived cell lines to investigate DE2C-binding activity in EMSA (Fig. 6A); cells representative of the corticotroph (AtT-20), somatolactotroph (GH3), and gonadotroph (αT3) lineages were used to prepare nuclear extracts.

In assays using anti-NeuroD1 antibodies, a complete supershift of the CUTE complexes present in AtT-20 cell extracts (Fig. 6A, lanes 8 and 9) was observed with a relatively small amount of antibodies (0.5 μg). In contrast, complexes observed with GH3 nuclear extracts did not show any supershift with the NeuroD1 antibodies (Fig. 6A, lanes 4 to 6), indicating that the DE2C-binding activity of these somatolactotroph cells does not contain NeuroD1. This observation was confirmed by the absence of NeuroD1 transcripts in these cells (Fig. 6B, lane 3). Complexes formed with αT3 nuclear extracts were partially blocked by NeuroD1 antibodies (Fig. 6A, lanes 1 to 3). However, complete blocking or supershift was not observed, even with antibody amounts sufficient to completely supershift the CUTE complexes of AtT-20 cells. These data suggest that NeuroD1 may account for part of the DE2C-binding activity of αT3 cells, as confirmed by Northern blotting (Fig. 6B, lane 2) showing NeuroD1 mRNA in these cells; however, the remainder of the DNA-binding activity is probably due to other bHLH proteins. These experiments suggest that NeuroD1 may not be ubiquitously expressed in the pituitary since it is not expressed in somatolactotroph cells, which represent around 50% of the cells.

To extend this conclusion to normal pituitary tissues and cells, we performed colocalization of NeuroD1 by in situ hybridization and hormone by immunohistochemistry. ACTH-producing cells and TSHβ-producing cells were identified with specific antibodies against ACTH and TSHβ. We used the TSHβ-positive cells as a control for colocalization in these experiments because they are the least abundant hormone-producing cells in the anterior pituitary. This was followed by

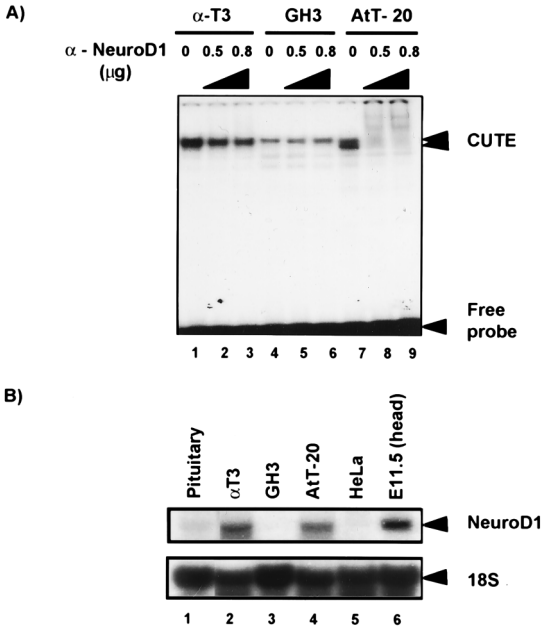

FIG. 6. DE2C-binding activity in different pituitary lineages (αT3, GH3, and AtT-20 cells). (A) DE2C-binding activity in extracts (10 μg) from αT3, GH3, and AtT-20 cells. Supershift experiments were performed as for Fig. 4 and 5, using antibodies (Santa Cruz) against NeuroD1 (α-NeuroD1) at 0, 0.5, and 0.8 μg per reaction. The binding activity detected in GH3 cells is bHLH, as it is supershifted by the E2A monoclonal antibodies (data not shown). (B) NeuroD1 mRNA expression in cells derived from different pituitary lineages, in adult pituitary and in head of an E11.5 embryo. 18S RNA was revealed as an internal control.

in situ hybridization using a [^{35}S]UTP-labeled NeuroD1 probe. A sense NeuroD1 probe was used as negative control, and it did not show significant labeling of any cell (data not shown). This approach was taken because the available anti-NeuroD antibodies were not sensitive enough to reveal a specific NeuroD signal in tissue sections.

At first glance, it appeared that NeuroD1-positive cells were also ACTH positive (Fig. 7A). To substantiate this, silver grains were counted over horseradish peroxidase-positive (i.e., ACTH-positive) and negative cells. This analysis (Fig. 7B) showed preferential labeling of ACTH-positive cells with the NeuroD1 probe. In contrast, we did not observe colocalization of NeuroD1 and TSHβ (Fig. 7C and D). Thus, it appears that NeuroD1 is preferentially expressed in corticotroph cells of the adult pituitary, although low-level expression in other cells would not be discriminated from background in these experiments.

NeuroD1 synergizes with Ptx1, a pituitary-restricted homeodomain transcription factor. We previously showed that two regulatory elements of the POMC promoter, DE2C and CE3, exert corticotroph-specific synergism in AtT-20 cells and not in GH3 or in L cells (51). This synergy likely depends on two transcription factors: Ptx1 acting on the CE3 element and NeuroD1-containing heterodimers acting on the DE2C E box. To assess the dependence on bHLH factors for DE2C-dependent synergism in AtT-20 cells, we used the dominant negative bHLH factor Id (4). Overexpression of Id blunted the activity of a DE2C-containing reporter but not that of reporters containing only the Ptx1 target site CE3 (Fig. 8A), in agreement with a model of NeuroD1 heterodimers acting on DE2C and with previous data showing blockade of CUTE DNA-binding activity by Id (51).

To test directly whether this synergism depends on NeuroD1

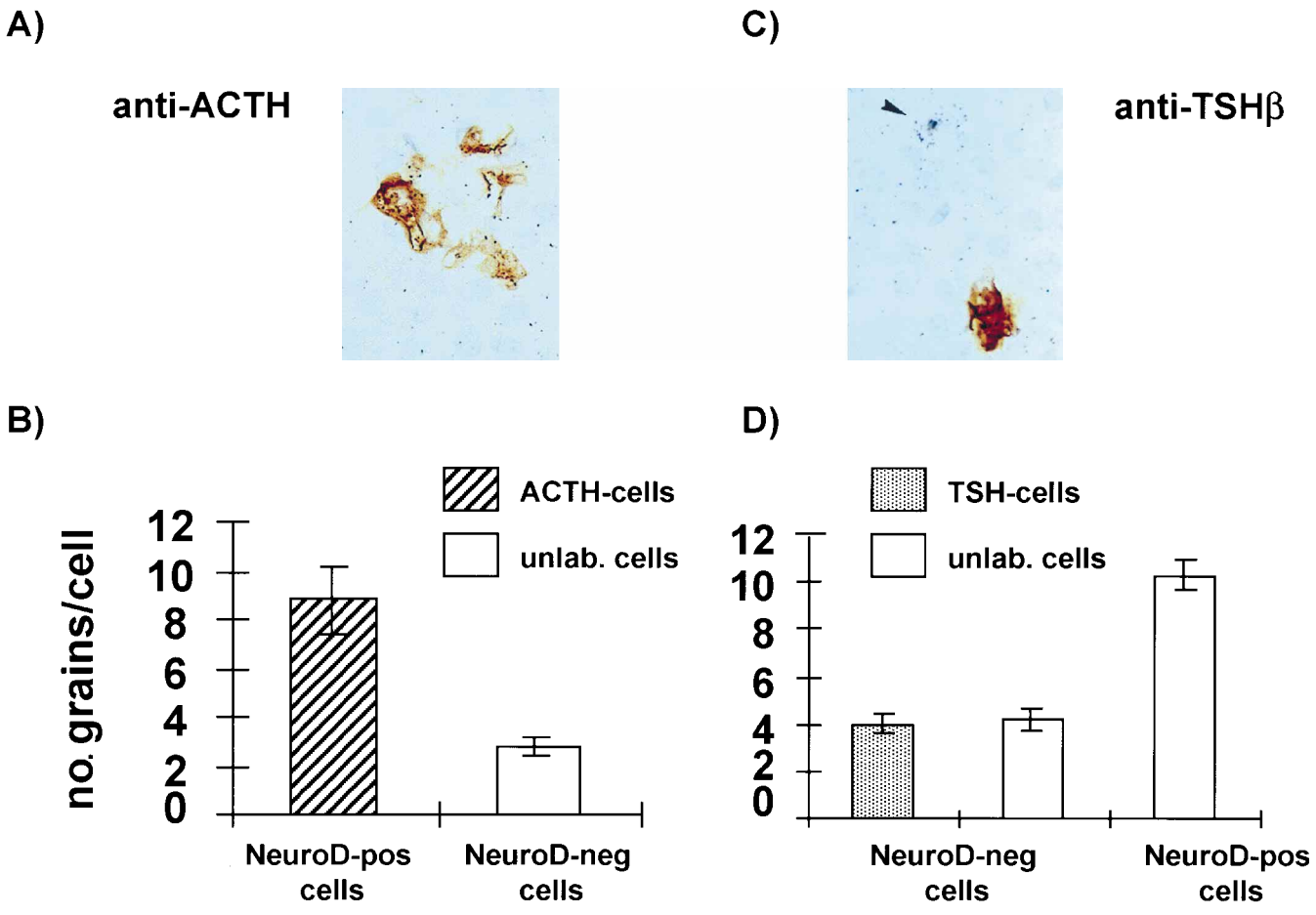

FIG. 7. Cellular localization of NeuroD1 mRNA in pituitary. (A) Colocalization of NeuroD1 with ACTH in adult male pituitary. Corticotroph cells were identified by ACTH immunohistochemistry (brown reaction product of horseradish peroxidase), and NeuroD1-expressing cells were revealed by in situ hybridization using a ^{35}S-labeled NeuroD1-specific antisense probe. (B) Histogram representing the distribution of NeuroD1 signal (mean silver grains/cell ± standard error of the mean) in ACTH-positive cells and other cells. Grain counts represent the average of about 150 cells. (C) Localization of NeuroD1-positive cells by in situ hybridization (arrowhead) and of TSHβ-positive cells by immunohistochemistry using anti-TSHβ antiserum on consecutive sections of the same pituitary as shown in panel A. (D) Silver grain counts of experiment shown in panel C.

and Ptx1, we tested the effect of their overexpression on the activity of the POMC promoter, either intact or deleted of its DE2C-containing distal domain (Fig. 8B). Overexpression of NeuroD1/Pan1 or Ptx1 produced a twofold increase of full-length POMC reporter activity, suggesting that each factor is limiting in AtT-20 cells. Together, they exhibited synergistic activation, and their synergism was lost in the DE2C-deleted promoter construct or in the absence of NeuroD1. Thus, the synergistic interaction between Ptx1 and NeuroD1/Pan1 depends primarily on the DE2C E box.

Further, we reconstituted the Ptx1 NeuroD1-Pan1 interaction in heterologous cells. Using the CE3 and DE2 reporter illustrated in Fig. 8A, we found that expression of NeuroD1/Pan1 and Ptx1 results in synergistic activation (Fig. 8C, lane 6 compared to lanes 2 and 5). Either bHLH factor alone had no effect (lanes 3 and 4), in agreement with previous data (Fig. 2A). Unrelated promoters like that of Rous sarcoma virus did not show this synergistic response (data not shown). Consistent with previous data (Fig. 8A and reference 51), overexpression of Id reversed the synergistic activity of NeuroD1/Pan1 and Ptx1 (lane 7). Interestingly, overexpression of Pan1 alone exhibited moderate synergism in the presence of Ptx1 (lanes 8 and 9) but not NeuroD1 (lane 10). These data suggest that the synergistic interaction between Ptx1 and the bHLH heterodimers is mediated through the Pan1 moiety. Taken together, these data indicate that transcription synergism between factors of two different structural families of DNA-binding proteins, a homeodomain and a bHLH factor, may form the basis for cell-specific activation of the POMC gene.

DISCUSSION

We have reported that the bHLH factor NeuroD1 is expressed in a subset of pituitary cells and that it contributes to cell-specific transcription of the POMC gene. NeuroD1 heterodimers, present in corticotroph cells of the pituitary and in AtT-20 cells, specifically activate transcription from one E box of the POMC promoter, the DE2C E box and discriminate this E box from other E boxes that are the targets of class I ubiquitous bHLH factors. Interestingly, NeuroD1-containing heterodimers exhibit transcriptional synergism with Ptx1, a Bicoid-related homeodomain protein. The joint action of the two factors in the pituitary corticotroph lineage may constitute the basis of a combinatorial usage of transcription factors with developmentally restricted patterns of expression in order to support cell-specific transcription programs and to drive cell differentiation during pituitary development.

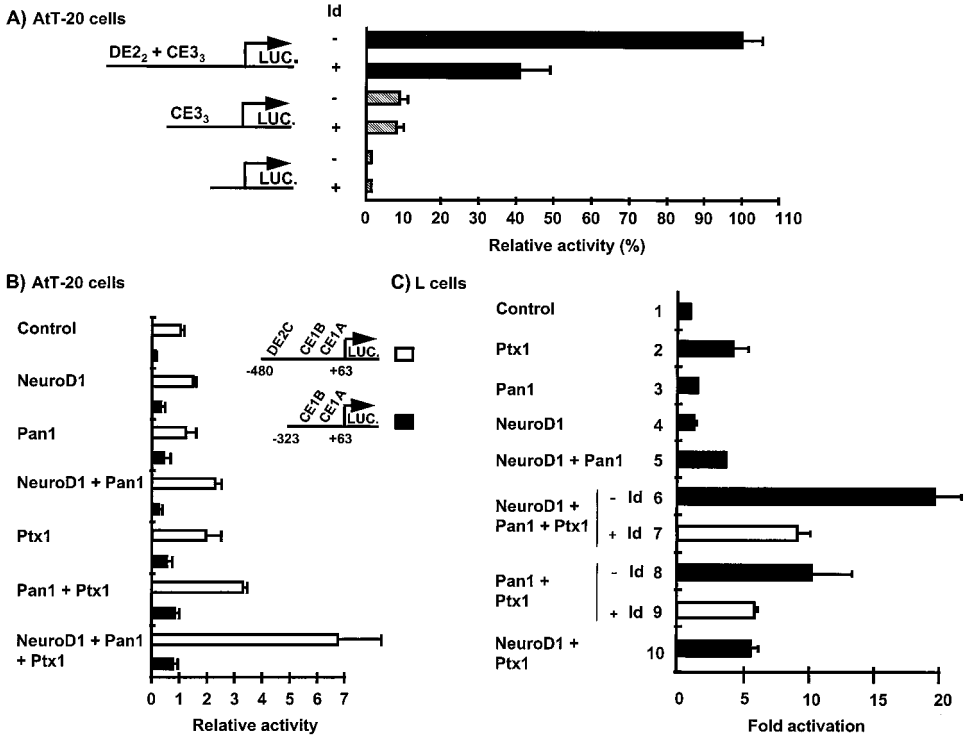

FIG. 8. Reconstitution of NeuroD1 synergism with Ptx1. (A) In NeuroD1- and Ptx1-expressing AtT-20 cells, both targets for NeuroD1 (DE2) and Ptx1 (CE3) are required for bHLH-dependent activity as demonstrated by Id overexpression. Data represent the average ± standard error of the mean of at least three experiments, each performed in duplicate. LUC., luciferase. (B) The NeuroD1/Pan1 and Ptx1 synergism requires the DE2C E box. AtT-20 cells were lipofected with the indicated expression plasmids and with either the full-length bp −480 POMC promoter or the promoter deleted of the DE2C E box (bp −323). The activity shown is relative to the basal activity of the full-length POMC reporter. (C) Reconstitution of NeuroD1 synergism with Ptx1 in heterologous cells (L). The DE2/CE3 reporter illustrated in panel A was cotransfected with expression vectors for the indicated factors. Ptx1 was described previously (27). Data represent the average ± standard deviation of two experiments, each performed in duplicate.

Specific activation of the DE2C E box. Both in vivo (transfection assays) and in vitro (EMSA) experiments have demonstrated that NeuroD1/Pan1 heterodimers discriminate the DE2C E box from other E boxes (Fig. 2 and 3). This specificity was previously attributed to the CUTE complexes, which are now shown to be NeuroD1 heterodimers (Fig. 4 and 5). bHLH factors interact with target sequences related to NCANNTGN (38). Previous work had identified specific DE2C residues that differentiate it from the CE1B and CE1A E boxes of the POMC promoter and that are likely sites of NeuroD1-specific interactions (51). On the basis of previous studies on MyoD, the recognition of the central N residues is highly dependent on lysine[122] situated in the junction region between the basic and HLH domains (10, 11, 31). NeuroD1 has a methionine at the same position instead of the lysine; this is characteristic of a subfamily of class II bHLH factors related to the *Drosophila atonal* gene product (30). This methionine is likely involved in discrimination of the central nucleotides of the POMC E boxes. In particular, it may prevent recognition of the CE1B E box by NeuroD1/Pan1 heterodimers (Fig. 2B). Interestingly, the central nucleotides of CE1A are the same as DE2C. However, previous work in our laboratory demonstrated that the CE1A E box, which differs from DE2C only by a T instead of C at the 3′ end of the E box, is almost inactive (51). Conversely, mutagenesis of this 3′ nucleotide of DE2C from C to T completely abolished its activity and decreased its in vitro DNA binding to CUTE complexes at least threefold (51). This discrimination was explained by a putative steric clash caused by a T at this position (12). This is in agreement with other studies that showed dependence on similar flanking residues for MyoD, Myc, and PHO4 (5, 13, 49). In fact, CE1A was not a good target for class I bHLH factors or for NeuroD1/Pan1 heterodimers (Fig. 2C). Specificity of NeuroD1 heterodimer recognition therefore relies as much on the central variant nucleotides as it does on the flanking residues.

CUTE is formed of NeuroD1 heterodimers. NeuroD1 does not form active homodimers (41). Using two different antibodies against NeuroD1 (Fig. 4 and 6), we have shown that NeuroD1 is present in both bands of the CUTE complexes of AtT-20 cells. These complexes must be heterodimers, and we have shown that they can be formed with class I bHLH factors like E2A, ITF2, and ME1 (Fig. 5B and C). All of the NeuroD1 heterodimers tested had similar transactivation potentials (Fig. 5B). It appears that NeuroD1/E2A heterodimers account for about 25% of the CUTE complexes (half of the upper band in Fig. 5A). Since only 50% of the upper CUTE complex is supershifted in conditions that did not appear to be limiting, we conclude that this band contains E2A gene products and other class I bHLH factors. The identity of the NeuroD1 partner in the lower CUTE complexes is still unknown, although it was previously shown that the two complexes have the same DNA-binding specificity (51). To verify if specific modification(s) of the class I bHLH factors might take place in AtT-20 cells and account for the faster migration of the lower CUTE complexes, we overexpressed these factors in AtT-20 cells and found that none of them could account for the faster CUTE complexes (data not shown). Formally, it might be possible that the lower CUTE complexes contain a splicing variant of

NeuroD1 which is unique to AtT-20 cells and therefore not observed in overexpression experiments in L cells (Fig. 4); however, this is not very likely, as in such a case, one might expect half of these faster-migrating complexes to also contain E2A gene products. Interestingly, NeuroD1 is expressed in hamster insulinoma tumor cells, and its complexes with DE2C comigrated with the upper CUTE complexes and not with the lower (51). Taken together, these data suggest that a class I-type bHLH factor different from the ones tested in this work may be expressed in AtT-20 cells. This unidentified factor would produce the faster-migrating CUTE complexes as heterodimers with NeuroD1.

NeuroD1 expression in pituitary. NeuroD1 is expressed in the nervous system and in α- and β-cell-derived lines of the pancreas (41). It is also expressed in enteroendocrine secretin cells (39). We now show that NeuroD1 is also expressed in a subset of pituitary cells (Fig. 7). These are predominantly the POMC-expressing corticotroph cells. However, the sensitivity of the in situ hybridization is such that it may not reveal low-level expression in other cells. The analysis of pituitary-derived cell lines has confirmed NeuroD1 expression in a model corticotroph line, AtT-20 cells (Fig. 4 and 6), but not in cells of the somatolactotroph lineage, GH3 cells (6) (Fig. 6A and B). The absence of NeuroD1 signal in TSHβ-positive pituitary cells (Fig. 7C and D) was also confirmed by Northern blot analysis of RNA from the αTSH (1) cell line (data not shown). In contrast, NeuroD1-related DNA-binding activity was detected in αT3 (54) cell extracts, a model of gonadotroph cells (Fig. 6A), and NeuroD1 expression was confirmed by Northern blot analysis (Fig. 6B, lane 2). Thus, NeuroD1 might be expressed below in situ detection in adult pituitary gonadotrophs; alternatively, the αT3 might be representative of a fetal differentiation intermediate of the gonadotroph lineage, and NeuroD1 expression might be temporally limited in this lineage during pituitary development.

Two types of POMC-expressing cells, corticotrophs and melanotrophs, are present in the pituitary. No expression of NeuroD1 is seen in the intermediate lobe melanotrophs (data not shown), indicating that NeuroD1 is not a marker of all POMC-expressing cells. This observation is strongly supportive of the conclusion that different transcriptional regulatory mechanisms are operative in the two POMC-expressing lineages. NeuroD1 is thus a marker of corticotrophs and could be useful to differentiate melanotrophs from corticotrophs, either during ontogeny or in human pituitary tumors.

NeuroD1 synergism with the homeobox factor Ptx1. We have previously documented the central role played by the NeuroD1 target sequence DE2C and the Ptx1 target sequence CE3 for cell-specific transcription of POMC (27, 50, 51). Formal promoter analysis had predicted that an interaction between NeuroD1 and Ptx1 might form the scaffold upon which other transcription factors become involved in POMC transcription. We now provide data showing transcriptional synergism between NeuroD1 heterodimers and Ptx1 (Fig. 8B and C). The NeuroD1-dependent synergism requires the DE2C E box, as it is not observed on a deleted POMC promoter that still contains the CE1A and CE1B E boxes (Fig. 8B). This interaction was reconstituted in cells that express neither factor, and its dependence on the bHLH dimers was supported by interference with the bHLH dominant negative Id. It had previously been shown that the CUTE complexes are disrupted by Id (51), presumably by competing potential partners. Similar dependence of the POMC promoter on bHLH activity was shown in AtT-20 cells (Fig. 8A and reference 51). Synergism between bHLH and homeobox-containing factors was observed on the insulin promoter. Indeed, the lim homeodomain factor *lmx-1* was shown to synergize with dimers of Pan1, and their interaction was dependent on the lim domain (14). Pan1 was also reported to activate transcription synergistically with another homeobox factor, STF-1 (Pdx1, IPF-1) in the same system (45). These experiments were performed before the cloning of NeuroD1/β2: if Pan1/NeuroD1 heterodimers are the active bHLH factors in β cells, we must conclude that Pan1 is the most likely interaction partner of the homeobox factor(s). Our data support the model that NeuroD1 heterodimers interact with Ptx1 through the class I bHLH moiety of the dimers, as suggested by the small synergism between Ptx1 and Pan1 (without NeuroD1 [Fig. 8C, lane 8]). Since Pan1 did not have any activity on its own (lane 3), it is possible that the Pan1 effect is due to its recruitment by protein-protein interaction with Ptx1; similar interactions were proposed between MyoD and MEF2A (36). Thus, it appears that NeuroD1 confers E-box selectivity to the heterodimers and that the ability to synergize with Ptx1 or other homeobox factors (like lmx-1 or STF-1) might be a property conferred by a class I bHLH partner like Pan1.

Putative role of NeuroD1 in the pituitary. NeuroD1 has a highly restricted pattern of expression: it is expressed only in the nervous system and in a subset of pancreatic and pituitary endocrine cells. Together with other neurogenic, bHLH factors like MASH-1, neurogenin, and HES-1, NeuroD1 (and its related factors NeuroD2 and NeuroD3) may constitute a developmental code in which each factor plays a role at specific times or/and places during neural development (15, 18, 19, 32, 34). These factors may have different functions, either in pattern formation or in cell commitment or differentiation. The current data on NeuroD1 function in *Xenopus* support a role in cell differentiation during the later stages of neurogenesis (30). The expression of NeuroD1 in a limited subset of pituitary cells suggests that NeuroD1 may play a similar role in pituitary lineage differentiation in addition to its role as transcription factor. In particular, the high-level NeuroD1 expression in corticotrophs, both in the pituitary (Fig. 7A and B) and in AtT-20 cells (Fig. 3), is suggestive of a function in this lineage. The only other lineage for which some expression was detected is the gonadotroph model αT3 cells (Fig. 6); however, αT3 cells also express other factors (as yet unidentified) with related DNA-binding specificity. Thus, the corticotrophs might be unique in the pituitary by their exclusive (of other bHLH) expression of NeuroD1. The synergistic action of NeuroD1 with Ptx1 and their joint expression in corticotrophs may form the basis of a combinatorial code for specification of this lineage.

In conclusion, we have shown that NeuroD1 is an essential determinant for the transcriptional specificity of the DE2C element of the POMC promoter. This specificity appears to be conferred by selective E-box recognition. Further, we have shown that the transcriptional activity of NeuroD1/Pan1 heterodimers is enhanced by interaction with a homeobox factor, Ptx1, that has a restricted pituitary pattern of expression. Taken together, these data support the model of a combinatorial action of transcription factors during pituitary development.

ACKNOWLEDGMENTS

We are grateful to Ming Tsai (Baylor College of Medicine, Houston, Tex.), Chris Nelson (Tularick), Toomas Neuman (Colorado State University, Fort Collins), and Scott McDonald and François Guillemot (Strasbourg, France) for the gift of plasmids encoding hamster NeuroD1 and Pan1/2, mouse ME1a/b, ITF-2, and NeuroD1, respectively. We are also grateful to Ming Tsai for the generous gift of NeuroD antiserum and to A. F. Parlow, Pituitary Hormones and An-

tisera Center, Torrance, Calif., for antisera against rat TSHβ. The expert advice of Christian Lanctôt was helpful in developing in situ hybridization and immunohistochemistry, and we thank Michel Chamberland for oligonucleotide synthesis. The expert secretarial assistance of Lise Laroche was greatly appreciated.

This work was funded by a grant from the National Cancer Institute of Canada with funds provided by the Canadian Cancer Society.

REFERENCES

1. **Akerblom, I. E., E. C. Ridgway, and P. L. Mellon.** 1990. An alpha-subunit-secreting cell line derived from a mouse thyrotrope tumor. Mol. Endocrinol. **4:**589–596.
2. **Andersen, B., and M. G. Rosenfeld.** 1994. Pit-1 determines cell types during development of the anterior pituitary gland. A model for transcriptional regulation of cell phenotypes in mammalian organogenesis. J. Biol. Chem. **269:**29335–29338.
3. **Aplan, P. D., K. Nakahara, S. H. Orkin, and I. R. Kirsch.** 1992. The SCL gene product: a positive regulator of erythroid differentiation. EMBO J. **11:**4073–4081.
4. **Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub.** 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61:**49–59.
5. **Blackwell, T. K., and H. Weintraub.** 1990. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science **250:**1104–1110.
6. **Boockfor, F. R., and L. K. Schwarz.** 1988. Cultures of GH3 cells contain both single and dual hormone secretors. Endocrinology **122:**762–764.
7. **Chiaramello, A., K. Neuman, K. Palm, M. Metsis, and T. Neuman.** 1995. Helix-loop-helix transcription factors mediate activation and repression of the p75LNGFR gene. Mol. Cell. Biol. **15:**6036–6044.
8. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thyocyanate-phenol-chloroform-extraction. Anal. Biochem. **162:**156–159.
9. **Cordle, S. R., E. Henderson, H. Masuoka, P. A. Weil, and R. Stein.** 1991. Pancreatic β-cell-type-specific transcription of the insulin gene is mediated by basic helix-loop-helix DNA-binding proteins. Mol. Cell. Biol. **11:**1734–1738.
10. **Davis, R. L., P.-F. Cheng, A. B. Lassar, and H. Weintraub.** 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell **60:**733–746.
11. **Davis, R. L., and H. Weintraub.** 1992. Acquisition of myogenic specificity by replacement of three amino acid residues from MyoD into E12. Science **256:**1027–1030.
12. **Ellenberger, T., D. Fass, M. Arnaud, and S. C. Harrison.** 1994. Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. Genes Dev. **8:**970–980.
13. **Fisher, F., and C. R. Goding.** 1992. Single amino acid substitutions alter helix-loop-helix protein specificity for bases flanking the core CANNTG motif. EMBO J. **11:**4103–4109.
14. **German, M. S., J. Wang, R. B. Chadwick, and W. J. Rutter.** 1992. Synergistic activation of the insulin gene by a LIM-homeo domain protein and a basic helix-loop-helix protein: building a functional insulin minienhancer complex. Genes Dev. **6:**2165–2176.
15. **Guillemot, F., L. C. Lo, J. E. Johnson, A. Auerbach, D. J. Anderson, and A. L. Joyner.** 1993. Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell **75:**463–476.
16. **Hasty, P., A. Bradley, J. Hsi Morris, D. G. Edmondson, J. M. Venuti, E. N. Olson, and W. H. Klein.** 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. Nature **364:**501–506.
17. **Henthorn, P., R. McCarrick-Walmsley, and T. Kadesch.** 1990. Sequence of the cDNA encoding ITF-1, a positive-acting transcription factor. Nucleic Acids Res. **18:**677.
18. **Ishibashi, M., S. L. Ang, K. Shiota, S. Nakanishi, R. Kageyama, and F. Guillemot.** 1995. Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev. **9:**3136–3148.
19. **Ishibashi, M., K. Moriyoshi, Y. Sasai, K. Shiota, S. Nakanishi, and R. Kageyama.** 1994. Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. EMBO J. **13:**1799–1805.
20. **Japon, M. A., M. Rubinstein, and M. J. Low.** 1994. In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. J. Histochem. Cytochem. **42:**1117–1125.
21. **Jeannotte, L., M. A. Trifiro, R. K. Plante, M. Chamberland, and J. Drouin.** 1987. Tissue-specific activity of the pro-opioomelanocortin gene promoter. Mol. Cell. Biol. **7:**4058–4064.
22. **Kageyama, R., Y. Sasai, C. Akazawa, M. Ishibashi, K. Takebayashi, C. Shimizu, K. Tomita, and S. Nakanishi.** 1995. Regulation of mammalian neural development by helix-loop-helix transcription factors. Crit. Rev. Neurobiol. **9:**177–188.
23. **Karlsson, O., T. Edlund, J. B. Moss, W. J. Rutter, and M. D. Walker.** 1987. A mutational analysis of the insulin gene transcription control region: expression in beta cells is dependent on two related sequences within the enhancer. Proc. Natl. Acad. Sci. USA **84:**8819–8823.
24. **Kawamura, K., and S. Kikuyama.** 1992. Evidence that hypophysis and hypothalamus constitute a single entity from the primary stage of histogenesis. Development **115:**1–9.
25. **Kawamura, K., and S. Kikuyama.** 1995. Induction from posterior hypothalamus is essential for the development of the pituitary proopiomelacortin (POMC) cells of the toad (Bufo japonicus). Cell Tissue Res. **279:**233–239.
26. **Kikuyama, S., H. Inaco, B. G. Jenks, and K. Kawamura.** 1993. Development of the ectopically transplanted primordium of epithelial hypophysis (anterior neural ridge) in Bufo japonicus embryos. J. Exp. Zool. **266:**216–220.
27. **Lamonerie, T., J. J. Tremblay, C. Lanctôt, M. Therrien, Y. Gauthier, and J. Drouin.** 1996. PTX1, a *bicoid*-related homeobox transcription factor involved in transcription of pro-opiomelanocortin (POMC) gene. Genes Dev. **10:**1284–1295.
28. **Lanctôt, C., B. Lamolet, and J. Drouin.** 1997. The *bicoid*-related homeoprotein Ptx1 defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. Development **124:**2817.
29. **Lassar, A. B., R. L. Davis, W. E. Wright, T. Kadesch, C. Murre, A. Voronova, D. Baltimore, and H. Weintraub.** 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell **66:**305–315.
30. **Lee, J. E., S. M. Hollenberg, L. Snider, D. L. Turner, N. Lipnick, and H. Weintraub.** 1995. Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science **268:**836–844.
31. **Ma, P. C., M. A. Rould, H. Weintraub, and C. O. Pabo.** 1994. Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. Cell **77:**451–459.
32. **Ma, Q. F., C. Kintner, and D. J. Anderson.** 1996. Identification of neurogenin, a vertebrate neuronal determination gene. Cell **87:**43–52.
33. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **McCormick, M. B., R. M. Tamimi, L. Snider, A. Asakura, D. Bergstrom, and S. J. Tapscott.** 1996. NeuroD2 and NeuroD3: distinct expression patterns and transcriptional activation potentials within the NeuroD gene family. Mol. Cell. Biol. **16:**5792–5800.
35. **McDonald, S., G. Poulin, T. Lamonerie, M. Therrien, and J. Drouin.** HLH transcription factors act at multiple sites to control POMC gene expression. Submitted for publication.
36. **Molkentin, J. D., B. L. Black, J. F. Martin, and E. N. Olson.** 1995. Cooperative activation of muscle gene expression by mef2 and myogenic bhlh proteins. Cell **83:**1125–1136.
37. **Murre, C., P. S. McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell **56:**777–783.
38. **Murre, C., P. S. McCaw, H. Vaessin, M. Caudy, L. Y. Jan, Y. N. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lassar, H. Weintraub, and D. Baltimore.** 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58:**537–544.
39. **Mutoh, H., B. P. Fung, F. J. Naya, M. J. Tsai, J. Nishitani, and A. B. Leiter.** 1997. The basic helix-loop-helix transcription factor beta2/neurod is expressed in mammalian enteroendocrine cells and activates secretin gene expression. Proc. Natl. Acad. Sci. USA **94:**3560–3564.
40. **Nabeshima, Y., K. Hanaoka, M. Hayasaka, E. Esumi, S. Li, and I. Nonaka.** 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. Nature **364:**532–535.
41. **Naya, F. J., C. M. M. Stellrecht, and M. J. Tsai.** 1995. Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. Genes Dev. **9:**1009–1019.
42. **Nelson, C., L. P. Shen, A. Meister, E. Fodor, and W. J. Rutter.** 1990. Pan: a transcriptional regulator that binds chymotrypsin, insulin, and AP-4 enhancer motifs. Genes Dev. **4:**1035–1043.
43. **Nordeen, S. K.** 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques **6:**454–456.
44. **Olson, E. N., and W. H. Klein.** 1994. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. Genes Dev. **8:**1–8.
45. **Peers, B., J. Leonard, S. Sharma, G. Teitelman, and M. R. Montminy.** 1994. Insulin expression in pancreatic islet cells relies on cooperative interactions between the helix loop helix factor E47 and the homeobox factor STF-1. Mol. Endocrinol. **8:**1798–1806.
46. **Rudnicki, M. A., P. N. Schnegelsberg, R. H. Stead, T. Braun, H. H. Arnold, and R. Jaenisch.** 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell **75:**1351–1359.
47. **Schwind, J.** 1928. The development of the hypophysis cerebri of the albino rat. Am. J. Anat. **41:**295–319.

48. **Shivdasani, R. A., E. L. Mayer, and S. H. Orkin.** 1995. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. Nature **373:**432–434.
49. **Sun, X.-H., and D. Baltimore.** 1991. An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. Cell **64:**459–470.
50. **Therrien, M., and J. Drouin.** 1991. Pituitary pro-opiomelanocortin gene expression requires synergistic interactions of several regulatory elements. Mol. Cell. Biol. **11:**3492–3503.
51. **Therrien, M., and J. Drouin.** 1993. Cell-specific helix-loop-helix factor required for pituitary expression of the pro-opiomelanocortin gene. Mol. Cell. Biol. **13:**2342–2353.
52. **Voss, J. W., and M. G. Rosenfeld.** 1992. Anterior pituitary development: short tales from dwarf mice. Cell **70:**527–530.
53. **Weintraub, H.** 1993. The MyoD family and myogenesis: redundancy, networks, and thresholds. Cell **75:**1241–1244.
54. **Windle, J. J., R. I. Weiner, and P. L. Mellon.** 1990. Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Mol. Endocrinol. **4:**597–603.
55. **Zhang, W., R. R. Behringer, and E. N. Olson.** 1995. Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. Genes Dev. **9:**1388–1399.