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The Ets Factor Etv1 Interacts with Tpit Protein for Pituitary Pro-opiomelanocortin (POMC) Gene Transcription*

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Pro-opiomelanocortin (POMC) is expressed in two lineages of the pituitary, the anterior lobe corticotrophs and the intermediate lobe melanotrophs. POMC expression in these two lineages is highly dependent on the cell-restricted transcription factor Tpit. As Tpit intervenes relatively late in differentiation of those lineages, we have been searching for other transcription factors that may participate in their gene expression program. On the basis of similarity with the Tpit expression profile, we identified Ets variant gene 1 (Etv1/Er81) as a putative POMC transcription factor. Using *Etv1-lacZ* knockin mice, we describe preferential Etv1 expression in pituitary POMC cells and also in posterior lobe pituicytes. We further show that Etv1 enhances POMC transcription on its own and in synergy with Tpit. The Ets-binding site located within the Tpit/Pitx regulatory element is necessary for Etv1 activity in POMC-expressing AtT-20 cells but dispensable for synergy with Tpit. Etv1 and Tpit interact together in coimmunoprecipitation experiments. Furthermore, Etv1 is present at the POMC promoter, and siRNA-mediated knockdown of Etv1 in AtT-20 cells produces a significant decrease in POMC expression. *Etv1* knockout pituitaries show normal POMC cell distribution and normal POMC mRNA abundance, suggesting compensation by other factors. The coordinate expression of Etv1 with POMC cell differentiation and its interaction with the highly cell-restricted Tpit factor indicate that Etv1 participates in a combinatorial code for pituitary cell-specific gene expression.

The adult rodent pituitary consists of three lobes. The posterior lobe contains mainly axonal projections from the hypothalamus and supporting pituicytes, whereas the intermediate and anterior lobes together enclose six distinct secretory cell types. Two of these lineages express pro-opiomelanocortin (POMC),[2] a precursor protein that is processed into ACTH in corticotrophs of the anterior lobe (AL) and melanostimulin (αMSH) in intermediate lobe (IL) melanotrophs. Corticotroph cells are the first to differentiate at about day 12.5 of embryonic development (E12.5) in mice. At E14.5, definitive thyrotrophs start expressing thyroid-stimulating hormone (TSH), and melanotrophs turn on POMC expression. At about E15.5, somatotrophs express growth hormone (GH). Lactotrophs and gonadotrophs are the latest cells to differentiate, respectively expressing prolactin (PRL) at E16.5 and gonadotropins (FSH and LH) from E17.5 (1).

Cell-specific transcription of hormone-coding genes relies on combinatorial expression of cell-specific and pituitary-specific transcription factors (for review, see Ref. 2). Among these, the Pitx1 and Pitx2 factors were shown to be important, but possibly redundant with each other, for transcription of most pituitary hormone-coding genes (3, 4). The Pitx are bicoid-related factors of the *paired* homeodomain subfamily that are expressed from e8 in the presumptive oral ectoderm (stomodeum). Their expression is required for pituitary development and is maintained in adult tissues. Inactivation of the *Pitx2* gene leads to arrest of pituitary development at the Rathke's pouch stage (5–8). Both homeodomain factors recognize the same response element and activate transcription at the gene promoters of POMC, α subunit of glycoprotein hormone, β-FSH, βLH, β subunit of thyroid stimulating hormone, PRL, and growth hormone. They act as pan-pituitary regulators of transcription and interact with cell-restricted factors such as Pit1 (9), SF1 (10), NeuroD1 (11), and Tpit (12) to activate promoter-specific transcription.

Our laboratory previously defined the roles of NeuroD1 and Tpit in POMC cell-specific expression and cell differentiation. NeuroD1 is a neurogenic basic helix-loop-helix factor of the atonal subfamily, originally isolated as β2, a cell-specific transcription factor of the insulin gene (13). We documented NeuroD1 expression in corticotroph cells and defined its interactions with Tpit and Pitx1 (11, 14, 15). Corticotroph expression of NeuroD1 is strongest between E12.5 and E15.5, and adult NeuroD1-null pituitaries contain a normal number of corticotrophs. However, POMC expression is delayed in NeuroD1-deficient mice, consistent with the developmental window of NeuroD1 expression in corticotrophs. This differentiation delay does not reflect a change of corticotroph commitment, as Tpit expression remains unchanged. NeuroD1 activates POMC transcription through dimer formation with various ubiquitous basic helix-loop-helix partners. It specifically recognizes and activates transcription from the POMC promoter $Ebox_{neuro}$ that confers transcriptional specificity. The *in vivo* importance of this promoter element was recently shown in transgenic mice (16).

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[2] The abbreviations used are: POMC, pro-opiomelanocortin; AL, anterior lobe; αMSH, melanostimulin; IL, intermediate lobe; E12.5, embryonic day 12.5; PRL, prolactin; LH, luteinizing hormone; P0, postnatal day 0; QPCR, quantitative PCR; CRH, corticotropin-releasing hormone; EGFP, enhanced GFP.

Tpit, a T-box containing transcription factor, was identified as a highly restricted T-box factor present in POMC-expressing AtT-20 cells, a corticotroph cell model (12). POMC transcription requires cooperation between Tpit and Pitx1, the two factors binding to contiguous sites within the same regulatory element. In gain of function experiments, Tpit induces POMC expression in undifferentiated pituitary cells, indicating that it can initiate differentiation into POMC-expressing lineages (17). Furthermore, *TPIT* gene mutations were found in patients with isolated deficiency of pituitary POMC-derived ACTH (12, 18, 19). The essential role of Tpit for differentiation of pituitary POMC lineages was ascertained in *Tpit* knockout mice that exhibit an almost complete loss of pituitary POMC-expressing cells (17). In these mice, the bulk of cells destined to become corticotrophs or melanotrophs remain undifferentiated, but a small number switch cell fate to become *bona fide* gonadotrophs (17). However, a small number of $Tpit^{-/-}$ pituitary cells do express POMC (17, 20), suggesting that other transcription factors may also contribute to cell-specific expression of POMC.

The goal of this work was to identify transcription factors that may contribute to POMC expression and that could positively influence melanotroph and corticotroph differentiation. Using developmental expression profiling on microarrays, we identified Etv1 (also known as Er81), an Ets-domain containing factor of the PEA3 subfamily (for review, see Refs. 21 and 22), as a novel activator of POMC expression with a Tpit-like pituitary expression profile. Indeed, the present report describes Etv1 expression in pituitary IL melanotrophs and in anterior lobe corticotroph cells but not in other endocrine lineages. Etv1 binds to and activates transcription from the POMC promoter through the Tpit/Pitx1 response element. There it collaborates with Tpit and Pitx1 for transcriptional activation mainly through protein-protein interaction with Tpit. Consistently, siRNA-mediated knockdown of Etv1 significantly diminished POMC expression in AtT-20 cells, illustrating its importance for full transcriptional activity. However, *Etv1* knockout mice display normal POMC cell and POMC mRNA content, suggesting a possible compensation by other related factors. These studies showed that Ets transcription factors participate in pituitary POMC expression.

EXPERIMENTAL PROCEDURES

Mice, Histology, and Immunohistochemistry—Etv1-lacZ mice were described previously (23) and generously provided by Dr. T. Jessel (Columbia University, NY). Animal experimentation was approved by the Institut de Recherches Cliniques de Montréal Animal Ethics Review Committee. For embryonic tissues, the morning when a vaginal plug was detected was considered E0.5. For postnatal tissues, day of birth was considered P0. Pregnant mice were killed by cervical dislocation, and embryos were isolated for dissection. The β-galactosidase activity was revealed as described (17). Tissues were then postfixed for 6 h and incubated in 20% sucrose containing PBS for cryoprotection. On the following days, they were imbedded in OCT compound (Sakura) and frozen on 99% EtOH-saturated dry ice. Histology blocks were stored at −80 °C until used to prepare 10-μm sections stained with nuclear fast red.

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections as described (24). The Tpit antibody was described previously (12). Antibodies were used at the following dilutions: rabbit anti-Pit1, 1:1000; rabbit anti-SF1, 1:100; rabbit anti-Etv1, 1:1000 (Abcam, ab36788); and rabbit anti-Etv5, 1:100 (X. Sun, University of Wisconsin, Madison, WI). For rabbit antibodies, slides were incubated with a biotinylated anti-rabbit antibody (Vector) and revealed with HRP or Alexa Fluor 488-coupled streptavidin (PerkinElmer Life Sciences). Mouse anti-POMC (Cortex Biochem) was used at 1:100, and detection was performed using HRP-coupled anti-mouse antibody (Sigma).

Cell Culture and Transfections—HEK293 cells were grown in DMEM with 10% fetal bovine serum and penicillin/streptomycin and maintained at 37 °C and 5% CO_2. AtT-20 cells were grown in the same conditions unless indicated otherwise. For both cell types, 250,000 cells/well were plated in 12-well plates. HEK293 cells were transfected by the calcium phosphate coprecipitation method (25). Each well was transfected with 1 μg of reporter construct along with varying amounts of expression vectors completed to equal amounts with empty vector. AtT-20 cells were transfected using Lipofectamine (Invitrogen) following the manufacturer's recommendations. Transfection experiments were performed in duplicates, and luciferase activity was measured 48 h post-transfection. Cells were lysed in 100 mM Tris (pH 8) containing 0.5% Nonidet P-40. Luciferin (Gold Biotechnology) was added to cell lysates at a final concentration of 0.5 mM before measurement of relative light units on a Glomax 96 luminometer (Promega). Data are presented as means ± S.E. of three to five separate experiments. Student's *t* test was used to assess statistical significance.

Plasmids—The different reporter plasmids constructed in vector pXP1-luciferase were described previously (12, 26, 27). Full-length coding sequences of mouse Etv1 were amplified from AtT-20-derived cDNA and cloned in the pLNCX2 expression vector (Clontech). Expression vectors for mouse Tpit and Pitx1 were obtained using the same strategy, starting with plasmids described previously (12). The flagged form of Etv1 was obtained by subcloning the wild-type version into a modified pLNCX2 containing three copies of the FLAG sequence at the 5′ end of the insert. All expression plasmids were sequenced.

Coimmunoprecipitation—Petri dishes (10 cm) were seeded with 7×10^6 HEK293 cells and transfected by the calcium phosphate method the next day for 8 h with 5 μg of each expression vector. Cells were harvested the following day, washed in PBS, pelleted, washed in 5 volumes, and then resuspended in two volumes of low-salt buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin). Samples were kept on ice for 5 min, and Nonidet P-40 was added at a final concentration of 0.1%. The mix was reincubated on ice for 5 min before addition of 1 volume of high-salt buffer (20 mM HEPES (pH 7.9), 6 mM $MgCl_2$, 1.2 mM KCl, 20% glycerol) and then left on ice for 30 min with occasional agitation. Lysates were centrifuged for 20 min at 13,000 rpm at 4 °C. 1.5 mg of protein from the supernatant was then incubated for 2 h at 4 °C for immunoprecipitation with anti-FLAG beads (Sigma). Beads were then washed four times in 20 mM HEPES (pH 7.9), 150 mM KCl, 10% glycerol, 0.05% Nonidet P-40, 0.2 mM EDTA by quick inversion. Beads

were boiled in 2.5% loading buffer, and SDS-PAGE was performed (18). After transfer, PVDF membranes (Millipore) were blotted with antibodies against FLAG M2 (Sigma) and Tpit or Pitx1 antibody (24). The signal was revealed using the TrueBlot system (eBioscience).

siRNA-mediated Knockdown—Knockdown of Etv1 and Etv5 was performed using onTARGET SMARTpool siRNA against Etv1 and/or Etv5 and a control non-target siRNA pool (Dharmacon). AtT-20 cells were plated in 6-well plates (500,000 cells/well) and transfected in triplicates with siRNAs the following day using Lipofectamine. RNA was collected using the RNeasy mini extraction kit (Qiagen) according to the manufacturer's recommendations, and protein extracts were prepared for Western blot analysis.

Direct read-out of mRNA abundance was assessed using the OneStep RT-PCR kit (Qiagen). The primers used were as follows: TATA-binding protein (TBP), 5′-TCTTGGCTCCTGTGCACACCATTT-3′ and 5′-CCGTGAATCTTGGCTGTAAACTTGACC-3′; Etv1, 5′-ATGGCTTGCTGAAGCTCAGGTAC-3′ and 5′-GCTGCTCTTGACTGCAAGCAGAC-3′; Etv5, 5′-TTTGATCTTGGTGGGAGGTGGAG-3′ and 5′-CTGATGATGAGCAGTTTGTCCCAG-3′; and POMC, 5′-GATGCAAGCCAGCAGGTTGCTCTC-3′ and 5′-TGGAAGATGCCGAGATTCTGCTACAGT-3′. Reactions were performed in triplicates on a MX-3005p (Stratagene), and quantitation was done using the accompanying software.

Efficacy of protein knockdown was verified using total protein extracts. Briefly, cells were rinsed in PBS, harvested, and resuspended in 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, and 20% glycerol. Samples were frozen on dry ice, thawed on ice, and centrifuged. From the supernatant, 50 μg of protein was submitted to SDS-PAGE. After transfer, PVDF membranes (Millipore) were blotted with rabbit antibodies against Etv1, Etv5, and Tpit. Signal detection was revealed using ECL reagents (GE Healthcare).

ChIP and QPCR—AtT-20 cells were grown to 60–70% confluence, and ChIP was performed as described previously (28) with the following modifications. Sonicated chromatin was immunoprecipitated with serum of rabbits immunized with Etv1 protein (23), and preimmune serum was used as control. Precipitated DNA was collected using protein A/G beads (Santa Cruz Biotechnology). After washes and de-cross-linking, DNA was purified using QIAquick columns following the manufacturer's directives. Specific enrichment at the POMC promoter was assessed relative to the MyoD promoter used as control reference by QPCR with QuantiTect SYBR Green (Qiagen). The primers used were as follows: MyoD, 5′-TGCTCCTTTGAGACAGCAGA-3′ and 5′-TTTCAGGAGGGCTCCCATGT-3′ and POMC, 5′-AAGGCAGATGGACGCACATA-3′ and 5′-CACTTAGAACTGGACAGAGGCT-3′.

FACS—EGFP-positive cells were obtained from POMC-EGFP mice (16) using the following procedure. Briefly, adult males were killed by dislocation, and the neurointermediate and anterior pituitary lobes were collected separately in DMEM/F12 1:1 medium containing 0.3% BSA and 200 units/ml DNaseI (Invitrogen). Trypsin was added at a final concentration of 5 mg/ml, and scalpel-minced pituitaries were dispersed at 37 °C in a hybridization oven for 30 min. Proteolysis was then stopped by adding FBS at a final concentration of 10%. The cell suspension was washed in 137 mM NaCl, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 0.22 mM KH_2PO_4, 0.13 mM Na_2HPO_4●$2H_2O$, 10 mM HEPES (pH6), and 5.6 mM glucose, supplemented with BME essential amino acids (Sigma). The first and second washes contained 2 mM EDTA and 1 mM EDTA, respectively. Samples were filtered on a 100-μm cell strainer (BD Falcon), and cells were sorted in ice-cold PBS containing 2% FBS.

Total RNA was prepared from EGFP-positive cells using the RNeasy mini extraction kit (Qiagen) according to the manufacturer's recommendations. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and purified using QIAquick columns following the manufacturer's directives. QPCR was performed using QuantiTect SYBR Green (Qiagen). The primers used were the ones mentioned above and the following: Etv2, 5′-ACT ACA CCA CCA CGT GGA ATA CTG-3′ and 5′-AGG AGG AAT TGC CAC AGC TGA ATG-3′ and Etv4, 5′ TAC CGG ACA GTG ATG AGC AGT TTG-3′ and 5′-GGA GTA AAG GCA CTG CTC TCC ATG-3′.

RESULTS

Etv1 Is Preferentially Expressed in Pituitary POMC Cells—In an attempt to identify new regulatory factors of pituitary development, we used expression arrays to analyze RNA from E10, E12, E14, and E16 embryonic pituitaries along with samples prepared from adult (P35) AL and IL. Detailed analyses of these experiments will be described elsewhere.[3] Using pattern recognition analyses, we screened this expression profiling database for transcription factors that display an expression pattern resembling that of Tpit (Fig. 1*A*). We rapidly focused on Etv1, an Ets-containing DNA-binding factor, because expression of the gene is similar to Tpit both in the developmental sequence and in established pituitary lineages (Fig. 1*B*). Indeed, Etv1 is highly expressed in AtT-20 cells but undetectable in αT3, LβT2, or GH3 cells. These data were confirmed using quantitative PCR (data not shown) and by histological analyses of *Etv1-lacZ* mice (originally described as Er81-nlslacZ) (23). Compared with Tpit, the developmental profile of pituitary Etv1 expression was higher at E10 and E12 (Fig. 1*A*), and this early expression appeared to be in the forming posterior lobe (*C*). In fact, expression of Etv1-lacZ in Rathke's pouch derivatives can only be detected at E16.5 in both IL and AL, and this was confirmed by immunohistochemistry with an anti-Etv1 antibody (Fig. 1*C*). In the adult pituitary, all melanotroph cells of the IL were lacZ-positive (Fig. 1*D*). In the AL, Tpit-expressing corticotrophs were also lacZ-positive, although Tpit and Etv1 colocalization was not complete (Fig. 1*D*). In fact, about 60–65% Tpit-positive cells were also Etv1-positive, and conversely, only about 50% of Etv1-positive cells were Tpit-positive (Fig. 1*D*, *insets*). This suggested that Etv1 may be expressed in other pituitary cells. These cells are most probably not endocrine because β-galactosidase staining did not colocalize with either Pit1 or SF1, two markers that, together with Tpit, stain all hormone-producing cells of the pituitary (Fig. 1*D*). These Etv1-positive cells may thus be support cells (pituicytes), as they are likely to be in the posterior lobe (29).

[3] L. Budry, A. Balsalobre, and J. Drouin, manuscript in preparation.

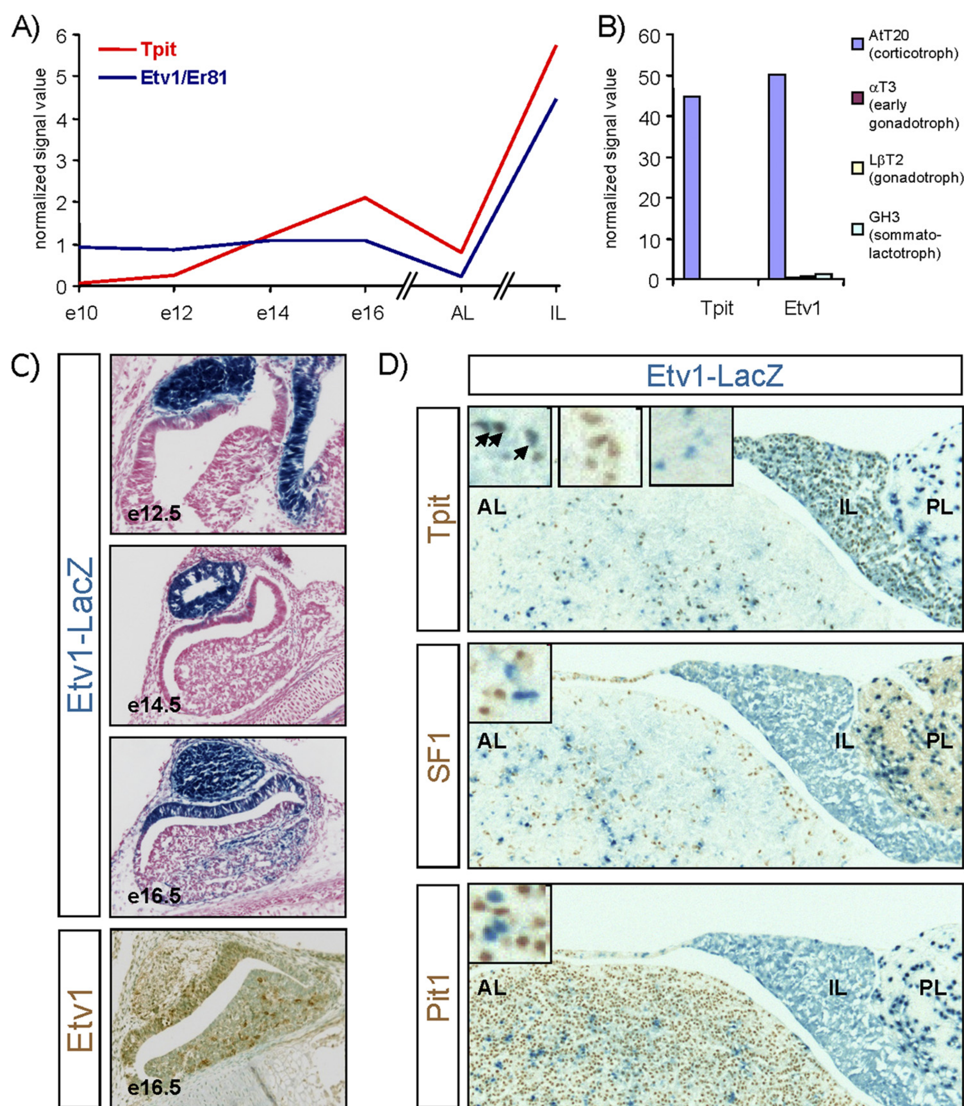

FIGURE 1. **Expression of Etv1 during development and in the adult pituitary.** *A*, expression profiles of Tpit (*red*) and Etv1 (*blue*) as revealed by expression on profiling microarrays. Signal values were normalized relative to the medians of all data per probe set. mRNA E12, E14, and E16 of development as well as in dissected adult AL and neurointermediate pituitaries (IL). The data are presented as continuous lines to reflect the pattern recognition analyses that were performed to identify transcription factors that have expression profiles similar to Tpit, such as Etv1. Complete analysis of these expression profiling data will be presented elsewhere.[3] *B*, expression of Tpit and Etv1 in established pituitary cell lines used as models of the indicated lineages. Tpit and Etv1 mRNAs are detected in AtT-20 but not in LβT2, αT3, or GH3 cells using Affymetrix expression microarrays. *C*, expression pattern of Etv1 during mouse pituitary development assessed in *Etv1-lacz* mice using β-galactosidase reporter activity (*blue*). Strong expression is detected in the forming posterior pituitary (infudibulum) from E12.5 onward. Etv1 (lacZ) is detected both in IL and AL from E16.5 on and confirmed by immunoperoxidase detection of Etv1 immunoreactivity (*bottom panel*). *D*, Etv1 expression in adult pituitary. Etv1 and Tpit are coexpressed in all cells of the IL, whereas the overlap (*arrowheads*) is partial in the AL. Some cells that express Tpit do not express Etv1 and vice versa (*insets*). Etv1 was not found to be expressed in either SF1- or Pit1-positive cells.

Etv1 Activates POMC Transcription through Tpit/PitxRE—The PEA3 group of Ets transcription factors are transcriptional activators (21, 22). Accordingly, Etv1 activated the full-length (-480/+63 bp) POMC promoter in HEK293 cells (Fig. 2*A*). Etv1 did not activate the minimal (-34/+63 bp) POMC promoter, indicating that Etv1 acted on upstream regulatory sequences. Consistent with the short Ets consensus DNA binding site (GGAA/T), the corresponding promoter region contains many putative Ets-binding sites. Ets factors generally display weak sequence specificity. They rather bind to their target sites in cooperation with other DNA-binding partners (30). In view of the Tpit-like expression pattern of Etv1, we focused our attention on regulatory elements that confer cell-specific expression to POMC. Two predicted Ets-binding sites are present within POMC cell-specific regulatory elements: one within the NurRE/Ebox$_{neuro}$ response element (16) and the other within the Tpit/Pitx response element (Tpit/PitxRE) (12). We tested Etv1 action on reporters containing multimers of these two regulatory element in POMC-expressing AtT-20 cells and found that Etv1 increased transcription from the Tpit/PitxRE but not from the NurRE/Ebox$_{neuro}$ (Fig. 2*B*). This result identified an Etv1 target site within the Tpit/PitxRE and prompted us to study its role with regard to POMC transcription and particularly in relation to Tpit and Pitx activity.

DNA Binding-dependent and Binding-independent Transcriptional Properties of Etv1—To determine the importance of DNA interactions for Etv1 activity, we used a version of the Tpit/PitxRE reporter construct bearing a mutated Ets-binding

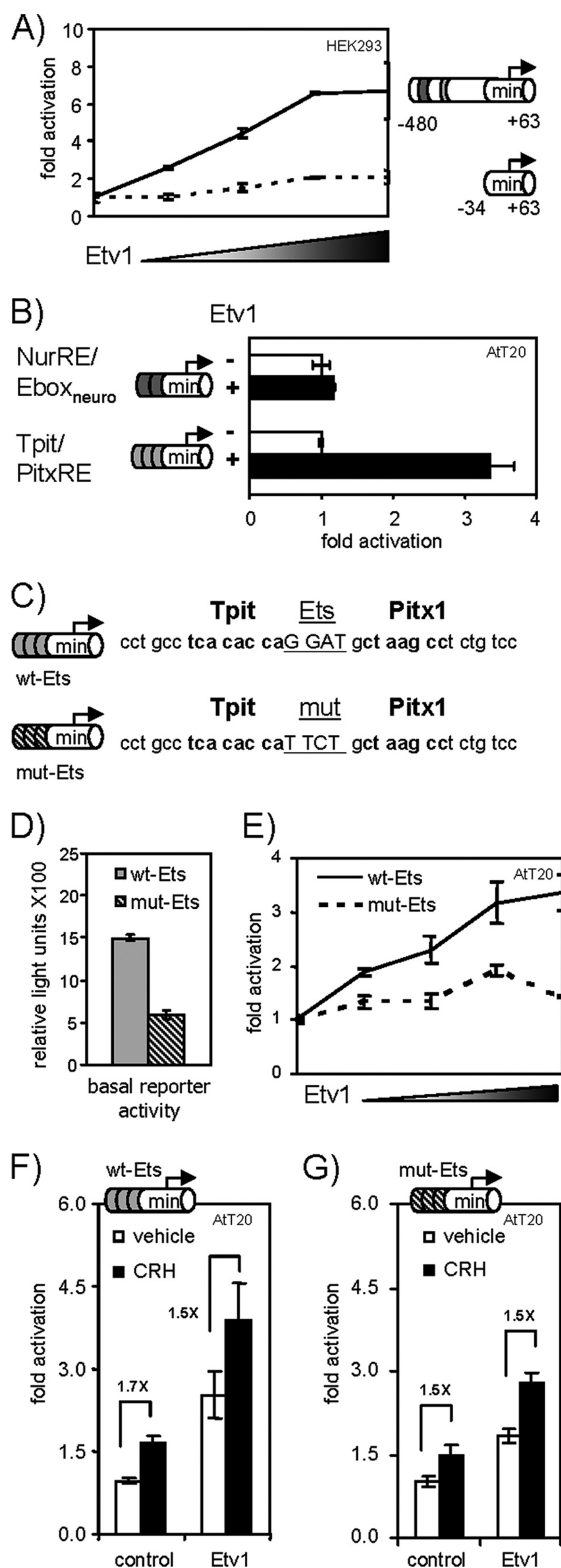

FIGURE 2. **Etv1 activates the POMC promoter.** *A*, reporter activity of the full-length −480/+63-bp POMC promoter (*solid line*) and −34/+63-bp minimal promoter (*dotted line*) in HEK293 cells expressing increasing amounts (25–100 ng expression vector) of Etv1 (representative of three experiments). *B*, Etv1 expression in AtT-20 cells increases Tpit/PitxRE reporter activity but not that of the NurRE/$Ebox_{neuro}$ reporter (representative of five experiments). *C*, sequences of wild-type (*wtEts*)- and mutant Ets (*mutEts*)-binding site of the Tpit/PitxRE. The mutation specifically targets the Ets-binding site (*underlined capitals*) but not the Tpit or Pitx binding sites (*bold*). *D*, basal activity of wild-type- and mutant Ets-binding sites of Tpit/PitxRE reporter constructs in AtT-20 cells. *E*, effect of increasing amounts of Etv1 (50–200 ng expression vector) on Tpit/PitxRE reporters. *F* and *G*, CRH response of the Tpit/PitxRE reporter is not affected by Etv1 (*F*) or by mutation of the Ets binding site (*G*). Data are shown as means ± S.E. for three to five experiments, each performed in duplicates.

site (Fig. 2*C*). Basal transcription of the Ets mutant reporter was reduced to almost one third of the wild-type reporter (Fig. 2*D*), underscoring the contribution of this promoter element to transcriptional activity in AtT-20 cells. Furthermore, the mutation ablated the response to increasing doses of Etv1 (Fig. 2*E*). Thus, the Ets-binding site within the Tpit/PitxRE is a target of Etv1, and action of Etv1 on this element is DNA-dependent in POMC-expressing AtT-20 cells. We showed previously that the Tpit/PitxRE confers responsiveness to CRH (31). We therefore tested a putative role of Etv1 as a downstream mediator of CRH signaling on the Tpit/PitxRE. The Tpit/PitxRE reporter showed increased transcriptional activity in response to CRH (Fig. 2*F*) or forskolin (data not shown). However, this increase was similar in both control and Etv1-stimulated cells as well as with the Ets site mutant (Fig. 2*G*), indicating that Etv1 is not limiting for full CRH/forskolin response and that it likely does not participate in this pathway.

The proximity of binding sites led us to test the combinatorial actions of Etv1, Tpit, and Pitx1 on Tpit/PitxRE-dependent transcription. Etv1 and Tpit synergistically enhanced transcription of the reporter, whereas Etv1 and Pitx1 only produced additive effects (Fig. 3*A*). The synergistic activity of Tpit and Pitx was also further enhanced by Etv1, indicating that the three proteins cooperate for maximal activity of the Tpit/PitxRE. We then tested the importance of the Ets-binding site for Etv1 synergy with Tpit. Mutation of the Ets-binding site consistently decreased Etv1 responsiveness (Fig. 3*C* compared with *B*). Tpit or Pitx1 activation were unaffected by the nucleotide changes in the Ets-binding site (Fig. 3*B* and data not shown), confirming the specificity of the mutation. However, the synergy between Tpit and Etv1 was similar both in the presence (Fig. 3*B*) or absence (*C*) of an intact Ets-binding site. Thus, Etv1 binding to its recognition element is dispensable for cooperative activity with Tpit in this overexpression paradigm, suggesting a possible physical interaction between the two factors. To test this idea, we cotransfected a tagged form of Etv1 (FLAG-Etv1) along with Tpit or Pitx1 in HEK293 cells (Fig. 4). Immunoprecipitation of FLAG-Etv1 using anti-FLAG antibody revealed coimmunoprecipitation of Tpit with Etv1 (Fig. 4*A*) but not Pitx1 under the same conditions (*B*), clearly suggesting that Tpit is the interacting partner of Etv1.

Etv1 Contributes to POMC Transcription—To directly assess the importance of Etv1 for POMC transcription, we targeted Etv1 mRNA for degradation in AtT-20 cells using a pool of siRNAs. In these experiments, a 50% knockdown of Etv1 mRNA resulted in a significant decrease in POMC mRNA (Fig. 5*A*), indicating that Etv1 contributes to transcription of the

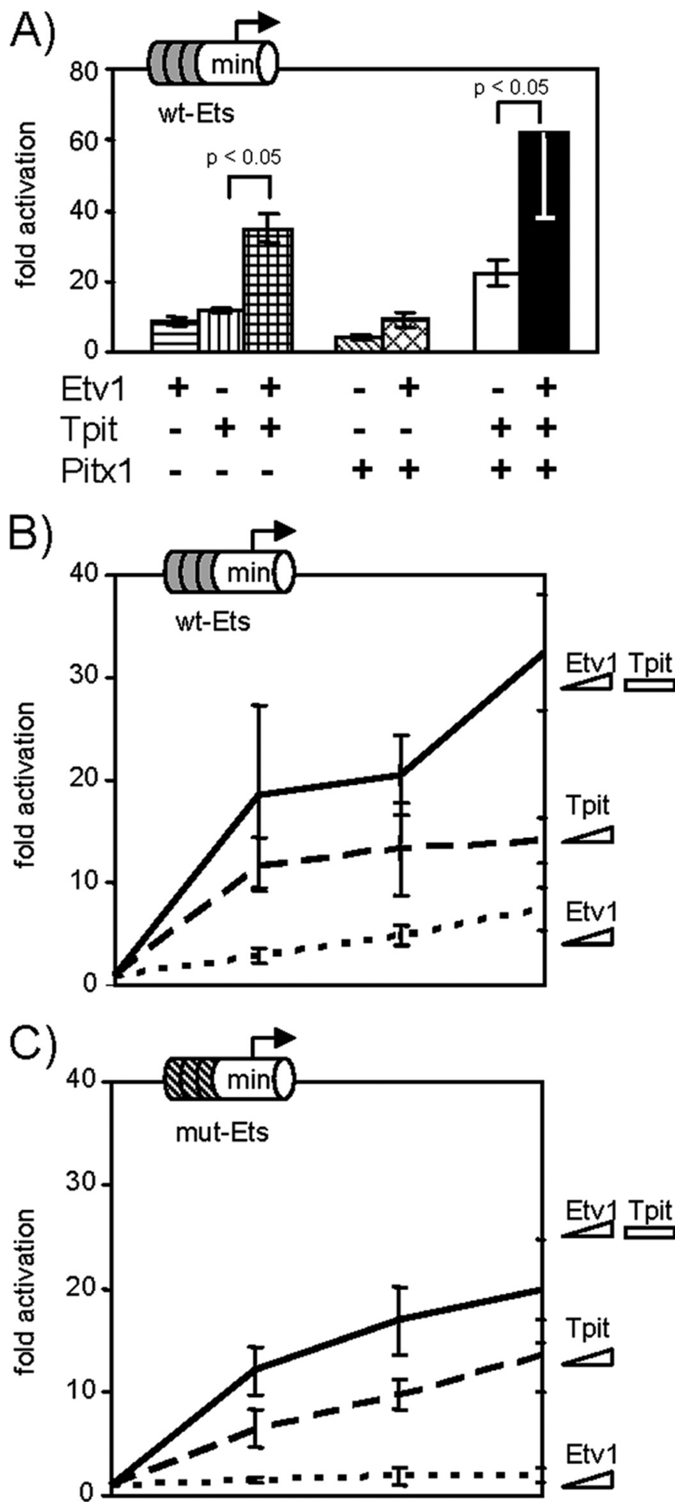

FIGURE 3. **Etv1 acts on Tpit/Pitx1RE.** *A*, Etv1 synergizes with Tpit but is additive with Pitx1 to activate the Tpit/PitxRE reporter in HEK293 cells. It also acts in synergy in the presence of both Tpit and Pitx1. *B* and *C*, dose-response curves for Etv1 and Tpit (25, 50, and 75 ng expression vectors with 100 ng of empty or Tpit vector as indicated) activation of Tpit/PitxRE reporters containing wild-type (*wt*) (*B*) or Ets-binding site mutant (*mut*) (*C*). Data are presented as means ± S.E. of three or more experiments, each performed in duplicates.

endogenous POMC gene. This contribution likely results from a direct effect because no change in Tpit expression was detected in these experiments (Fig. 5*B*). We next wanted to ascertain the presence of Etv1 on the endogenous POMC promoter. We thus performed ChIP experiments using AtT-20 nuclear extracts. As assessed by QPCR, ChIP with the Etv1 antiserum produced a 4-fold enrichment of the POMC promoter sequence relative to a control preimmune antiserum (Fig. 5*C*), clearly establishing the presence of Etv1 protein at the POMC locus.

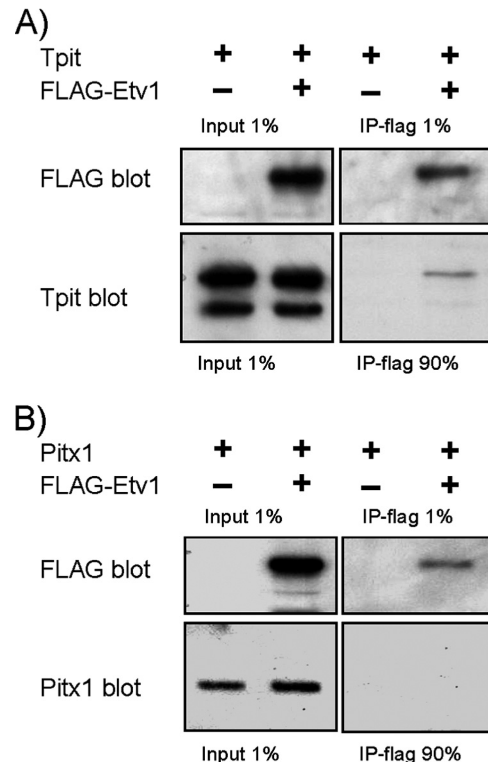

FIGURE 4. **Coimmunoprecipitation (*IP*) of Etv1 with Tpit but not Pitx1.** *A*, Tpit expression vector was transfected in HEK293 cells alone or in combination with vector for FLAG-Etv1 that contains three copies of the FLAG sequence. The Tpit blot (*lower right panel*) reveals the interaction between Tpit and Etv1. *B*, immunoprecipitation of FLAG-Etv1 does not bring down Pitx1 in similar IP experiments.

These data indicate an important role of Etv1 for POMC transcription in cultured cells. We next inquired whether the *Etv1* gene is similarly important *in vivo* using *Etv1-lacZ* knockout mice that are born at normal Mendelian frequencies. Homozygous mice for the *Etv1-lacZ* allele develop limb ataxia and abnormal posturing of the limbs and die by 3–5 weeks of age (23). No obvious pituitary phenotype was reported before we undertook the present study. We measured POMC pituitary content in these mice by immunohistochemistry and QPCR. Surprisingly, no difference between homozygous and wild-type animals was observed. POMC cell distribution is identical for both genotypes at E16.5 (data not shown) and at 20 days after birth (Fig. 6*A*). Accordingly, there is no significant difference in POMC mRNA levels between homozygous and wild-type animals at birth (Fig. 6*B*). This apparent discrepancy between cell culture data and *Etv1* gene inactivation could be due to compensation by another Ets factor.

The PEA3 subfamily of Ets factors (22) consists of four members (Etv1/Er81, Etv2/Er71, Etv4/Pea3, and Etv5/Erm), of which only Etv1 displayed a Tpit-like expression profile (Fig. 1*A*). We used RT-QPCR to assess pituitary expression of Etv2/Er71, Etv4/PEA3, and Etv5/Erm. Etv5 and Etv4, but not Etv2,

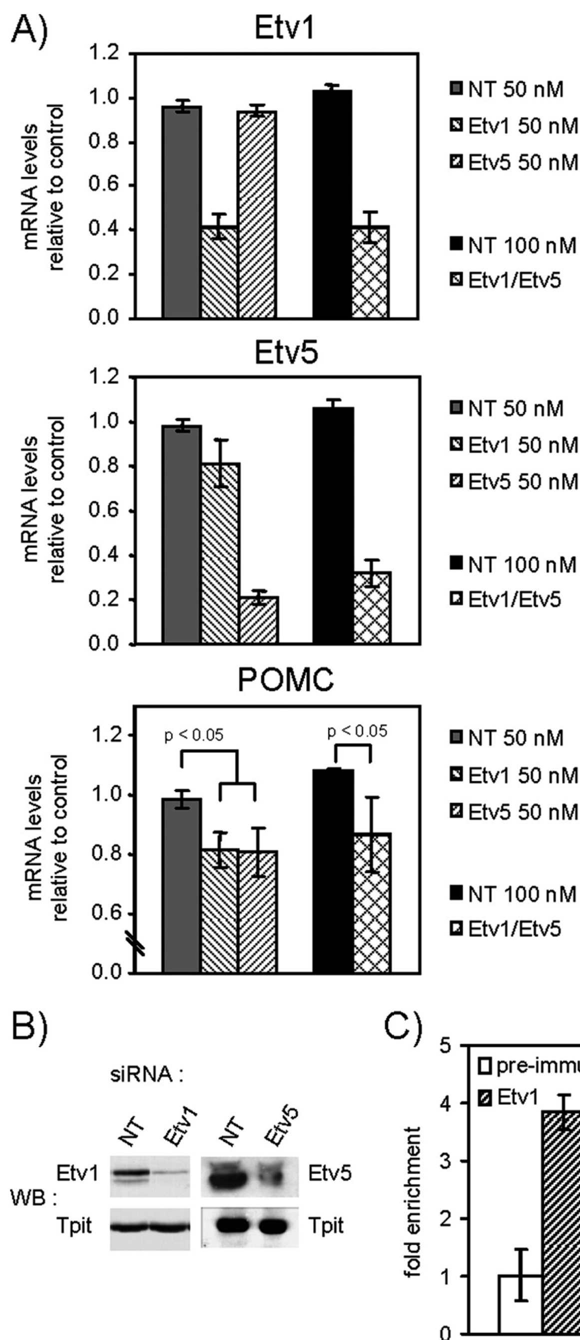

FIGURE 5. **POMC expression requires Etv1 that occupies the POMC promoter.** *A*, siRNA knockdown of Etv1 and Etv5 in AtT-20 cells results in approximately 50 or 80% decreases in Etv1 or Etv5 mRNAs, respectively, as measured by RT-QPCR. This is accompanied by a significant decrease in POMC ($p < 0.05$) mRNA. Control (non-target) siRNA (*siRNA NT*) did not affect these mRNAs. All data were normalized relative to TBP mRNA. *B*, Western blot (*WB*) analyses of Etv1, Etv5, and Tpit reveal the effect of knockdowns on cognate proteins but not on Tpit. *C*, ChIP using Etv1 antibody reveals enrichment of Etv1 at the POMC promoter by comparison to IgG control.

mRNAs are clearly detectable by QPCR in newborn pituitaries (Fig. 6*B*). However, homozygous and wild-type pituitaries of *Etv1-lacZ* newborns contained similar levels of Etv5 and Etv4 mRNAs, ruling out an increase of these factors as a mechanism of compensation for Etv1 loss. Etv4/5 mRNA levels could differ between AtT-20 and pituitary POMC cells to explain a different sensitivity to Etv1 loss. To test this idea, we prepared RNA from FACS-sorted cells obtained after dispersion of POMC-EGFP transgenic pituitaries (16). QPCR analysis revealed expression of Etv5 both in AtT-20 and pituitary POMC cells. Etv5 expression levels were slightly higher in melanotrophs (MSH+) and in corticotrophs (ACTH+) compared with AtT-20 (Fig. 6*C*). The cell distribution of Etv5 expression was further supported by immunohistofluorescence colabeling of adult pituitary sections for Etv5 and POMC (Fig. 6*D*). All melanotrophs of the intermediate lobe stained positive for nuclear Etv5, whereas Etv5-positive cells of the anterior lobe exhibited predominant cytoplasmic staining. Some of these cells are also positive for POMC but not all. Etv5 thus appeared to be present in a subset of corticotrophs as well as in other cells. Etv4 expression was extremely low in mouse pituitary POMC cells (Fig. 6*C*), and Etv2 was undetectable. It is thus possible that Etv5 may compensate for the loss of Etv1 because these factors may share redundant activities.

Because AtT-20 cells express Etv5 together with Etv1, we used these cells to test the ability of Etv5 to act on POMC expression through siRNA knockdown. As for Etv1, knockdown of Etv5 (Fig. 5*B*) significantly reduced POMC mRNA levels in AtT-20 cells (*A*), but their combined knockdown did not produce any greater effect than individually. The data thus indicate that both contribute to POMC transcription and suggest that their action may depend on a common target, such as a coregulatory protein or a transcription complex that is present in limiting amount at POMC regulatory sequences.

DISCUSSION

The present work documents a role for Etv1, an Ets transcription factor of the PEA3 subfamily, in POMC gene transcription. This function is consistent with the restricted expression of this transcription factor in the POMC-expressing pituitary lineages, the corticotrophs and melanotrophs (Fig. 1). In particular, we identified a critical Ets-binding site located between the Tpit and Pitx1 binding sites of the POMC promoter (Fig. 2). In addition to direct DNA interactions, the action of Etv1 appears to rely on protein interactions with Tpit (Figs. 3 and 4). Because the cognate DNA sites for Tpit, Etv1, and Pitx1 are very close to each other, we used computed modeling to verify that all three factors can interact concomitantly with Tpit/PitxRE (Fig. 7). Interestingly, the model suggests a direct interface between the Ets and T-box domains that may mediate the interaction between Tpit and Etv1 (Fig. 4*A*). Although Etv1 is present at the POMC promoter in AtT-20 cells and contributes to basal POMC expression (Fig. 5), knockout of the mouse *Etv1* gene is not sufficient to impair differentiation of pituitary POMC lineages (Fig. 6). The function of Etv1 thus appears to be redundant with other transcription factors of the Ets family, as was observed in other systems, including other pituitary lineages (22).

The restricted expression of Etv1 in POMC lineages suggests that this factor may contribute to cell-specific transcription in these cells. This idea is reinforced by the parallel expression with Tpit and by the direct interactions between Tpit and Etv1 (Fig. 4). The joint action of Etv1 interacting with Tpit for cell-specific transcription is reminiscent of the interaction of other Ets transcription factors with Pit1 for lactotroph-specific transcription of the PRL gene (30, 32). Indeed, Ets transcription

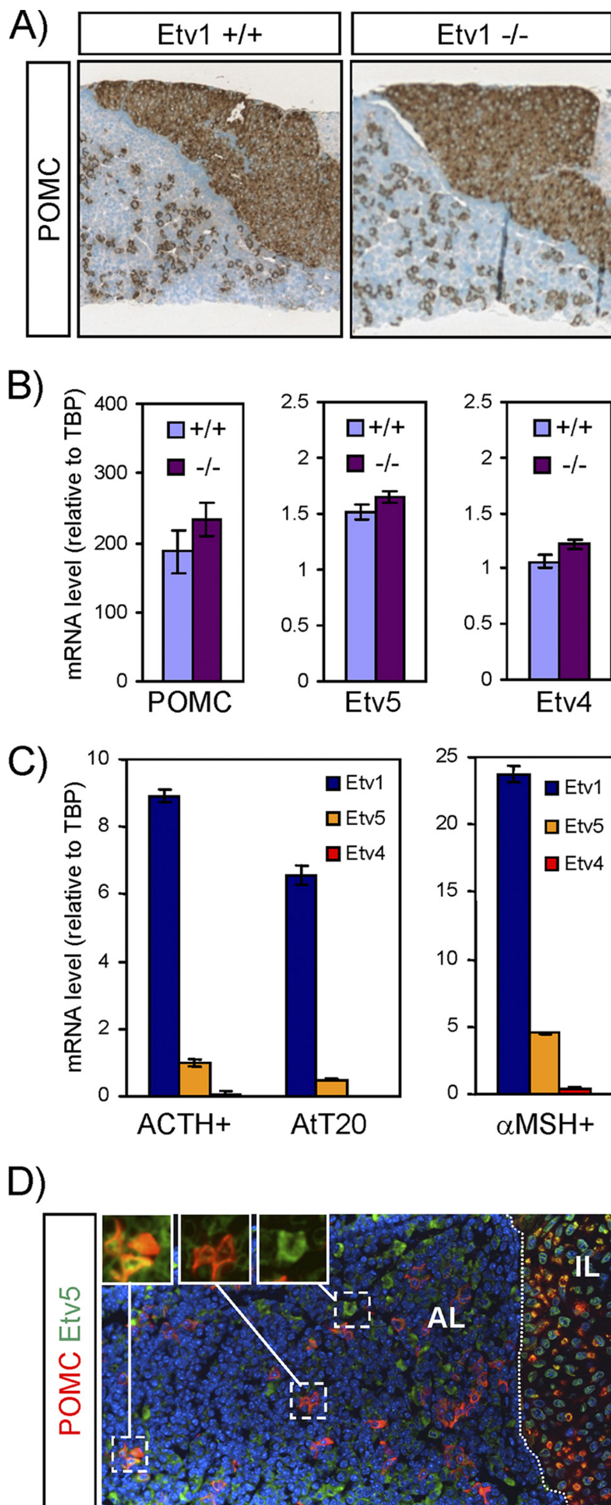

FIGURE 6. **Normal POMC expression in *Etv1* knockout mice.** *A*, ACTH (POMC) immunohistochemical analysis of P20 pituitaries from wild-type and *Etv1* knockout mice reveals similar expression patterns and histology. *B*, RT-QPCR quantitation of POMC, Etv5, and Etv4 mRNA levels in newborn pituitaries from control and $Etv1^{-/-}$ mice. All data are expressed relative to TBP mRNA. Etv2 mRNA was not expressed at significant levels (data not shown). *C*, RT-QPCR analysis of Etv1, Etv5, and Etv4 mRNAs in FACS-sorted cells from adult POMC-EGFP mice and AtT-20 cells. Corticotrophs (*ACTH+*) and melanotrophs (*αMSH+*) were derived from the same pool of 20 male pituitaries. Mouse ACTH and αMSH cells express significantly more Etv5 than AtT-20 ($p = 0.038$ and $p < 0.002$, respectively). Etv2 was undetectable in all cell preparations as was Etv4 in AtT-20 cells. Data are shown as mean ± S.E. of measurements performed in triplicate. *D*, coimmunofluorescence labeling of adult pituitary section for Etv5 (*green*) and POMC (*red*). IL cells show nuclear colabeling, whereas AL cells exhibit mostly cytoplasmic staining for Etv5. The *insets* show examples of AL cells colabeled for Etv5 and POMC or labeled by only one marker.

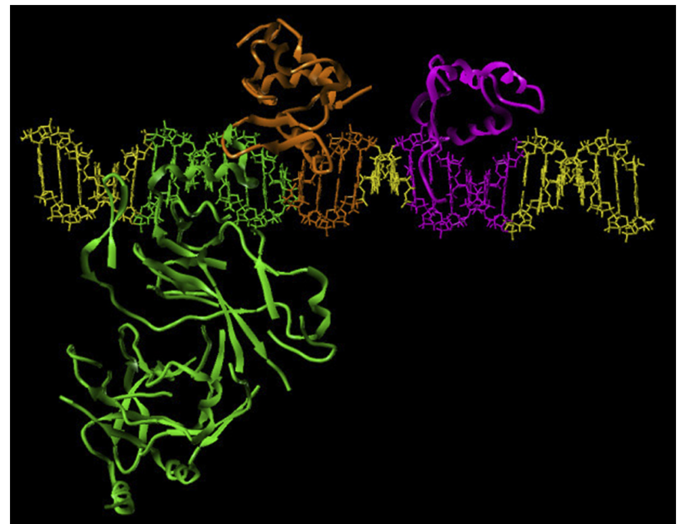

FIGURE 7. **Computer modeling of interactions between Tpit, Etv1, Pitx1, and their cognate binding sites (*colored*) within the POMC gene Tpit/PitxRE.** Modeling was achieved using crystal structures for full-length human Tbx5 (42) (*green*) and Ets DNA-binding domain (amino acids 331–440) of mouse Ets-1 (43) (*orange*) together with a solution structure of the human Pitx2 (44) homeodomain (amino acids 85–144) (*purple*).

factors interact with Pit1 for cell-specific transcription of the PRL gene, but they also contribute to basal transcription through binding to a basal transcription element (33). Various members of the Ets family were shown to act on PRL transcription, but direct affinity purification of Ets factors from GH3 cells identified GA-binding protein α and $\beta 1$ as predominant factors (34). Etv1 was also shown to be an androgen receptor-dependent gene in prostate cancer cells (35) and to be a survival factor for gastrointestinal stromal tumors (36).

Etv1 may also contribute to signal-dependent activation of POMC transcription by analogy, once again, with the action of other Ets factors on PRL gene expression. Indeed, basal PRL transcription-dependent on GA-binding protein α and $\beta 1$ is responsive to growth factors such as FGF2, FGF4, insulin, insulin growth factor, and EGF, and the action of these factors has been shown to be mediated through Ets factors (33, 34). In addition, the action of the Ras-MAP kinase pathway on PRL gene transcription was mapped to Ets factors (30) and shown to depend on both Ets and Pit1 binding sites (32). Ets transcription factors were also involved in function of another pituitary lineage, the gonadotrophs, and there it is the action of hypothalamic gonadotropin-releasing hormone through the MAP kinase pathway that was shown to be mediated through Ets factors. In this instance, signaling through the Ets factors acts primarily on the promoter of the *Egr1* immediate early gene and transcription factor (37) that itself acts on the LHβ gene (38). Interestingly, we did not find evidence that CRH signaling is enhanced through Etv1 or its binding site (Fig. 2, *F* and *G*), but Etv1 may nonetheless be involved in signaling initiated by other hormones or growth factors. Indeed, POMC expression is modulated by a variety of growth factors or cytokines (39). Fur-

ther, Etv1 was implicated in signaling initiated at the HER2/neu receptor in breast cancer cells (40). In these cells, this EGF-related receptor/pathway leads to activation of telomerase and MMP-1 metalloproteinase genes (41). As in pituitary lactotrophs cells, these actions are mediated through the MAP kinase pathway.

The Ets family of transcription factors includes many different transcription factors, with the PEA3 subfamily itself containing four related factors (21, 22). There are thus a large number of possible factors that may compensate for the loss of Etv1 and account for the absence of a pituitary phenotype in *Etv1*$^{-/-}$ mice (Fig. 6). We have assessed expression and putative up-regulation of the closely related factors Etv2, Etv4, and Etv5, some of which are also expressed in the mouse pituitary and AtT-20 cells (Fig. 6). Clearly, Etv4 and Etv5 are not up-regulated in absence of Etv1 (Fig. 6*B*), but they may nonetheless compensate for the absence of Etv1. Indeed, knockdown of Etv5 was as effective as knockdown of Etv1 to reduce POMC expression in AtT-20 cells (Fig. 5), thus supporting a role for both Etv1 and Etv5 in POMC transcription. A quantitative assessment of Etv4 and Etv5 relative to Etv1 mRNAs suggests that they are expressed at much lower levels compared with Etv1. However, mRNA comparisons cannot predict protein accumulation, and thus the Etv4 and/or Etv5 proteins might be more abundant than suggested by their relative mRNA levels. It is noteworthy that the Etv5 protein could be detected by immunofluorescence in pituitary melanotrophs and corticotrophs (Fig. 6*D*). The nuclear *versus* cytoplasmic localization of Etv5 in these different cells may be indicative of differential regulation. A similar pattern was also observed for Etv1 (Fig. 1*C*). These Ets factors may therefore act differently in the two POMC lineages. Although the current studies were conducted in AtT-20 cells, a corticotroph model, there is no model cell line for melanotrophs. Notwithstanding this, it is also possible that other members of the Ets family may compensate for some function normally fulfilled by Etv1 or Etv5.

Taken collectively, the present work clearly identified Etv1 as a transcription factor for POMC gene expression. The well documented responsiveness of this class of transcription factors to various signaling pathways clearly highlights Etv1 as a candidate to account for signal-dependent regulation of POMC transcription.

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