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BETA3, a Novel Helix-Loop-Helix Protein, Can Act as a Negative Regulator of BETA2 and MyoD-Responsive Genes

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Using degenerate PCR cloning we have identified a novel basic helix-loop-helix (bHLH) transcription factor, BETA3, from a hamster insulin tumor (HIT) cell cDNA library. Sequence analysis revealed that this factor belongs to the class B bHLH family and has the highest degree of homology with another bHLH transcription factor recently isolated in our laboratory, BETA2 (neuroD) (J. E. Lee, S. M. Hollenberg, L. Snider, D. L. Turner, N. Lipnick, and H. Weintraub, Science 268:836–844, 1995; F. J. Naya, C. M. M. Stellrecht, and M.-J. Tsai, Genes Dev. 8:1009–1019, 1995). BETA2 is a brain- and pancreatic-islet-specific bHLH transcription factor and is largely responsible for the tissue-specific expression of the insulin gene. *BETA3* was found to be tissue restricted, with the highest levels of expression in HIT, lung, kidney, and brain cells. Surprisingly, despite the homology between BETA2 and BETA3 and its intact basic region, BETA3 is unable to bind the insulin E box in bandshift analysis as a homodimer or as a heterodimer with the class A bHLH factors E12, E47, or BETA1. Instead, BETA3 inhibited both the E47 homodimer and the E47/BETA2 heterodimer binding to the insulin E box. In addition, BETA3 greatly repressed the BETA2/E47 induction of the insulin enhancer in HIT cells as well as the MyoD/E47 induction of a muscle-specific E box in the myoblast cell line C2C12. In contrast, expression of BETA3 had no significant effect on the GAL4-VP16 transcriptional activity. Immunoprecipitation analysis demonstrates that the mechanism of repression is via direct protein-protein interaction, presumably by heterodimerization between BETA3 and class A bHLH factors.

Factors involved in regulating tissue-specific gene expression play a major role in cellular differentiation. Work in our laboratory has focused on understanding the tissue-specific expression of the insulin gene (12, 32, 47, 55). We have shown that the insulin enhancer RIPE3 (rat insulin promoter element 3) can confer tissue specificity to a heterologous reporter in transient-transfection assays (32) and can bind at least four distinct factors, two of which are tissue specific (55). A subregion of RIPE3, RIPE3a (also variously known as E1, Nir box, IEB1, and ICE), includes a 9-bp sequence (GCCATCTGC) conserved in all characterized mammalian insulin genes (7, 12, 19, 36, 56, 67) and harbors a consensus E box (CANNTG) motif.

In general, tissue-specific factors which bind E boxes have been shown by in vitro and in vivo studies to consist of heterodimers between a ubiquitous class A and a tissue-specific class B family member of the basic helix-loop-helix (bHLH) transcriptional activators (28, 34, 38, 45, 46, 51). The conserved features of the bHLH domain are a basic region required for specific DNA binding and two amphipathic helices, which are required for dimerization, separated by a variable loop (14, 17, 44, 64). Examples of class A bHLH family members are the E2A gene products (E12 and E47), ITF2, and HEB (HTF4 and BETA1) (25, 30, 44, 50, 59, 71). Class B members include BETA2 (neuroD), MyoD, myogenin, achaete-scute, MASH-1 and MASH-2, SCL, lyl-1, twist, and NSCL 1 and 2 (2, 4, 5, 13, 16, 22, 27, 35, 39, 43, 47, 60, 63, 69). A specialized subset of the class B genes encode dominant negative forms of the protein. These factors behave like class B proteins by heterodimerizing with class A proteins, but they lack a functional basic domain and hence cannot bind DNA. Thus, expression of dominant negative genes inhibits the ability of class A and B proteins to carry out their function(s). In addition, another group of negative bHLH proteins which contain proline residues in their basic region have been shown to directly bind DNA and repress transcription. The dominant negative and repressor forms include Id, emc, and hairy (6, 49, 61, 62).

Several lines of evidence demonstrate that bHLH proteins play an important role in controlling insulin gene expression. Previously, it was shown the insulin E box binding complex contained various class A bHLH family members (3, 11, 20, 48, 50, 55, 65). More notably, our laboratory has recently isolated BETA2, the class B bHLH component of the insulin enhancer E box binding complex (47). *BETA2* is expressed primarily in pancreatic-islet cells and in the brain, two regions which demonstrate insulin gene expression (15). Independently, Lee and coworkers isolated the mouse homolog of *BETA2*, which they termed *neuroD*, and demonstrated that it is involved in neuronal differentiation (39).

In our quest to further identify class B bHLH genes present in hamster insulin tumor (HIT) cells, we isolated *BETA3* through PCR using degenerate oligonucleotides in a procedure which should be generally applicable to isolating novel class B members of the bHLH superfamily. *BETA3* is expressed in a tissue-restricted manner and encodes a protein which inhibits E47 and BETA2 from binding DNA and acts as a strong repressor of BETA2 and MyoD-responsive genes.

MATERIALS AND METHODS

Reverse transcriptase PCR of BETA3. cDNA was prepared from 5 μg of total HIT cell RNA using 1 mg of oligo(dT) and 400 U of Moloney murine leukemia virus reverse transcriptase (RNase H minus; Promega). The entire cDNA preparation was used for PCR (52) under the following conditions: 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dideoxynucleoside triphosphates, 1 μg of each primer, and 7.5 U of *Taq* polymerase in a 100-μl final volume. The primer sequences were 5′-CAGCATGC(CA)GGGAGCGC(CA)

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(GA)(GC)(CA)G-3′ and 5′-CCAAGCTTCAGG(CA)(TAC)(GC)T(GC)GAT GTA-3′, with the *Sph*I and *Hin*dIII cloning sites underlined, respectively. The cDNA was amplified with a PCR profile of 95°C for 30 s, 50°C for 1 min, and 72°C for 1 min repeated for 30 cycles followed by 5 min at 72°C. As a positive PCR control, 100 ng of EMSV-MyoD plasmid was assayed in a duplicate reaction.

Screening of a HIT cell cDNA library. A novel bHLH PCR subclone, pbHLH3 was ^{32}P labeled and used to screen ~500,000 recombinant phage (42) from a HIT cell λgt11 cDNA library (20).

Plasmids and sequencing. PCR fragments and phage inserts were cloned into *Sph*I-*Hin*dIII- or *Eco*RI-digested pGEM7Zf(+), respectively. The sequencing strategy was a combination of deletion constructs and primer walking. Sequencing was carried out with the Sequenase version 2.0 kit (U.S. Biochemicals) according to the manufacturer's instructions. The largest cDNA subcloned into pGEM7Zf(+) is referred to as pBETA3. The mammalian expression plasmid for BETA3 was constructed by inserting a *Kpn*I-to-*Xba*I fragment of pBETA3 into pCMV. The BETA2 plasmids are described by Naya et al. (47), and the BETA1 plasmid is described by Peyton et al. (50). The in vitro translation plasmid for shPan1 is as described previously (20) as are the expression plasmids EMSV-MyoD (13), pSVE2-5 (25), and GAL4-VP16 (66) and the reporter plasmids RIPE3 (32), pCKCATE4+ (58), and 17×4-TATA-CAT (66).

Cell culture and CAT assays. The HIT-T15 M2.2.2 cells (53) and the C2C12 myoblasts (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 mg of streptomycin per ml at 37°C under 5% CO_2. HIT cells were plated onto 60-mm-diameter tissue culture dishes and transfected by the calcium phosphate coprecipitation method (31) with 1 μg of reporter and expression plasmids with the final 4 μg of DNA equilibrated with an empty pCMV expression vector. The C2C12 cells were plated onto 100-mm-diameter tissue culture dishes and transfected by the polybrene method (10) with 4 μg of reporter plasmid and 2 μg of expression plasmids with the final 9 μg of DNA equilibrated with an empty pCMV expression vector. When the C2C12 cells became confluent (1 to 2 days after transfection) they were switched to differentiation medium (Dulbecco's modified Eagle's medium with 5% horse serum) and harvested 2 days later. Chloramphenicol acetyltransferase (CAT) assays were performed as described previously (54).

Bandshift assays. The RIPE3 (−126 to −86) double-stranded oligonucleotide was end labeled with [α-^{32}P]dGTP by using the Klenow fragment to a specific activity of ~1.0 × 10^8 cpm/μg. The RIPE3 sequence used was as follows: 5′-GATCTGGAAACTGCAGCTTCAGCCCCTCTGGCCATCTGCTGATCCG-3′. Binding reaction mixtures contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 200 ng of poly(dI-dC), ~3 × 10^4 cpm of RIPE3 probe, and various amounts of in vitro-translated protein (50). Protein-DNA complexes were resolved by electrophoresis on nondenaturing 5% polyacrylamide gels. Proteins were synthesized with the Promega TNT coupled transcription-translation kit according to the manufacturer's instructions. Parallel reactions with [^{35}S]methionine or unlabeled methionine were carried out. Labeled proteins were subjected to sodium dodecyl sulfate–10% polyacrylamide electrophoresis (SDS–10% PAGE) to ensure proper synthesis.

RNase protection analysis. RNA was extracted with RNazol B (Cinna/Biotecx Laboratories Inc., Houston, Tex.). The BETA1 and BETA3 ^{32}P-labeled riboprobes were synthesized with a *Sau*3AI-digested pBETA1 plasmid and a *Ban*I-digested pbHLH3 plasmid, respectively, according to the manufacturer's procedure. The synthesized products were isolated from a 7 M urea–5% polyacrylamide gel by eluting at 37°C overnight in 0.5 M ammonium acetate–0.1% SDS–1 mM EDTA. The riboprobes were then ethanol precipitated and resuspended in hybridization buffer (80% formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.4], 400 mM NaCl, 1 mM EDTA). RNA samples were dried in a Speed Vac and hybridized with 5 × 10^5 cpm of probe in 30 μl of hybridization buffer overnight at 60°C (50 μg of RNA for the BETA3 probe and 5 μg of RNA for the BETA1 probe). Samples were digested with 350 μl of 2-mg/ml RNase A in RNase digestion buffer (10 mM Tris-HCl [pH 7.5], 300 mM NaCl, 5 mM EDTA) for 1 h at 30°C. A total of 10 μl of 20% SDS and 2.5 μl of 20-mg/ml proteinase K were added and incubated at 37°C for 15 min, phenol extracted, and ethanol precipitated. Samples were resuspended in 3 μl of 80% formamide–1 mM EDTA and run on a 7 M urea–5% polyacrylamide sequencing gel (21). The 425-nucleotide (nt) BETA3 riboprobe was predicted to yield a protected fragment of 121 to 141 nt, depending on the degree of homology between the primer region of the pbHLH probe and the endogenous mRNA. The 208-nt BETA1 riboprobe was predicted to protect a 165-nt fragment.

Immunoprecipitation. Aliquots of the proteins transcribed and translated in vitro with [^{35}S]methionine or unlabeled methionine were mixed at 37°C for 15 min and then added to 500 μl of immunoprecipitation buffer containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 100 mM NaCl, 0.5 mM EDTA, 5% glycerol, 0.5% Nonidet P-40, and 0.5 mM dithiothreitol. Immunoglobulin G-purified rabbit polyclonal anti-E47 antibody (6 μl) (Santa Cruz Biotechnology) or 2 μl of anti-BETA2 (47) was added along with 20 μl of protein A-Sepharose beads (Pharmacia). The beads were incubated on a rocker at 4°C for 4 h and then washed five times, in 200 μl each time, with immunoprecipitation buffer. The beads were then resuspended in 20 μl of SDS-PAGE sample buffer and boiled for 5 min. Supernatants were analyzed by SDS–10% PAGE and autoradiography.

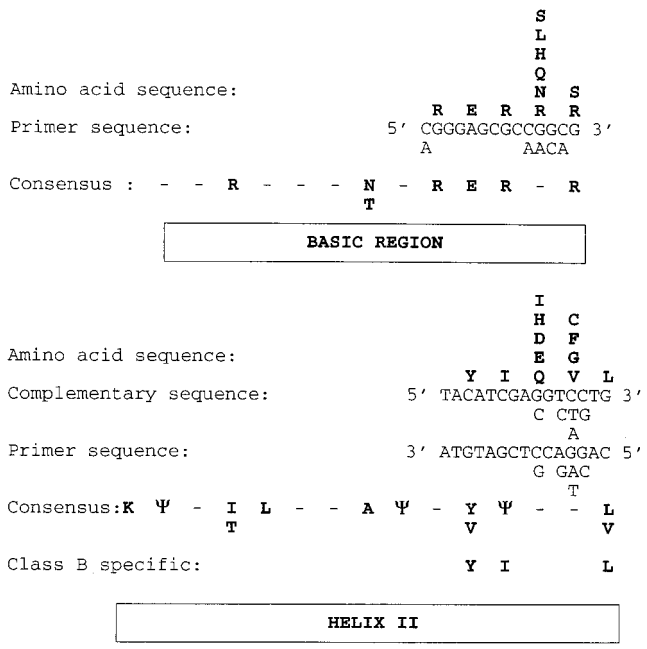

FIG. 1. Primer design strategy for PCR amplifying novel bHLH proteins. The location of the upstream (top) and downstream (bottom) primer binding sites are shown in relation to the bHLH domain (compare Fig. 2B). The possible encoded amino acid sequences as well as the consensus amino acid sequences are shown in boldface type. Ψ denotes a hydrophobic residue. The nucleotide primer sequences as well as the complementary codon sequence of the downstream promoter are as indicated.

RESULTS

PCR cloning of BETA3. Partially degenerate oligonucleotides were designed by taking advantage of conserved class B-specific amino acid sequences (Fig. 1). The upstream primer was prepared against the RER-R consensus sequence found in the basic region of all bHLH factors (Fig. 2B). The nucleotide sequences at the nonconserved amino acid in this region were partially randomized to contain preferred codon sequences for arginine, asparagine, glutamine, serine, lysine, and histidine. The amino acid in this position is often either arginine, asparagine, or glutamine (Fig. 2B). The downstream primer was designed to be specific for class B bHLH proteins. This is based on the observation that the consensus sequence (Y/V)ΨXX(L/V) (Ψ signifies a hydrophobic residue) found in the C terminus of the second helix is primarily YIXXL for class B molecules. The nonconserved amino acids were also partially randomized to contain preferred sequences. These oligonucleotides were used in a PCR to amplify cDNA from a hamster insulinoma cell line (HIT). Bands corresponding to the predicted size of approximately 150 bp were isolated from a polyacrylamide gel, reamplified, and subcloned. Sequence analysis of 22 subclones revealed the subclone pbHLH, which encoded a product with the characteristics of a novel class B bHLH family member.

Over 500,000 recombinant phage were screened with radiolabeled pbHLH inserts, yielding 18 positive clones. Several of these clones were picked at random, purified, and subcloned. All of these clones were analyzed by restriction mapping and determined to be overlapping clones.

Sequence analysis. The longest clone, pBETA3, containing a 2.6-kb insert, was completely sequenced on both strands (Fig. 2A). The sequence was found to be extremely GC rich (73% up to the stop of translation). It contains a region of 100% ho-

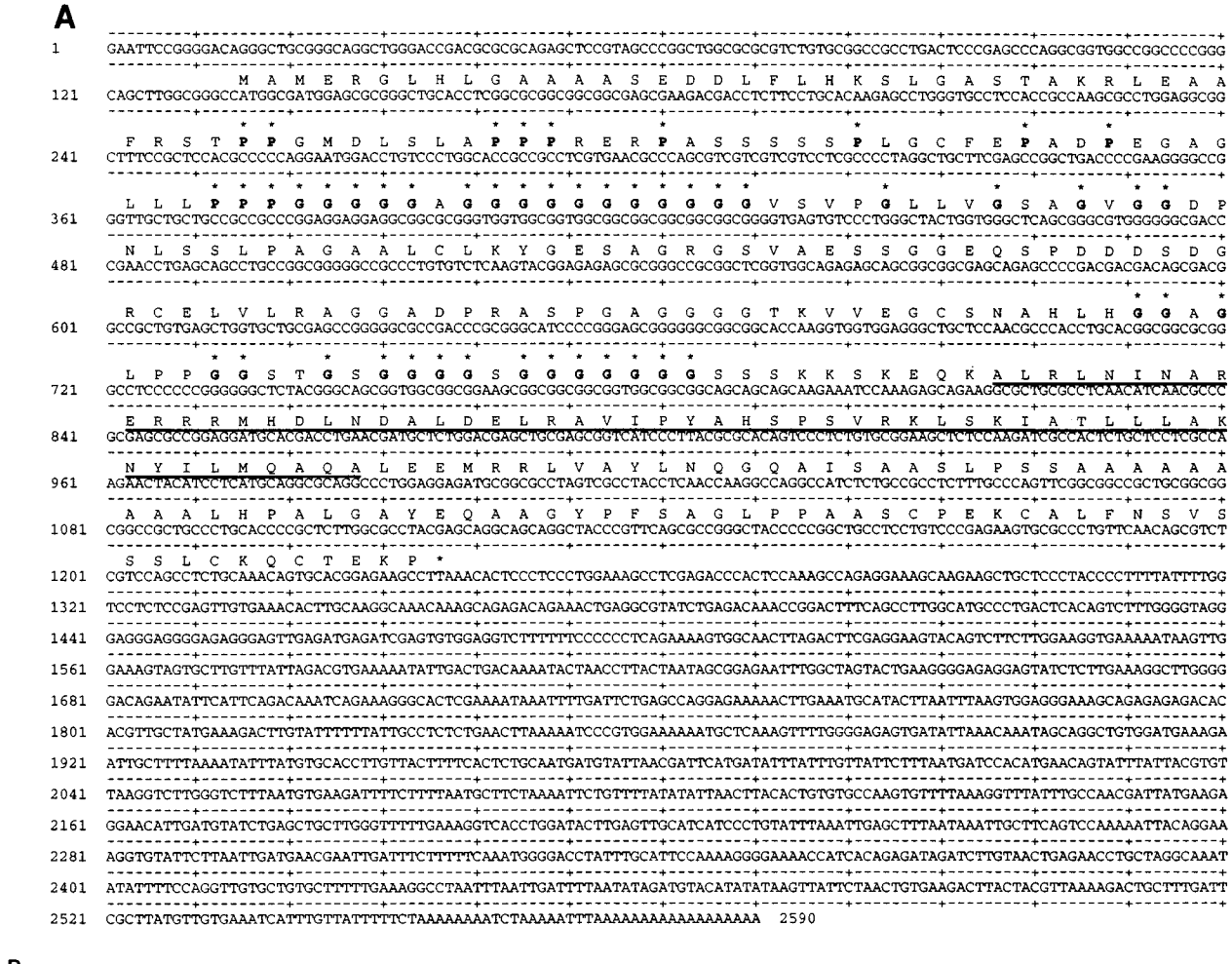

FIG. 2. *BETA3* cDNA sequence and alignment to sequences of class B bHLH domains. (A) Sequence analysis of *BETA3*. The nucleotides are numbered on the left and marked with a plus every 10 nt. The deduced amino acid sequence is shown in single-letter code, with an asterisk for the stop codon. The bHLH domain is underlined. The prolines and glycines of the proline- and glycine-rich regions are shown in boldface and marked with an asterisk. (B) Alignment of the BETA3 bHLH domain with other class B bHLH domains. The consensus bHLH sequence for all three groups of family members is from reference 44. Dashes denote sequence identity. Ψ denotes a hydrophobic residue. The various bHLH domains aligned with BETA3 are BETA2 (47), mtwist (68), xtwist (27), atonal (33), lyl (43), tal1(scl) (4), tal 2 (70), MASH-2 and MASH-1 (35), and MyoD (13).

mology to the PCR fragment which was originally isolated. The longest open reading frame is contiguous with the bHLH domain coding sequence and encodes a protein of 367 amino acids with a molecular weight of ~40,000. This open reading frame begins with two in-frame ATG codons, both of which show high degrees of sequence identity to the Kozak consensus sequence for ribosome binding sites (36). Upstream of these ATGs is an in-frame stop codon. The other two reading frames have numerous stop codons throughout the entire sequence.

The putative bHLH domain of BETA3 is located between residues 228 and 285 (Fig. 2A, underlined). In addition, it contains a proline-rich region between amino acids 41 and 82, followed by two glycine-rich regions, amino acids 83 to 99 and 193 to 217, N terminal to the bHLH domain (Fig. 2A, asterisks and boldface type). Alignment of the BETA3 bHLH domain with other members of the bHLH family indicates that it belongs to the tissue-specific class B bHLH subgroup (Fig. 2B). Note that BETA3 contains an intact basic region, indicating that it is not a member of the dominant negative subgroup of the class B molecules (i.e., an Id-like molecule). BETA3 has

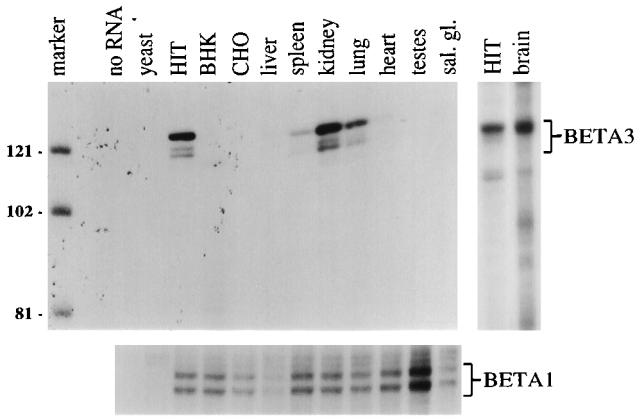

FIG. 3. RNase protection analysis of *BETA3* tissue and cell expression pattern. Aliquots (50 μg) of total RNA from various hamster tissues and cell lines were hybridized to *BETA3*-specific riboprobes and assayed by RNase protection. To assess RNA integrity, 5-μg aliquots of duplicate RNA samples were assayed with a ubiquitous *BETA1*-specific riboprobe. The lanes and bands are as marked. Sizes in nucleotides are indicated to the left of the gel. sal. gl., salivary gland.

the most homology with BETA2, with 53.4% amino acid identity in the bHLH domain (47), followed by 48.3% identity with the mouse *twist* gene product (68) (Fig. 2B).

Tissue distribution. Class B bHLH transcription factors have been found to be involved in the differentiation and development of the specific tissue in which they are expressed. To determine the tissue distribution of *BETA3*, we used RNase protection to assess its expression pattern. The pbHLH plasmid was used to produce a 425-nt ^{32}P-labeled riboprobe and was predicted to protect a 121- to 141-nt fragment, depending on the degree of homology between the primer region of the probe and the endogenous mRNA. The highest levels of expression were observed in HIT, kidney, brain, and lung cells. Much lower levels were found in liver, heart, and spleen cells. No specific bands were seen in BHK, CHO, testis, salivary gland, and muscle cells (Fig. 3 and data not shown). A riboprobe against ubiquitously expressed *BETA1* was used to assess RNA integrity.

DNA binding and dimerization. Because of the homology BETA3 has to the insulin E box binding protein BETA2 and because BETA3 contains an intact consensus basic domain, we predicted it would heterodimerize with E47 and bind the insulin E box containing sequence, RIPE3. To assess this, pBETA3 and shPan1 (a full-length hamster homolog of E47) were transcribed and translated in vitro. To examine the integrity of the translated products, duplicate reactions were carried out in the presence of [^{35}S]methionine and assessed by SDS-PAGE. The translation of BETA3 repeatedly was approximately one-third less efficient than the Pan1 and BETA2 translations (57). The translated products were assayed as a mix or alone by incubating with the radiolabeled RIPE3 oligonucleotide and examined by bandshift analysis. Surprisingly, though the Pan1 homodimer (Fig. 4A) and the BETA2/Pan1 heterodimer (Fig. 4B) bind this oligonucleotide, BETA3 does not bind as a homodimer or as a heterodimer with Pan1. Similar results were obtained with shPan2 (the hamster E12 homolog) and BETA1 (the hamster HEB homolog) (57). Even

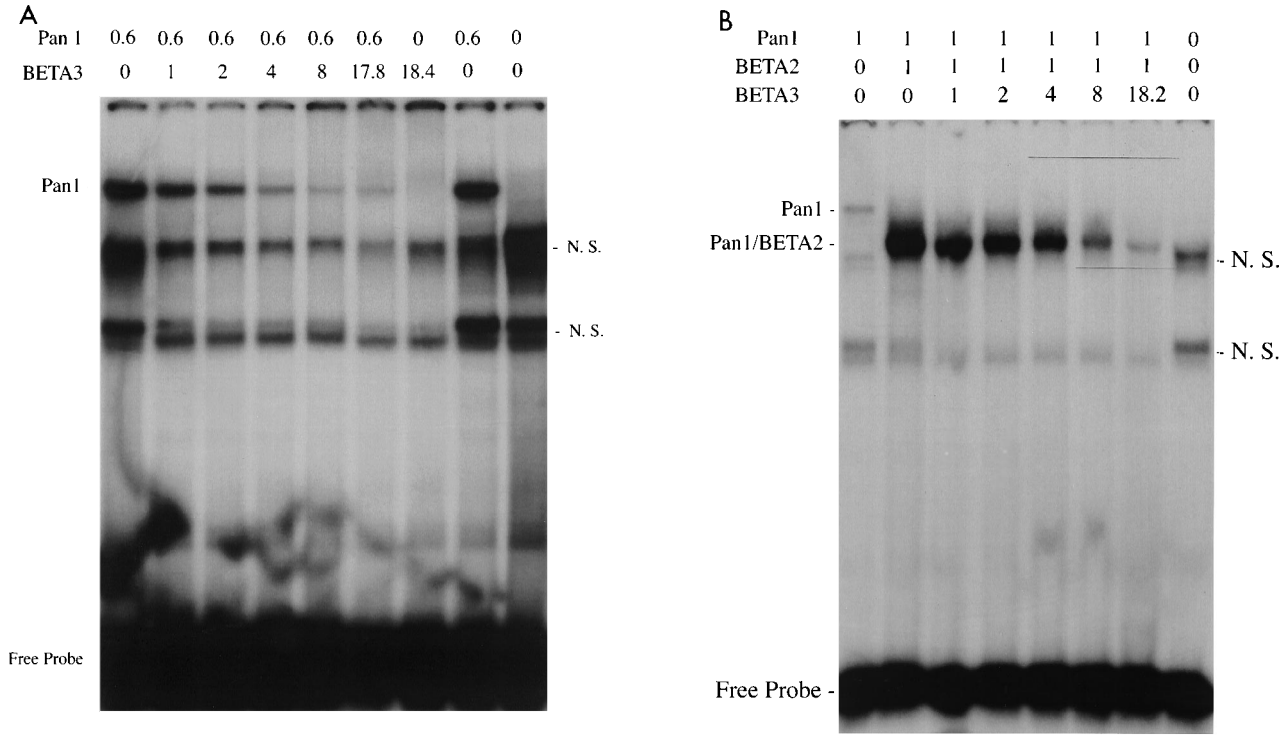

FIG. 4. BETA3 inhibition of E box binding factors. Bandshift analyses were performed with radiolabeled RIPE3 oligonucleotides and the indicated volumes (in microliters) of translation products. All lanes were equilibrated by a mock translation reaction in which no DNA vector was added to the transcription-translation reaction mixture to ensure that equal amounts of protein, salts, etc. were included in the assay in each lane. N. S., nonspecific or endogenous E box binding factors present in the reticulocyte lysate. (A) BETA3 inhibition of the Pan1 homodimer. Increasing amounts of BETA3 interact with Pan1 and thus decrease the amount of the Pan1 homodimer binding DNA. (B) BETA3 inhibition of the BETA2/Pan1 heterodimer. Increasing amounts of BETA3 interact with Pan1 and/or BETA2 and thus decrease the amount of the BETA2/Pan1 heterodimer formation, resulting in less DNA binding.

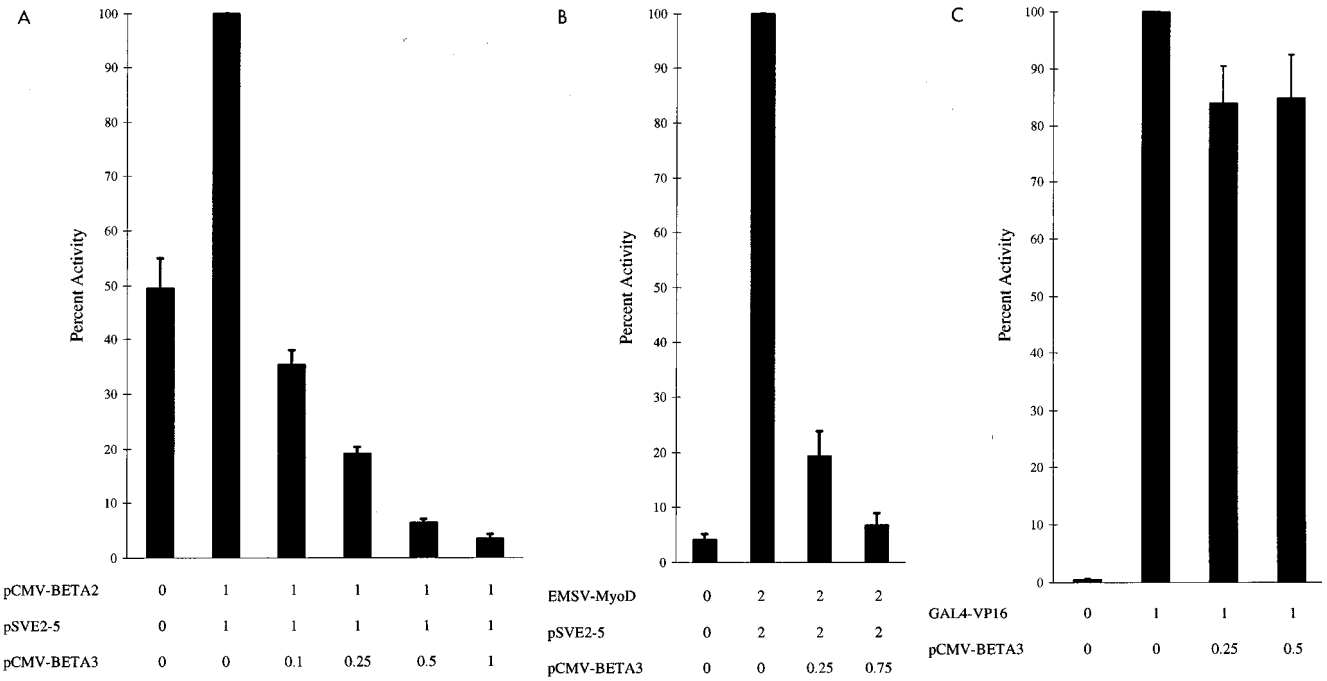

FIG. 5. Repressor activity of BETA3. Results are the averages of 4 to 10 transfections. All transfections were equilibrated with the pCMV expression vector. Bars indicate standard errors of the means. Values below bars are amounts (in micrograms) of expression plasmids. (A) BETA3 inhibits BETA2/E47 transactivation. HIT cells were cotransfected with 1 μg of RIPE3 reporter plasmid (described in the text and in reference 32) and the various expression plasmids as indicated. Activity is expressed as a percentage of the activity of RIPE3 obtained with cotransfection of pCMV-BETA2 and pSVE2-5. (B) BETA3 inhibits MyoD/E47 transactivation. C2C12 cells were cotransfected with 2 μg of pCKCATE4+ reporter plasmid (described in reference 58) and the various expression plasmids as indicated. Activity is expressed as a percentage of the activity of pCKCATE4+ obtained with cotransfection of EMSV-MyoD and pSVE2-5. (C) Effect of BETA3 on GAL-VP16 transactivation. HIT cells were cotransfected with 1 μg of 17X4-TATA-CAT reporter plasmid and the various expression plasmids as indicated. Activity is expressed as a percentage of the activity of 17X4-TATA-CAT obtained with cotransfection of GAL-VP16 alone.

though the complex does not bind RIPE3, BETA3 does appear to interact with Pan1, resulting in the depletion of the Pan1 homodimer (Fig. 4A). In titration analysis, increasing amounts of the BETA3 translation product augmented the depletion of the Pan1 homodimer while a mock translation reaction did not, further indicating that BETA3 interacts with Pan1.

BETA3 can inhibit BETA2/E47 transcription activation. Both BETA2 and BETA3 are expressed in HIT cells and in brain cells, and both appear to interact with E47, but only the BETA2/E47 heterodimer can bind the RIPE3 E box. Thus, the scenario exists that BETA3 could also inhibit BETA2/E47 DNA binding and the transcriptional activation of a RIPE3 reporter construct. To test this, we assessed the ability of BE TA3 to inhibit the binding of the BETA2/Pan1 heterodimer to the RIPE3 sequence (Fig. 4B). As shown, BETA2/Pan1 forms a strong DNA binding heterodimer complex. Like the Pan1 homodimer, this complex can be titrated off the DNA with increasing amounts of BETA3. To test for inhibition activity in vivo, we cotransfected HIT cells with 1 μg of the RIPE3 reporter construct, 1 μg of each of the expression vectors, pCMV-BETA2 and pSVE2-5 (E47), and with various amounts of the pCMV-BETA3 expression vector. The RIPE3 reporter construct consists of three copies of the E box containing the insulin enhancer nucleotide sequence −126 to −86 inserted at the −50 site of the ovalbumin minimal promoter CAT construct, pOVCAT-50 (32).

As shown previously, cotransfection of BETA2 and E47 stimulates expression of the RIPE3 reporter (Fig. 5A) (47). In a control experiment E47 also stimulated expression of the RIPE3 reporter (data not shown). The addition of 1 μg of BETA3 completely blocked this effect and repressed the transcription activity to a level ~10-fold lower than that seen with the reporter construct alone. Titration analysis revealed that cotransfection with as little as 0.1 μg of pCMV-BETA3 reduced CAT activity to levels lower than those with the reporter alone. Similar results were obtained with a RIPE3/ovalbumin promoter construct driving the human growth hormone reporter gene (57). In addition, BETA3 also inhibits the MyoD/E47 induction of the pCKCATE4+ construct, containing the muscle creatine kinase promoter and enhancer (58), when transfected in the C2C12 myoblast cell line (Fig. 5B). Thus, BETA3 appears to be a strong inhibitor of bHLH transcription factors.

To determine if the repressive activity of BETA3 on bHLH factors is a general phenomenon for all transcription factors, we cotransfected HIT cells with pCMV-BETA3 and GAL4-VP16. This construct consists of the transcriptional activator from herpes simplex virus, VP16, fused to the GAL4 DNA binding domain (66). As shown before, GAL4-VP16 transactivates the 17 × 4-TATA-CAT reporter, which consists of four copies of the GAL4 DNA response element linked to the adenovirus major late *E1B* TATA box to drive the CAT reporter (66). BETA3 did not have a significant effect on GAL4-VP16 transactivation activity. Thus, BETA3 does not appear to have general repressor activity.

BETA3 acts as a bHLH-specific repressor by heterodimerizing with E47. The hypothesis that BETA3 acts as a repressor of bHLH activity via direct interaction with class A bHLH proteins was tested by immunoprecipitation analysis. To examine this, BETA3 and Pan1 were transcribed and translated

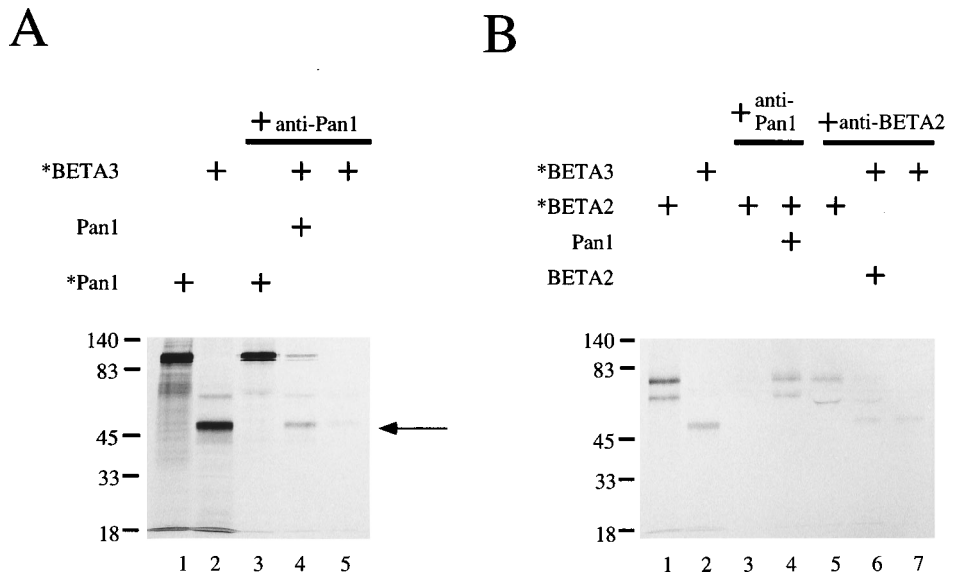

FIG. 6. In vitro interaction between BETA3 and Pan1. (A) Coimmunoprecipitation of BETA3 with Pan1 by anti-E47 antibody. BETA3 and Pan1 were transcribed and translated separately with [^{35}S]methionine or unlabeled methionine. Labeled proteins are indicated with an asterisk. Amounts of lysate used are as follows: *BETA3, 30 μl; *Pan1, 3 μl; Pan1, 15 μl. Aliquots of the proteins were subjected to immunoprecipitation with anti-E47 antibody (lanes 3 to 5). Values to the left of each gel are molecular weights (in thousands). (B) Absence of interaction between BETA3 and BETA2. BETA3 and BETA2 were transcribed and translated separately with [^{35}S]methionine or unlabeled methionine. Amounts of lysate used are as follows: *BETA3, 20 μl; *BETA2, 4 μl (except 0.7 μl used for lane 5); BETA2, 6 μl; Pan1, 6 μl. Aliquots of the proteins were subjected to immunoprecipitation with an antibody against BETA2 (lanes 5 to 7).

separately in the presence of [^{35}S]methionine. Additionally, Pan1 was transcribed and translated with unlabeled methionine. Translated products were then analyzed by SDS-PAGE.

The molecular weights of the E47 and BETA3 translation products are ~83,000 and ~50,000, respectively (Fig. 6A, lanes 1 and 2). Significantly, the BETA3 translation product could be immunoprecipitated with antibody against Pan1 when coincubated with unlabeled Pan1 (Fig. 6A, lane 4) but not in its absence (lane 5). As a positive control, [^{35}S]methionine-labeled Pan1 could be immunoprecipitated with antibody against Pan1 (lane 3).

To examine whether the nature of this interaction could be extended to include the class B bHLH subclass, the unlabeled BETA2 translation product was coincubated with [^{35}S]methionine-labeled BETA3 and immunoprecipitated with antibody against BETA2. As shown in Fig. 6B, BETA2-specific antibodies could not significantly immunoprecipitate BETA3 in the presence (lane 6) or absence (lane 7) of BETA2. However, BETA2-specific antibodies could immunoprecipitate labeled BETA2 (Fig. 6B, lane 5). As a positive control, Pan1-specific antibodies could immunoprecipitate labeled BETA2 when coincubated with unlabeled Pan1 (Fig. 6B, lane 4) but not in the absence of Pan1 (Fig. 6B, lane 3).

DISCUSSION

We have successfully used degenerate reverse transcriptase PCR to isolate a novel member of the bHLH class B family, BETA3. This family member has a high degree of homology with BETA2 (neuroD), the insulin E box transcription factor and neuron differentiating factor. BETA3 displays a tissue-restricted pattern of expression and appears to interact with class A bHLH family members. Though it has homology with BETA2 and has a conserved intact basic region, BETA3 is unable to bind a BETA2 binding site. However, it appears to interact with E47, thus allowing it to inhibit the DNA binding and transcriptional activity of BETA2.

The reverse transcriptase PCR method which we used should prove useful for isolating additional novel class B bHLH cDNAs. In general, other attempts at PCR cloning class B bHLH family members have been unsuccessful; only attempts at cloning specific subfamily members have met with any success (1, 35). The reasons for the success of this method are likely attributable to the choices of degenerate nucleotides, binding sites for the primers, and reaction conditions (i.e., salt concentration and temperature). In control experiments, we found the salt concentration to be a crucial parameter, as altered salt concentrations allowed these primers to amplify class A bHLH products as well.

Sequence analysis reveals that pBETA3 encodes a 367-amino-acid protein with a predicted molecular weight of ~40,000; however, the in vitro-translated product migrates at an apparent molecular weight of ~50,000 when subjected to SDS-PAGE. Alignment of the bHLH domain of BETA3 with other bHLH family members shows that it exhibits the highest degree of homology with the class B subgroup and contains a tyrosine residue within helix II that is absolutely conserved among class B family members; class A members contain a valine at this residue. BETA3 contains an intact basic region, as it has all of the conserved amino acid residues found in the other members of the superfamily (44) and does not contain proline residues, which are found in the basic region of some of the bHLH repressor proteins (49, 62).

Sequence analysis also revealed a 28% proline-rich region followed by 65 and 68% glycine-rich regions in the amino terminus. The functional significance of these sequences in BETA3 is unknown at this time, but proline- and glycine-rich regions have been shown to be required for the repressor activity of the homeobox gene product Msx-1; the Wilms' tumor suppressor gene product, WT-1; and the *Drosophila* proteins engrailed, even-skipped, and krüppel (9, 23, 24, 40, 41).

The tissue distribution reveals that the *BETA3* gene has a restricted pattern of expression, consistent with the expression pattern of genes in the class B subfamily. However, the expres-

sion of *BETA3* is not confined to a single tissue type; rather it is expressed in a select group of tissues, primarily the lung, kidney, and brain. It has not yet been determined if there is a common cell type expressing *BETA3* within these tissues.

Though BETA3 has the most homology with the insulin enhancer E box binding protein BETA2, BETA3 is not able to bind the insulin E box. Instead, it acts as a repressor by inhibiting the BETA2/Pan1 heterodimer's DNA binding and transcriptional activity. There are several possible mechanisms for this inhibition. One is that BETA3 is a non-DNA binding factor which forms a stable heterodimer with the class A bHLH factors. A second possible mechanism is that BETA3 may in fact bind other DNA elements and positively regulate transcription but is unable to bind the insulin E box. Both scenarios would allow BETA3 to effectively inhibit the DNA binding of BETA2 by competing for the class A molecules. Another possible mechanism for this inhibition is by affecting the dimer equilibrium of the bHLH molecules. Id has been shown to inhibit the MyoD/E47 dimer from binding DNA by driving MyoD to form tetrameric complexes which cannot bind DNA, thus reducing the amount of available DNA binding dimers (18). The results of the immunoprecipitation analysis indicate that BETA3 acts as a repressor, presumably by heterodimerizing with the class A bHLH factors. However, the precise in vivo function of BETA3 remains unclear.

The role of the dual expression of *BETA2* and *BETA3* in HIT and brain cells is not clear, though, presumably, the level of abundance of these genes is critical. The expression levels of the two genes have not been directly compared, but in general we have observed *BETA2* to be expressed at much higher levels than *BETA3* in HIT and brain cells, as *BETA2* is readily detectable in total RNA on Northern (RNA) blots while *BETA3* is not. Though BETA3 is a very strong repressor of BETA2 in transient-transfection assays, the endogenous levels of BETA2 in HIT cells is high enough to overcome this competition. Unfortunately, we have not been successful in comparing the expression levels of these two bHLH factors in pancreatic-islet cells, in which both are believed to be expressed. In addition, it would be interesting to compare the expression patterns of these two genes during development as it is conceivable that varying levels of BETA2 and BETA3 may be an important regulatory mechanism of the activity of BETA2 and thus neuronal differentiation and insulin gene expression. Interestingly, Lee et al. discovered a possible competition between *Xenopus* BETA2 (neuroD) and Xtwist (the *Xenopus* homolog of the *Drosophila twist* gene product). Though the nature of this competition is unknown, it defines the identity of the different cell types derived from the neural crest (39). It is intriguing that after BETA2, BETA3 is most homologous to the mouse *twist* gene product.

While BETA3 appears to be a bona fide class B member of the bHLH family, we have not been able to demonstrate its ability to bind DNA. In addition to trying the E box sequence, we have tried the noncanonical hairy binding site CACGCG as well as CACGAG, which is present in the *Drosophilia* E(spl) m8 protein binding site, but these attempts were to no avail (57). It is quite probable that BETA3 will be like some of the other various members of the bHLH superfamily that have distinct binding sites (8, 26, 29) and will require binding site selection to ascertain its favored DNA binding site. Also, one cannot rule out that BETA3 may require a novel class A bHLH partner for high-affinity binding to the insulin gene E box. Once the binding site is determined, BETA3 can then be assessed for activation or repression of transcriptional activity when it binds DNA.

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