THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 36, Issue of September 8, pp. 27989–27999, 2000

Printed in U.S.A.

Heterodimeric Pbx-Prep1 Homeodomain Protein Binding to the Glucagon Gene Restricting Transcription in a Cell Type-dependent Manner*

Received for publication, April 19, 2000, and in revised form, June 22, 2000
Published, JBC Papers in Press, June 26, 2000, DOI 10.1074/jbc.M003345200

Stephan Herzig‡, Laszlo Füzesi§, and Willhart Knepel‡¶

From the ‡Department of Molecular Pharmacology and §Department of Gastroenteropathology, University of Göttingen, 37075 Göttingen, Germany

Homeodomain proteins specify developmental pathways and cell-specific gene transcription whereby proteins of the PBC subclass can direct target gene specificity of Hox proteins. Proteins encoded by nonclustered homeobox genes have been shown to be essential for cell lineage differentiation and gene expression in pancreatic islets. Using specific antiserum in an electrophoretic mobility shift assay and *in vitro* transcribed/translated proteins, the nuclear proteins binding domain B of the G3 enhancer-like element of the glucagon gene were identified in the present study as heterodimers consisting of the ubiquitously expressed homeodomain protein Prep1 and the also widely expressed PBC homeoprotein Pbx (isoform 1a, 1b, or 2). These heterodimeric complexes were found to bind also to the glucagon cAMP response element and to a newly identified element termed G5 (from −169 to −140). Whereas the expression of Prep1 or Pbx forms alone had no effect, coexpression of Pbx1a/1b-Prep1 inhibited the glucagon promoter when activated by cotransfected Pax6 or another transcription factor in non-glucagon-producing cells. In contrast, in glucagon-producing pancreatic islet cells, Pbx-Prep1 had no effect on GAL4-Pax6-induced mutant glucagon promoter activity or on Pax6-dependent wild-type glucagon promoter activity. Furthermore, 5′-deletion of G5 enhanced glucagon promoter activity in a non-glucagon-producing cell line but not in glucagon-producing islet cells. This study thus identifies a novel target and Hox-independent function of Pbx-Prep1 heterodimers that, through repression of glucagon gene transcription in non-glucagon-producing cells, may help to establish islet cell-specific expression of the glucagon gene.

The homeodomain protein superfamily comprises a large number of sequence-specific transcription factors sharing a highly conserved DNA-binding homeodomain. Homeodomain proteins of the HOM/Hox gene clusters in *Drosophila* and vertebrates are critical regulators of cell fate and segment identity, differentiation, and cellular transformation during early development (1–3). Also, the nonclustered homeobox genes play critical roles in directing and maintaining cellular differentiation in many organs, including the pancreatic islets. The pancreatic islets of Langerhans are composed of four different cell types, α-, β-, δ-, and pancreatic polypeptide cells, which produce the hormones glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively (4, 5). Phenotypic analysis of null mutant mice bearing targeted disruptions of homeobox genes established an essential role for homeodomain factors in pancreas organogenesis, including the homeodomain proteins Pdx1, Hlxb9, and Nkx2.2, the LIM-homeodomain protein lsl-1, and the paired homeodomain proteins Pax4 and Pax6 (6–14). These proteins are also expressed in the adult pancreas, and it is becoming clear that most homeodomain proteins possess early functions involved in the differentiation of the various islet cell types as well as late functions involved in maintenance, function, and the optimal expression of hormone genes in mature islet cells (15, 16).

Glucagon is a functional antagonist of insulin. It raises blood glucose levels by stimulating glycogenolysis and gluconeogenesis (5). In diabetes mellitus, relative hyperglucagonemia contributes to hyperglycemia (17). The glucagon gene is expressed early in development at embryonic day 9.5 in the mouse, concomitantly with the appearance of the dorsal pancreatic bud (4). The α-cell specificity of glucagon gene expression is conferred by the 5′-flanking region of the glucagon gene (18–22) and depends on homeodomain protein binding to several regulatory DNA control elements (23). The glucagon G1 promoter element is bound by the POU homeodomain protein Brain-4 up-regulating glucagon gene transcription (24). Additional positively acting G1-binding homeodomain factors have been identified as lsl-1 (25) and the caudal-related homeodomain protein Cdx-2/3 (26–28). One of the major islet-specific glucagon enhancer elements, G3, comprises two distinct protein binding domains, A and B (29). The glucagon G3A-binding protein has been identified as Pax6 (30, 31). In addition to its role as G3-stimulating factor, Pax6 functionally synergizes with Cdx-2/3 on G1 (29, 32–34). Thus, homeodomain proteins binding to the glucagon gene seem to play a bivalent role as cell lineage-determining factors during embryonic islet cell differentiation and as transcriptional regulators critically involved in the maintenance of α-cell-specific cellular phenotype and glucagon gene transcription in the adult islet.

In this study, the nuclear proteins binding to domain B of the G3 enhancer-like element of the glucagon gene are molecularly identified as heterodimers consisting of Pbx1/2, which are members of the PBC family of homeodomain proteins, and Prep1, which is related to the *Drosophila* HTH and vertebrate Meis homeodomain proteins. Pbx-Prep1 act as transcriptional repressors of glucagon gene transcription in non-glucagon-producing cells but not in glucagon-expressing pancreatic islet cells. These proteins have previously been shown to form com-

* This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 402/A3. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Molecular Pharmacology, University of Göttingen, Robert-Koch-Str. 40, Postfach 3742, D-37070 Göttingen, Germany. Tel.: 49-551-395787; Fax: 49-551-399652; E-mail: wknepel@med.uni-goettingen.de.

plexes with various Hox proteins and thereby play important roles in regulating the specific activity of homeotic selector proteins (35). The results of the present study define a novel target and Hox-independent function of Pbx-Prep1 heterodimers that may help to establish α-cell-specific expression of the glucagon gene.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Plasmids 4xG3T81Luc (36), 4xG3AT81Luc, −136GluLuc (37), 4xG3BT81Luc (38), −350GluLuc (39), −200GluLuc, and −169GluLuc (40) have been described previously. Transcription factors encoding expression vector pBAT14-mPax6 (31), pCMV-HNF-3β (41), pSG5c-ets-1p68 (42), pSG5-Prep1, or pSG5-Pbx1b (43, 44) have also been described in previous reports. An expression vector encoding a GAL4-Pax6 fusion protein and plasmid −350(mutG1/G3)GluLuc has been described elsewhere.[1] To construct the plasmid −350(mutG3B)-GluLuc base pairs −240 to −245 of the glucagon promoter within −350GluLuc were replaced by an *Eco*RI restriction site using a commercial PCR kit (*Taq* PCR Core Kit, Qiagen, Hilden, Germany). Two fragments were obtained by using −350GluLuc as PCR template in combination with the following primer pairs (recognition sites of the indicated restriction enzyme are underlined): primer pair 1, upstream primer 5′-CGTAC<u>TCGAG</u>ATGGCCAAATAGCACATCAAGG-3′ (*Xho*I) and downstream primer 5′-CGG<u>GAATTC</u>TCTCAGTCAGGCGTGAAA-AAC-3′ (*Eco*RI); primer pair 2, upstream primer 5-CGG<u>GAATTC</u>GGT-GTATTTCAAACTACCCTT-3′ (*Eco*RI), downstream primer 5′-GT<u>AGA-TCT</u>AGACAGGTGGAGCTCCTTTGG-3′ (*Bgl*II). PCR was performed according to the manufacturer's instructions with an annealing temperature of 70 °C, respectively. PCR products were gel-purified, digested with the corresponding restriction enzymes, and cloned into the *Xho*I–*Bgl*II sites of pXP2 (45). Expression vector pBK-Pbx1a was constructed by inserting an *Eco*RI fragment of pSP65-Pbx1a (46) containing the entire Pbx1a cDNA (1819 bp)[2] into the *Eco*RI site of the pBK-CMV expression vector (Stratagene, Heidelberg, Germany). According to manufacturer's instructions, *lac* promoter sequences of pBK-CMV were excised by *Spe*I/*Nhe*I restriction enzyme digestion, and the linear vector was religated in order to get optimized eucaryotic expression of Pbx1a under control of a CMV promoter. All constructs were sequenced by the enzymatic method to confirm the identity and the orientation of the inserts.

Cell Culture and Transfection of DNA—JEG-3 choriocarcinoma cells (47) were grown in Dulbecco's modified Eagle's medium (4.5 g of glucose/liter) supplemented with 10% fetal calf serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml. Glucagon-producing pancreatic αTC2 cells (48) were grown in Dulbecco's modified Eagle's medium (4.5 g of glucose/liter) supplemented with 2.5% fetal calf serum, 15% horse serum, and antibiotics as described above. Glucagon-producing pancreatic InR1-G9 cells (49) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics as described above. JEG-3 cells were transfected by the calcium phosphate precipitation method (3 μg of indicator plasmid/6-cm dish), and αTC2 and InR1-G9 cells were transfected by the DEAE-dextran method (2 μg of indicator plasmid/6-cm dish). Where indicated, expression plasmid pBAT14-Pax6 (12 or 23 ng/6-cm dish), pGAL4-Pax6 (900 ng/6-cm dish), pCMV-HNF-3β (12 ng/6-cm dish), pSG5c-ets1p68 (750 ng/6-cm dish), pSG5-Prep1 (750 or 2250 ng/6-cm dish), pSG5-Pbx1b (1 μg/6-cm dish), or pBK-Pbx1a (23 or 75 ng/6-cm dish) was cotransfected. These cotransfections were done with a constant DNA concentration, which was maintained by adding Bluescript (Stratagene, Heidelberg, Germany). The transfection efficiency and possible unspecific squelching effects were checked by cotransfection of pGFPtpz-cmv[R] vector (Canberra-Packard, Dreieich, Germany) encoding a green fluorescent protein mutant (50 or 500 ng/6-cm dish). Cell extracts were prepared 48 h after transfection, and the luciferase assay was performed as described previously (39). Fluorescence signals were determined by using 50 μl of cell extract per well in a 96-well microplate (Greiner, Frickenhausen, Germany), which was analyzed in a Fluorocount fluorometer (Canberra-Packard) equipped with a 485-nm (excitation)/530-nm (emission) filter pair.

Nuclear Extracts—Nuclear extracts were prepared from αTC2 cells by the method of Dignam *et al.* (50) with the modification that after the chromatin extraction step an ammonium sulfate (0.3 g/ml) precipitation was included to concentrate proteins before dialysis.

In Vitro Transcription/Translation—For use in *in vitro* transcription/translation reactions, the following cDNA constructs under the control of the SP6 or T7 promoter were used: pSP65-Pbx1a, pSP64-Pbx2, pSP65-Pbx3a (46), pSG5-Prep1, pSG5-Pbx1b (43, 44). The cDNA expression vectors were transcribed and translated *in vitro* using the TNT coupled transcription-translation reticulocyte lysate system (Promega) according to the manufacturer's instructions, including SP6 or T7 polymerase and [^{35}S]methionine (Amersham Pharmacia Biotech). Plasmids were added either alone (1 μg of plasmid/reaction) or co-translated by mixing 750 ng of pSG5-Prep1 and 1000 ng of Pbx-encoding plasmids/reaction. Translation products were analyzed by SDS-polyacrylamide gel electrophoresis and visualized with a Fuji PhosphorImager.

Electrophoretic Mobility Shift Assay—Synthetic complementary oligonucleotides with 5′-GATC overhangs were annealed and labeled by a fill-in reaction with [α-^{32}P]dCTP (ICN, Eschwege, Germany) and Klenow enzyme. Nuclear extracts (20 μg of protein) or 2 μl of reticulocyte lysate were incubated with 2 μg of poly(dI-dC) as nonspecific competitor and with specific competitors, if indicated, in 20 μl (total volume) of 20 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM dithiothreitol, 140 mM KCl, and 10% glycerol for 10 min on ice. The assay was then performed as described (29). In some binding reactions, specific antibodies to various homeodomain proteins were used. Nuclear extracts were incubated with 2 μl of antibody in 20 μl (total volume) of 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol for 30 min at room temperature. Following a 10-min incubation on ice with 2 μg of poly(dI-dC), the labeled oligodeoxynucleotides were added, and the assay was then performed as described (29).

Oligonucleotides—The sequences of the G3, G3B, TTR-HNF3, TTR-HNF4, and glucagon cAMP-responsive element (GluCRE) oligonucleotides used as probes or competitors in electrophoretic mobility shift assays have been described before (36, 51). The sequences of the Pbx-HoxCon oligonucleotide containing the Pbx-responsive sequence (52) and of the 136/169 oligonucleotide-containing glucagon gene 5′-flanking sequences from bp −133 to −170 were as follows (only one strand with the 5′-GATC overhang is shown): 5′-GATCCCGCATCAATCAATTTC-GA-3′ (PbxHoxCon) and 5′-GATCCTCTGAGGTCTCACCCCGGTATC-AGCGTGAGGAGCAGA-3′ (136/169).

Antibodies—Homeodomain protein-specific antibodies against Pax6 (αPax6) (53), Meis1 (αMeis1) (54), Cdx-2 (αCdx-2) (55), and Engrailed-1/2 (αEnhb-1) (56) have been described previously. An anti-glucagon antibody was obtained from Biotrend Chemikalien (Cologne, Germany). Antibodies against Prep1 and Pbx proteins were obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). The antibodies αPbx1, αPbx2, and αPbx3 are directed against isoform-specific N-terminal parts of the corresponding protein; αPbx1 and αPbx3 thereby simultaneously recognize the long and short splice variants 1a/1b and 3a/3b, respectively. αPbx1/2/3 specifically detects a C-terminal peptide common to long Pbx1a, Pbx2, and Pbx3a isoforms.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)—Poly(A)$^+$ RNA was extracted from αTC2 cells using a commercial kit (Fast Track 2.0™; Invitrogen). The quality of the RNA was assessed by electrophoresis in a 1% formaldehyde-agarose gel. RT-PCR was performed using a rTth DNA polymerase-including commercial kit (Gene Amp™ Thermostable rTth Reverse Transcriptase RNA PCR Kit, Roche Molecular Systems) with primers as follows: primer pair Prep1, upstream primer 5′-AAGATCTCAGCATCTTGC-3′ and downstream primer 5′-TGTTGACTTGGAGTAGTGTC-3′ (size of the expected product: 183 bp); primer pair Pbx1, upstream primer 5′-CACTGCTAC-CAATGTGTC-3′ and downstream primer 5′-TCCATCACTGTATC-CTCC-3′ (size of the expected products: 229 bp (Pbx1a) and 116 bp (Pbx1b)); primer pair Pbx2, upstream primer 5′-GCCACAGCCGCAC-CAGCTCC-3′ and downstream primer 5′-CCTTAGAGGCCCCATTCT-TCC-3′ (size of the expected product: 503 bp) (46); primer pair Pbx3, upstream primer 5′-GCACACGCAGTAGCAGCAGC-3′ and downstream primer 5′-TGTGGCCAGAGATTAGTTAG-3′ (size of the expected products: 381 bp (Pbx3a) and 269 bp (Pbx3b)) (46). The PCRs were conducted as follows: 1 min at 95 °C, 1 min at 55 °C, and 1.5 min at 72 °C for 35 cycles. Control for DNA contamination was performed by omitting the reverse transcriptase step in parallel samples. After agarose gel electrophoresis, the products obtained were verified by extraction, subcloning (pCR® 2.1, Invitrogen), and cycle sequencing (Thermo sequenase fluorescent labeled primer cycle sequencing kit, Amersham Pharmacia Biotech; IRD-800 labeled primers, MWG Biotech (Ebersberg, Germany)).

[1] Grzeskowiak, R., Amin, J., Oetjen, E., and Knepel, W. (2000) *J. Biol. Chem.*, in press.

[2] The abbreviations used are: CRE, cAMP-response element; CMV, cytomegalovirus; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; HNF, hepatocyte nuclear factor.

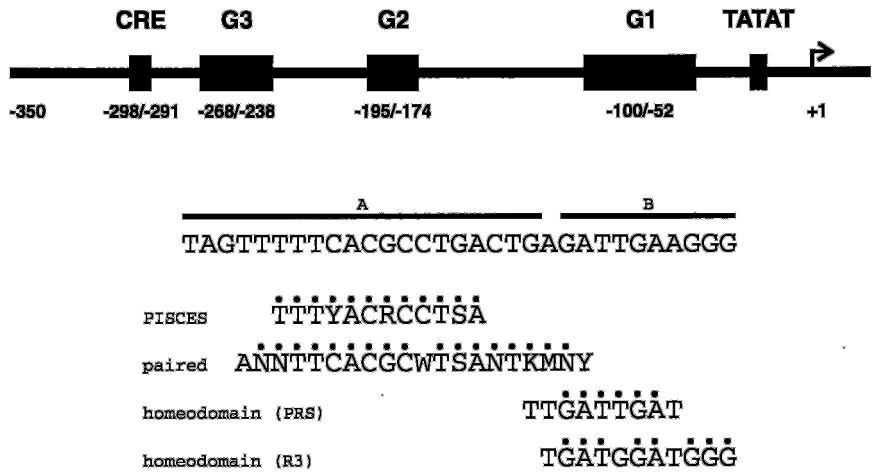

FIG. 1. **DNA sequences within the rat glucagon gene 5′-flanking region display similarities with consensus binding sites for three families of interacting homeodomain proteins, the Hox, PBC, and HTH/Meis/Prep1 families of homeodomain proteins.** *Upper panel*, control elements in the 5′-flanking region of the glucagon gene. *+1* indicates the transcription start site. Relative positions of control elements are indicated by bp numbers with regard to the transcription start site. *Lower panel*, sequence of the G3 enhancer-like element (from bp −238 to −268), which contains two distinct protein binding domains, A and B (29). Domain A of G3 contains a sequence motif, PISCES (pancreatic islet cell-specific enhancer sequence), that is also found in the G1 element as well as in the rat somatostatin and insulin I genes (29, 33); it defines a subclass of binding sites of the transcription factor Pax6 (30, 31). The PISCES consensus sequence (33) is shown together with the consensus binding site of the Pax6 paired domain (paired) (100). The sequence of domain B of G3 is similar to known binding sites of distinct members of the homeodomain family of transcription factors: homeodomain (*PRS*), Pbx-responsive sequence (52); homeodomain (R3), sequence of the third repeat of a Pbx-Hoxb1 binding site in the autoregulatory b1-ARE element of the Hoxb1 gene (102). *Dots* (●) indicate sequence identities. *CRE*, octameric core motif of the CRE; *G1*, glucagon element 1; *G2*, glucagon element 2; *G3*, glucagon element 3.

Immunohistochemistry—Healthy tissue of a partially inflamed human pancreas was formalin-fixed, paraffin-embedded, and cut into 2-μm-thick sections. Prior to incubation with primary antibodies, sections to be stained were placed in 10 mM citrate buffer at pH 6.0 and irradiated by microwaves at 750 W for 15 min. Every 5 min, evaporated citrate buffer was replaced by fresh buffer. Subsequently, sections were incubated for 30 min at room temperature with antibodies against glucagon (α-glucagon), Prep1 (αPrep1), or Pbx1a, -2, and -3a (αPbx1/2/3) at a dilution of 1:10. Unbound primary antibodies were then rinsed off with Tris, pH 7.4, before being stained with a commercial ChemMate kit (Dako, Hamburg, Germany). The slides were then rinsed with water and counterstained with hematoxylin for 2 min at room temperature. Finally, slides were rinsed again with water and coverslipped. For a negative control, incubation with the primary antibody was omitted.

RESULTS

Nuclear Proteins Binding to Domain B of the G3 Enhancer-like Element Are Recognized by Antiserum Directed against Pbx and Prep1 Homeodomain Proteins—G3B-binding proteins (G3B-BP) have been characterized in nuclear extracts from various islet cell lines as well as non-islet cells. In the electrophoretic mobility shift assay, G3B-BPs typically form two protein complexes with slightly different mobilities (29, 33). Their binding to a labeled G3B probe is competed by an oligonucleotide containing a winged helix protein binding site from the transthyretin promoter (TTR-HNF3) (36). Using mRNA from pancreatic islet cells (αTC2, InR1-G9, HIT, or mouse islets) and a pair of degenerate primers targeting highly conserved sequences within the winged helix DNA binding domain (57), cDNAs encoding the members of the winged helix family of transcription factors HNF-3β, HNF-3γ, ILF, MNF, and FKH4 were obtained by RT-PCR. After expression in bacteria, these proteins did not bind G3B in the electrophoretic mobility shift assay (data not shown), suggesting that the nuclear G3B-BPs may belong to a different class of DNA-binding proteins. Sequence comparison reveals that the sequence of domain B of the glucagon G3 element is similar to known DNA-binding sites of three families of interacting homeodomain proteins, the Hox, PBC, and HTH/Meis/Prep1 families of homeoproteins (Fig. 1) (35). As shown in Fig. 2*A*, an oligonucleotide (PbxHoxCon), containing a consensus binding site (Pbx-responsive sequence) for Pbx-Hox or Pbx-Prep1 heterodimers (52), competed for the binding of G3B-BPs to labeled G3B in the electrophoretic mobility shift assay. It did so with higher potency than TTR-HNF3 (Fig. 2*A*). To test more directly whether nuclear G3B-BPs contain homeodomain proteins, specific antibodies were used. The Pbx proteins are members of the PBC family of homeodomain proteins and comprise three highly related proteins, Pbx1, -2, and -3, that are encoded by distinct genes. Two forms of Pbx1 and Pbx3, designated a and b, result from differences in splicing in the 3′-portion of their transcripts (46, 58, 59). The alternative b forms of Pbx1 and Pbx3 are C-terminally truncated and thus shorter (about 40 kDa) than Pbx1a, Pbx2, and Pbx3a (all about 50 kDa) (46). As shown in Fig. 2*B*, the addition of a Pbx-specific antibody (αPbx1/2/3), which recognizes the 50-kDa Pbx isoforms 1a, 2, and 3a, completely disrupted the binding of the slower migrating G3B-BP complex, whereas the faster migrating complex remained unaffected. This suggests the presence of a long Pbx form within the slower G3B-BP complex (Fig. 2*B*, *lane 3*). In contrast, the binding of nuclear G3B-BPs was not affected by antiserum directed against the homeodomain protein Cdx2 (60) or the G3A-binding protein Pax6, which in addition to a paired domain also contains a homeodomain (61) (Fig. 2*B*, *lanes 2* and *7*). The mammalian Pbx proteins and their homologue EXD from *Drosophila* have been shown to bind certain DNA elements cooperatively with other homeodomain proteins including Engrailed-2 (62–64), Meis1, or Prep1 in mammals (43, 53, 65, 66) and EN or HTH in *Drosophila* (67, 68). This prompted us to test whether one of these proteins may be contained in the nuclear G3B-BP complexes. Whereas preincubation of binding reactions with antibodies against Meis1 or Engrailed-1/2 (αMeis1, αEnhb1) had no effect on the binding of G3B-BP (Fig. 2*B*, *lanes 5* and *6*), the addition of Prep1 antibodies completely abolished the binding of the slower migrating G3B-BP complex and strongly reduced the intensity of the faster migrating G3B-BP complex (Fig. 2*B*, *lane 4*). These results indicate that Prep1 proteins are present in both G3B-binding complexes and (in the case of the slower migrating complex) combine with a long Pbx variant to form binding activity on G3B.

To further define the Pbx isoforms binding to G3B, addi-

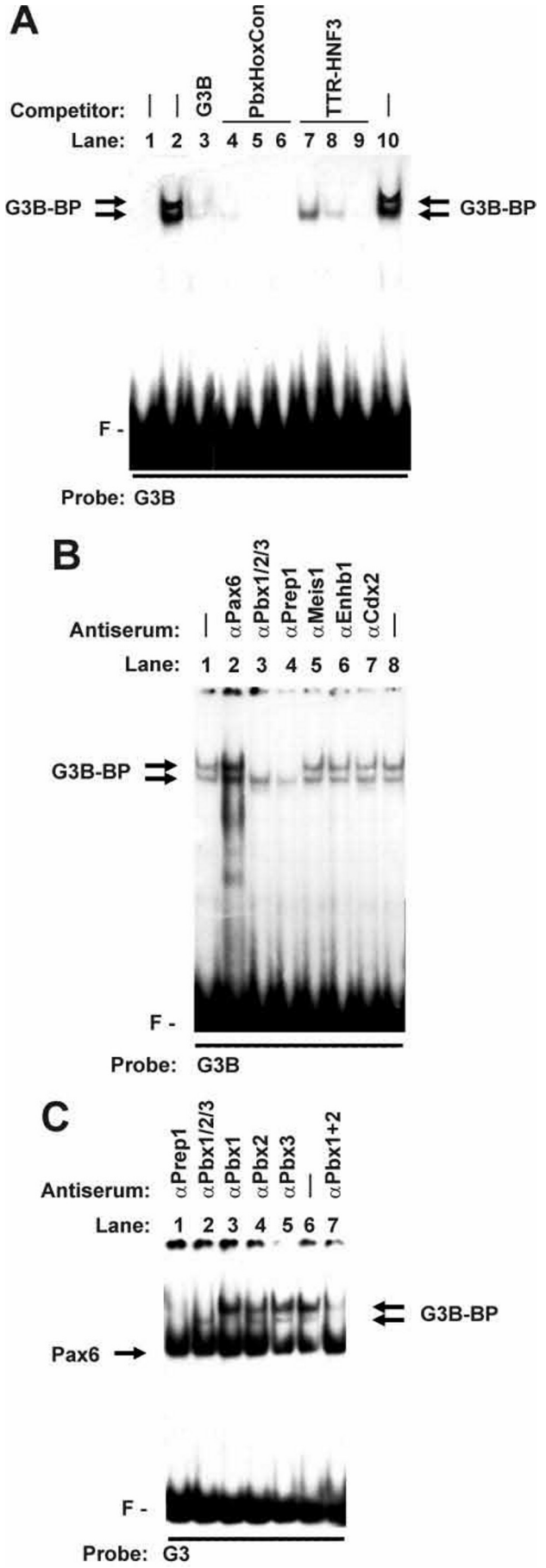

FIG. 2. **Domain B of the glucagon G3 enhancer element is recognized by Pbx and Prep1 homeodomain proteins as revealed by the electrophoretic mobility shift assay.** *A*, competition experiments. Labeled G3B oligonucleotide was incubated with nuclear extracts from the glucagon-producing pancreatic islet cell line αTC2. Protein complexes formed specifically with labeled G3B are marked as G3B-BP. Indicated competitors were added at a 50-fold (*lanes 4* and *7*), 250-fold (*lanes 3*, *5*, and *8*), and 1250-fold (*lanes 6* and *9*) molar excess, respectively. *F*, free probe. *Lane 1*, no nuclear extract. *B*, G3B-binding protein complexes are specifically detected by antibodies directed against long isoforms of homeodomain proteins Pbx and by antibodies directed against the homeodomain protein Prep1. Labeled G3B oligonucleotide was incubated with nuclear extracts from αTC2 cells. Protein complexes formed specifically with labeled G3B are marked as *G3B-BP*. The indicated antibodies were directed against Pax6 paired domain (αPax6), long Pbx isoforms 1a, 2, and 3a (αPbx1/2/3), Prep1 (αPrep), Meis1 (αMeis1), Engrailed-1/2 (αEnhb1), and Cdx-2 (αCdx2), respectively, and were incubated with nuclear extracts prior to the addition of the labeled G3B probe. *F*, free probe. *C*, Prep1 forms slower migrating complexes with Pbx long isoforms 1a and 2 and a faster migrating complex with Pbx isoform 1b. Labeled G3 oligonucleotide was incubated with nuclear extracts from αTC2 cells. Bands representing protein binding to domain B are marked as G3B-BP. Pax6 binding to domain A is indicated as Pax6. Specific antibodies were directed against Prep1 (αPrep1), long Pbx isoforms 1a, 2, and 3a (αPbx1/2/3), Pbx isoform 1 (long splice variant 1a and short splice variant 1b; αPbx1), Pbx isoform 2 (αPbx2), and Pbx isoform 3 (long splice variant 3a and short splice variant 3b; αPbx3), respectively, and were incubated with nuclear extracts prior to the addition of the labeled G3 probe. The binding reaction in lane 7 contained αPbx1 and αPbx2 antibodies. *F*, free probe.

tional gel shift experiments were performed using antibodies that are specifically directed against N-terminal parts of Pbx1, -2, or -3 (αPbx1, αPbx2, or αPbx3, respectively). Thus, αPbx1 and αPbx3 recognize both the long and short splice variants Pbx1a/1b and Pbx3a/3b, respectively. When an oligonucleotide (G3) containing domain A and B of the G3 element (Fig. 1) was used as probe, three retarded bands were detected (Fig. 2*C*, *lane 6*). The complex in addition to the G3B-BPs represents the binding of Pax6 through its paired domain to domain A of G3 (15, 29, 30) as confirmed by the addition of a Pax6 antiserum (data not shown). Both G3B-BP complexes were disrupted by Prep1-specific antibodies (Fig. 2*C*, *lane 1*), whereas antibodies specifically detecting the long Pbx forms interfered only with the slower migrating G3B-BP complex (αPx1/2/3; Fig. 2*C*, *lane 2*), thus confirming the results obtained by using G3B as probe (Fig. 2*B*). Formation of the faster migrating G3B-BP complex was prevented by αPbx1 directed against Pbx1a and -1b, suggesting the presence of Pbx1b within this complex (Fig. 2*C*, *lane 3*). On the other hand, antibodies against Pbx2 (αPbx2) markedly reduced the intensity of the slower complex but did not affect the faster one (Fig. 2*C*, *lane 4*). Although αPbx1 alone did not inhibit the binding of the slower migrating G3B-BP complex (Fig. 2*C*, *lane 3*), αPbx1 decreased the intensity of this complex when added in addition to αPbx2 (Fig. 2*C*, compare *lanes 7* and *4*), suggesting that the slower migrating G3B-BP complex may contain also some Pbx1a protein in addition to Pbx2. Antibodies against Pbx3a and 3b (αPbx3) had no effect on the binding of G3B-BP (Fig. 2*C*, *lane 5*). None of the Prep1 or Pbx antibodies affected the binding of Pax6 (Fig. 2*C*), demonstrating their specificity. Taken together, these experiments suggest that the nuclear G3B-BPs are heterodimers consisting of the homeodomain proteins Prep1 and Pbx1a/2 (slower migrating complex) or Pbx1b (faster migrating complex).

In Vitro Transcribed and Translated Pbx-Prep1 Heterodimers Bind G3B and Comigrate with Nuclear G3B-BP—To further support this conclusion, Prep1 and Pbx proteins were transcribed and translated *in vitro* using a reticulocyte lysate system, and their binding to G3B was investigated in electrophoretic mobility shift assays. As shown in Fig. 3*A*, the translated proteins migrated on an SDS gel as expected. When added alone, neither Prep1 nor any of the Pbx proteins bound to labeled G3 or labeled PbxHoxCon (Fig. 3*B*, *lanes 12–21*). In

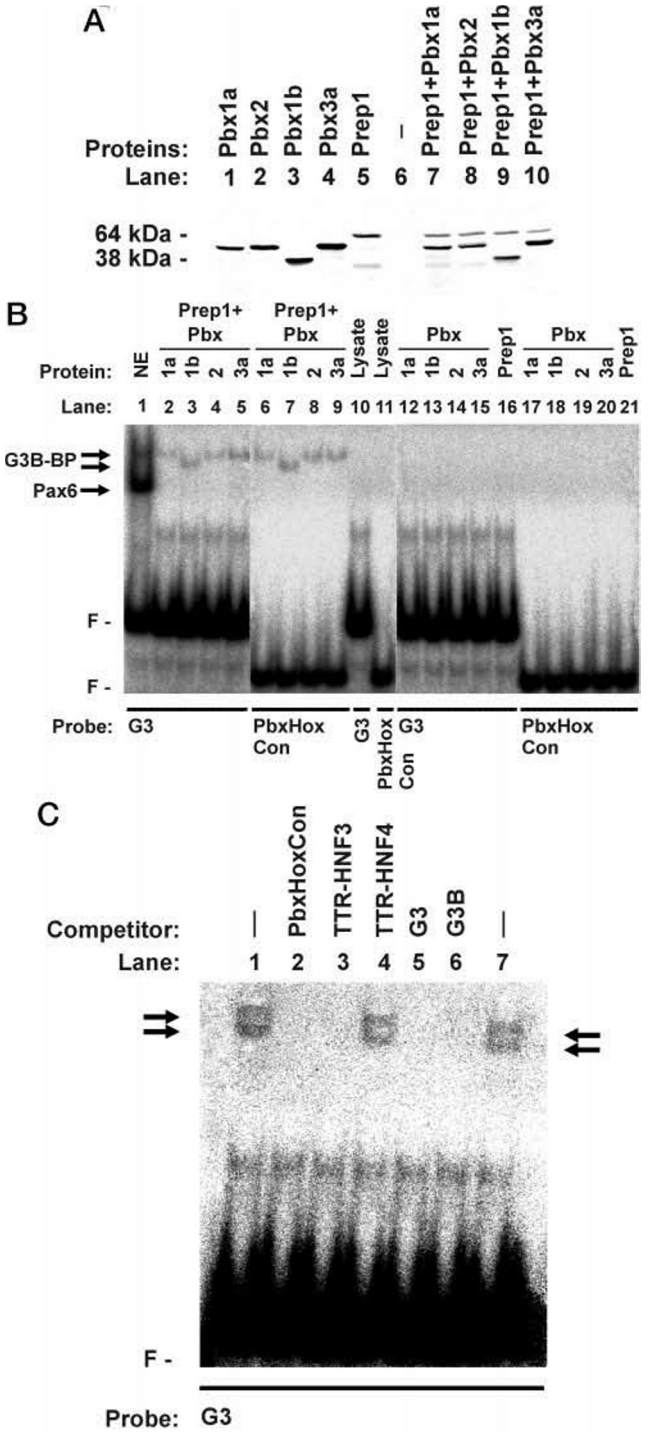

FIG. 3. **Binding to G3B of *in vitro* transcribed and translated Pbx and Prep1 proteins in electrophoretic mobility shift assays.** *A*, analysis of *in vitro* translated Pbx and Prep1 proteins by SDS-polyacrylamide gel electrophoresis. Pbx isoforms 1a, 2, 1b, and 3a and Prep1 were either translated separately (*lanes 1–5*) or co-translated by mixing expression plasmids encoding Prep1 and the indicated Pbx protein in the same translation reaction using reticulocyte lysate (*lanes 7–10*). A degradation product is visible in Prep1-containing reactions as observed previously (*lanes 5* and *7–10*) (65). *Lane 6* shows a control reaction containing only lysate with no expression plasmid added. *B*, binding to G3 requires both heterodimerization partners, Pbx and Prep1. The labeled oligonucleotides G3 or PbxHoxCon (containing a consensus binding site (PRS) for Pbx-Hox or Pbx-Prep1 heterodimers) were incubated with *in vitro* translation reactions containing either co-translated Pbx-Prep1 proteins (*lanes 2–9*) or Pbx proteins or Prep1 alone (*lanes 12–21*). Translation reactions containing only lysate were incubated with G3 or PbxHoxCon as specificity controls (*lanes 10* and *11*). *Lane 1* shows a binding reaction containing nuclear extracts from αTC2 cells incubated with labeled G3. Bands representing nuclear protein binding to G3B are marked as *G3B-BP*. Pax6 binding to G3A is indicated as Pax6. *F*, free probe; *NE*, αTC2 nuclear extract. *C,* competition experiments. *In vitro* co-translated Pbx2-Prep1 and Pbx1b-Prep1 were incubated with labeled G3 oligonucleotide. Protein complexes representing Pbx-Prep1 binding are indicated by *arrows*. The indicated competitors were added at a 500-fold molar excess. *F*, free probe.

contrast, *in vitro* cotranslated Pbx1a-Prep1, Pbx1b-Prep1, Pbx2-Prep1, and Pbx3a-Prep1 gave rise to specific retarded complexes on labeled G3 (Fig. 3*B*, *lanes 2–5*) as well as on labeled PbxHoxCon (Fig. 3*B*, *lanes 6–9*), indicating that Pbx-Prep1 heterodimers can bind to G3. Noteworthy, Pbx1a-Prep1 and Pbx2-Prep1 complexes on labeled G3 comigrated with the slower migrating nuclear G3B-BP complex, whereas the Pbx1b-Prep1 complex on G3 comigrated with the faster migrating nuclear G3B-BP complex (Fig. 3*B*, compare *lanes 2–4* with *lane 1*). Furthermore, the binding of *in vitro* transcribed and translated Pbx2-Prep1 and Pbx1b-Prep1 heterodimers to labeled G3 (Fig. 3*C*, *lanes 1* and *7*) was competed for by unlabeled G3, G3B, TTR-HNF3, and PbxHoxCon but not by an unrelated oligonucleotide (TTR-HNF4) (Fig. 3*C*, *lanes 2–6*), consistent with the DNA binding specificity of the nuclear G3B-binding proteins (Fig. 2*A*). When taken together, these results support the conclusion that the nuclear G3B-BP complexes consist of Pbx1a/1b/2-Prep1 heterodimers and may contain no additional proteins.

Pbx-Prep1 Expression as Revealed by RT-PCR in a Glucagon-producing Pancreatic Islet Cell Line and by Immunohistochemistry in Normal Human Pancreatic Islets—The use of antiserum in the electrophoretic mobility shift assay indicated (Fig. 2, *B* and *C*) that Prep1, Pbx1a, Pbx1b, and Pbx2 are expressed in the glucagon-producing pancreatic islet cell line αTC2 and bind as Pbx-Prep1 heterodimers to the glucagon G3 element. To examine the expression of Prep1 and Pbx isoforms in these cells independently of antiserum, an RT-PCR analysis was performed. Primer pairs specific for Prep1, Pbx1, Pbx2, and Pbx3 were used. The primer pairs for Pbx1 and Pbx3 amplified fragments that include or exclude the alternatively spliced exon, depending on the splice variants a and b (46). As shown in Fig. 4, fragments of the expected size were obtained for Prep1, Pbx1a, Pbx1b, and Pbx2 but not for Pbx3. The cDNA fragments were verified by subcloning and sequencing (not shown). No cDNA fragments were obtained when in parallel control reactions the reverse transcription step was omitted (Fig. 4). Thus, these results confirm at the mRNA level the expression of Prep1 and the Pbx isoforms 1a, 1b, and 2 in these glucagon-producing pancreatic islet cells and thereby support their identification as glucagon G3B-binding proteins.

To examine Pbx-Prep1 expression in human pancreatic islets, consecutive sections of normal human pancreatic tissue were immunohistochemically stained with antibodies detecting glucagon, Prep1, or Pbx (recognizing the isoforms 1a, 2, and 3a; αPbx1/2/3). In controls, no primary antibodies were used. As shown in Fig. 5, specific staining for Prep1 and Pbx was found in cells throughout the pancreatic islets. Glucagon immunoreactivity was localized to discrete cells within the islets (Fig. 5). While the staining for Prep1 and Pbx in islets was specific, the subcellular localization of Pbx-Prep1 within islet cells and their expression in the exocrine pancreas remain unclear due to technical limitations when collecting pancreatic tissue from human donors. Taken together, these results demonstrate that the G3B-binding proteins Pbx-Prep1 are expressed in normal human pancreatic islets most likely in glucagon-producing but clearly also in non-glucagon-producing cells.

Pbx-Prep1 Heterodimers Can Inhibit Glucagon Gene Tran-

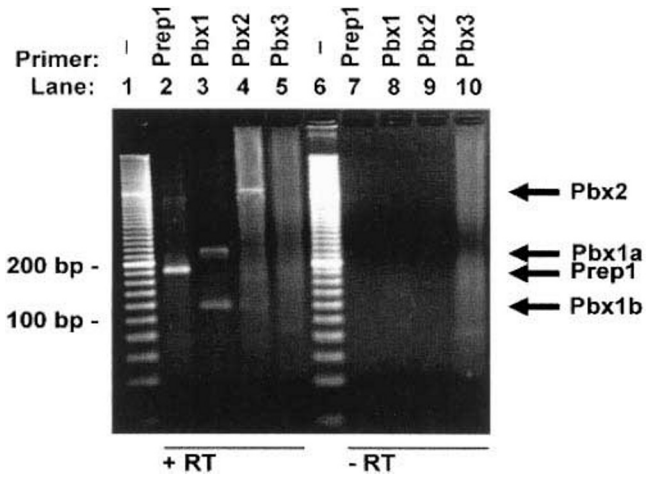

FIG. 4. **Prep1 and Pbx isoforms 1a, 1b, and 2 are expressed in the glucagon-producing pancreatic islet cell line αTC2 as revealed by RT-PCR.** Poly(A)$^+$ RNA from αTC2 cells was used with primer pairs specific for Prep1 or Pbx 1, 2, or 3. Specific RT-PCR products with the expected size for Prep1, Pbx1a/1b, and Pbx2 are indicated by *arrows*. The RT-PCR products were verified by subcloning and sequencing. *200 bp/100 bp*, sizes of the corresponding molecular weight marker (*lanes 1* and *6*); + *RT*, reactions were performed with a reverse transcriptase step (*lanes 2–5*); −RT, reverse transcriptase step was omitted (*lanes 7–10*).

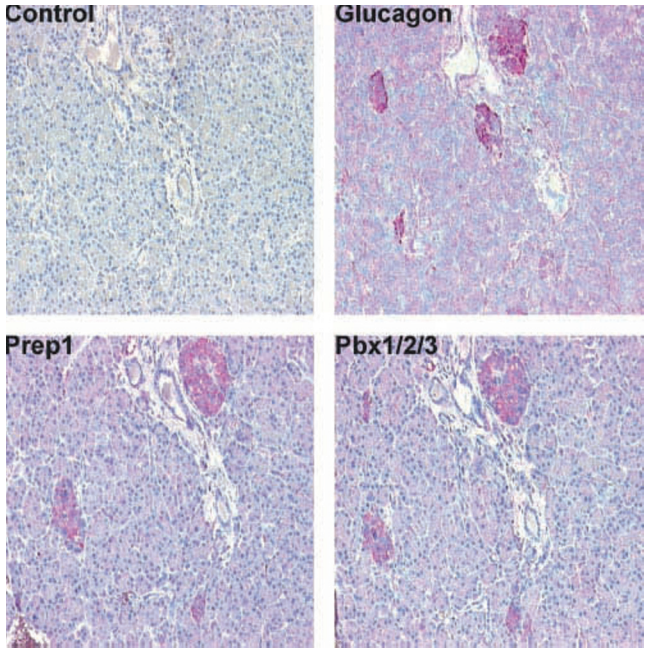

FIG. 5. **Pbx and Prep1 are expressed in normal human pancreatic islets as demonstrated by immunohistochemistry.** Parallel sections of formalin-fixed and paraffin-embedded human pancreatic tissue were incubated with primary antibodies directed against glucagon (*upper right panel*), Prep1 (*lower left panel*), or long Pbx isoforms 1a, 2, and 3a (*lower right panel*). Binding of primary antibody was detected with Fast Red color (Dako, Germany). Sections were counterstained with hematoxylin. *Control*, incubation with primary antibody was omitted (*upper left panel*). Magnification was × 80.

scription—To study the transcriptional activity conferred by Pbx-Prep1 heterodimers binding to glucagon gene sequences, four copies of an oligonucleotide containing domain B of the G3 enhancer-like element were placed in front of the truncated thymidine kinase promoter (from −81 to +52) of herpes simplex virus linked to the luciferase reporter gene (construct 4xG3BT81Luc). After transfection into the non-glucagon-producing choriocarcinoma cell line JEG-3, G3B did not confer transcriptional activity to the promoter, nor did overexpression

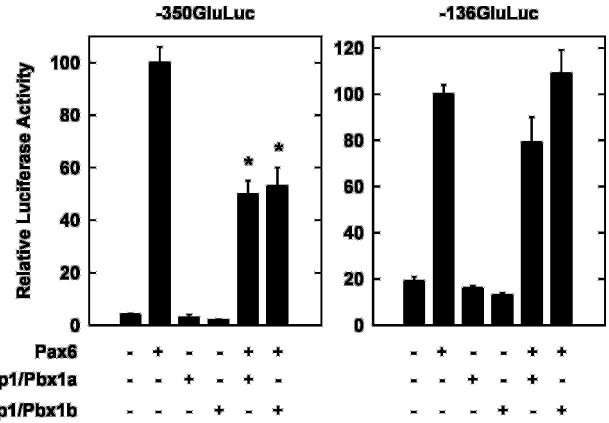

FIG. 6. **Pbx-Prep1 inhibit glucagon gene transcription.** Plasmids −350GluLuc or −136GluLuc were transfected together with expression plasmids pBAT14-mPax6, pSG5-Prep1, pBK-Pbx1a, and pSG5-Pbx1b, as indicated, into the nonpancreatic choriocarcinoma cell line JEG-3. Luciferase activity is expressed relative to Pax6-induced reporter gene activity. Values are means ± S.E. of three independent experiments, each done in duplicate. *, $p < 0.05$ compared with Pax6-induced reporter gene activity (one-way analysis of variance, Bonferroni's method).

of Pbx1a plus Prep1 stimulate G3B activity (not shown). To study the effect of Pbx-Prep1 on glucagon promoter activity, the transcription start site and part of the 5′-flanking region of the rat glucagon gene from −350 to +58 were fused with the luciferase reporter gene (construct −350GluLuc). These glucagon gene sequences have been shown to confer islet cell-specific gene expression in cell lines (22). Multiple transcription factors, whose expression is not restricted to glucagon-producing pancreatic islet α-cells, are known to bind to the glucagon promoter including Pax6, which binds to a sequence motif (PISCES) within domain A of the G3 element and also within the G1 element (Refs. 23, 29–31, and 33; see also Fig. 1). After transfection into JEG-3 cells, glucagon promoter activity was stimulated 25-fold by cotransfection of an expression vector encoding Pax6 (Fig. 6, *left panel*). The overexpression of only Prep1 or Pbx1b had no effect on −350GluLuc activity in the absence or presence of Pax6 (not shown). However, when Prep1 and Pbx1b were expressed together, they inhibited basal and Pax6-dependent glucagon promoter activity to about 50% of controls (Fig. 6, *left panel*). The coexpression of Prep1 with Pbx1a was similarly effective (Fig. 6, *left panel*). In contrast, Pbx1b-Prep1 and Pbx1a-Prep1 had no effect on Pax6-dependent activity of the truncated glucagon promoter (−136GluLuc) (Fig. 6, *right panel*). These results indicate that Pbx-Prep1 heterodimers can inhibit glucagon promoter activity.

Pbx-Prep1 Heterodimers Inhibit Glucagon Gene Transcription through Domain B of the G3 Element as Well as through Additional Elements—Four copies of oligonucleotides containing the G3 element or domain A of the G3 element were placed in front of the truncated thymidine kinase promoter linked to the luciferase reporter gene (constructs 4xG3T81Luc and 4xG3AT81Luc, respectively). The activity of both constructs was stimulated more than 10-fold by cotransfection of Pax6 in JEG-3 cells (Fig. 7*A*). The expression of Prep1 plus Pbx1a or Prep1 plus Pbx1b inhibited Pax6-dependent G3 activity by about 25% (Fig. 7*A*, *left panel*), whereas G3A activity remained unaffected (Fig. 7*A*, *right panel*), indicating that Pbx-Prep1 heterodimers can inhibit transcription through domain B of the G3 element. To study the role of domain B of the G3 element in the inhibition by Pbx-Prep1 of Pax6-dependent glucagon promoter activity, 6 bp of domain B of the G3 element were mutated inside the glucagon promoter (from TTGAAG into GAATTC; see Fig. 1), yielding the construct −350(mutG3B)-

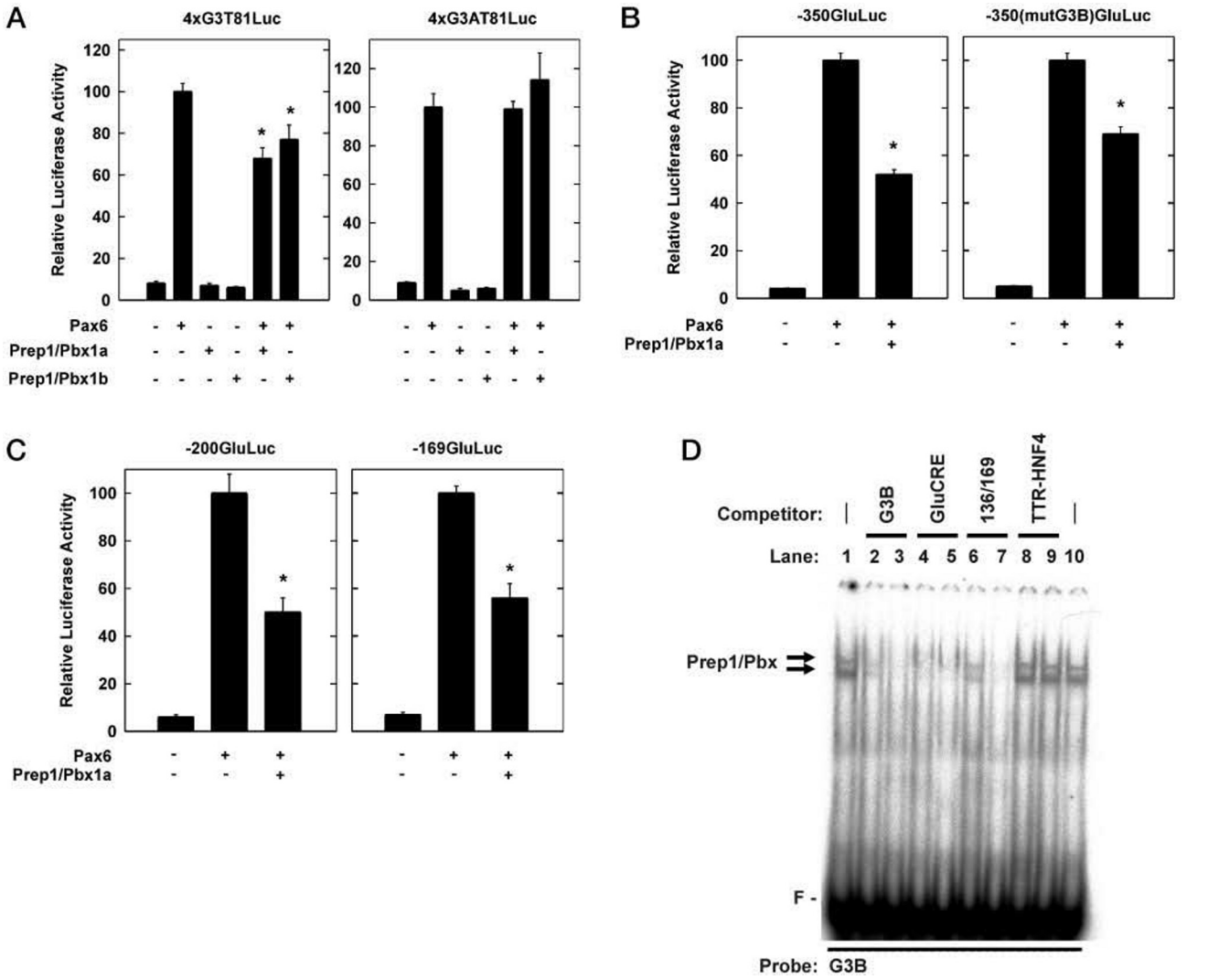

FIG. 7. **Pbx-Prep1 inhibit glucagon gene transcription through domain B of G3 and through additional elements of the glucagon gene.** *A*, Pbx-Prep1 negatively regulate Pax6-mediated G3 but not G3A multimerized minienhancer transcriptional activity in JEG-3 cells. Plasmid 4xG3T81Luc or 4xG3AT81Luc was transfected together with expression plasmids pBAT14-mPax6, pSG5-Prep1, pBK-Pbx1a, and pSG5-Pbx1b, as indicated, into JEG-3 cells. Luciferase activity is expressed relative to Pax6-induced reporter gene activity. Values are means ± S.E. of three independent experiments, each done in duplicate. *, $p < 0.05$ compared with Pax6-induced reporter gene activity (one-way analysis of variance, Bonferroni's method). *B*, mutation of domain B of G3 diminishes inhibition of glucagon gene transcription by Pbx-Prep1. Plasmid −350GluLuc or −350(mutG3B)GluLuc was transfected together with expression plasmids pBAT14-mPax6, pSG5-Prep1, and pBK-Pbx1a, as indicated, into JEG-3 cells. Luciferase activity is expressed relative to Pax6-induced reporter gene activity. Values are means ± S.E. of three independent experiments, each done in duplicate. *, $p < 0.05$ compared with Pax6-induced reporter gene activity (one-way analysis of variance, Bonferroni's method). *C*, Pbx-Prep1 can repress glucagon gene transcription through DNA sequences between −169 and −136 of the glucagon gene 5′-flanking region. Plasmid −200GluLuc or −169GluLuc was transfected together with expression plasmids pBAT14-mPax6, pSG5-Prep1, and pBK-Pbx1a, as indicated, into JEG-3 cells. Luciferase activity is expressed relative to Pax6-induced reporter gene activity. Values are means ± S.E. of three independent experiments, each done in duplicate. *, $p < 0.05$ compared with Pax6-induced reporter gene activity (one-way analysis of variance, Bonferroni's method). *D*, Pbx-Prep1 binding to the −136/−169 fragment of the glucagon gene 5′-flanking region and the GluCRE as revealed by the electrophoretic mobility shift assay. In competition experiments, labeled G3B oligonucleotide was incubated with nuclear extracts from αTC2 cells. Protein complexes representing Pbx-Prep1 binding are marked with *arrows*. Indicated competitors were added at a 50-fold (*lanes 2*, *4*, *6*, and *8*) and 500-fold (*lanes 3*, *5*, *7*, and *9*) molar excess. *F*, free probe.

GluLuc. These bp have been shown before to be essential for G3B binding (29). As shown in Fig. 7*B*, the inhibition by Pbx1a-Prep1 of Pax6-induced glucagon promoter activity was reduced but not abolished by mutating G3B (inhibition to 69 ± 3% as compared with inhibition to 52 ± 2% in the wild type). This suggests that, although domain B of the G3 element mediates part of the inhibition, other elements may also be involved.

To functionally characterize additional Pbx-Prep1-responsive elements in the glucagon gene 5′-flanking region, 5′-deleted glucagon promoter constructs were used. As shown in Fig. 7*C*, the expression of Pbx1a and Prep1 inhibited Pax6-dependent glucagon promoter activity by about 50% when the glucagon promoter was deleted from its 5′-end to −200 or −169. Since −136GluLuc was not inhibited (Fig. 6), these results suggest that an additional Pbx-Prep1-responsive element may be located between −169 and −136. To test whether this Pbx-Prep1 response may include binding of Pbx-Prep1 to the −169/−136 fragment, electrophoretic mobility shift assays were performed. As shown in Fig. 7*D*, the binding of nuclear Pbx-Prep1 to labeled G3B was competed for by an oligonucleotide containing the sequence from −169 to −136 of the glucagon promoter, whereas an unrelated oligonucleotide (TTR-HNF4) did not compete. When taken together with the functional data, these results indicate that Pbx-Prep1 can inhibit glucagon promoter activity not only through binding to G3B but also through binding to a sequence between −169 and −136. This region

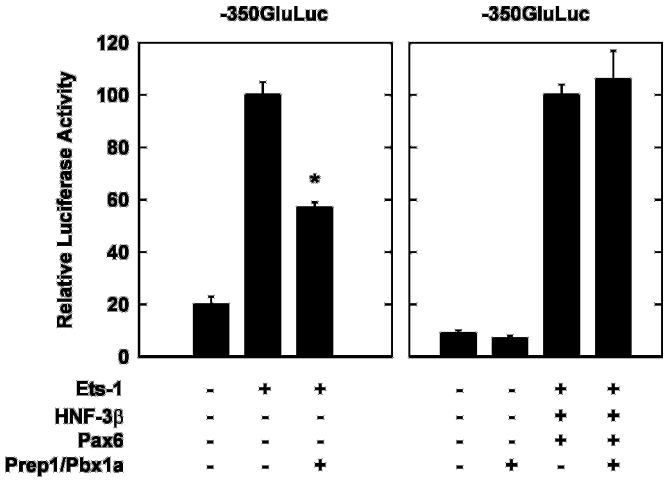

FIG. 8. **Effect of Pbx-Prep1 on glucagon gene transcription activated by Ets-1 or by a combination of Ets-1, HNF-3β, and Pax6 in JEG-3 cells.** Plasmid −350GluLuc was transfected together with expression plasmids pBAT14-mPax6, pCMV-HNF-3β, pSG5c-ets1p68, pSG5-Prep1, and pBK-Pbx1a, as indicated, into the nonpancreatic choriocarcinoma cell line JEG-3. Luciferase activity is expressed relative to reporter gene activity induced by Ets-1 (*left panel*) or by a combination of Ets-1, HNF-3β, and Pax6 (*right panel*). Values are means ± S.E. of three independent experiments, each done in duplicate. *, $p < 0.05$ compared with Ets-1-induced reporter gene activity (one-way analysis of variance, Bonferroni's method).

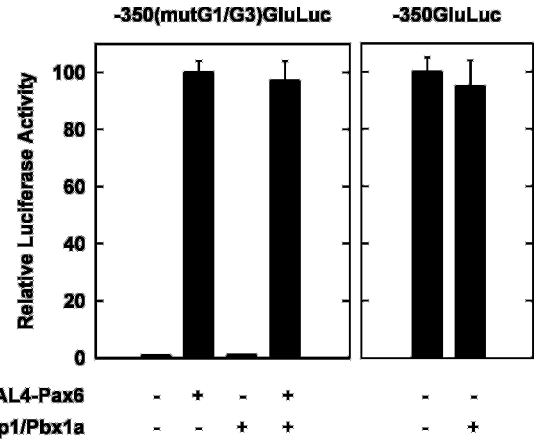

FIG. 9. **Lack of inhibition of glucagon gene transcription by Pbx-Prep1 in glucagon-producing pancreatic islet αTC2 cells.** Plasmid −350GluLuc or −350(mutG1/G3)GluLuc was transfected together with expression plasmids pGAL4-Pax6, pSG5-Prep1, and pBK-Pbx1a, as indicated, into αTC2 cells. In the plasmid −350(mutG1/G3)GluLuc, the Pax6 binding sites in the G1 and G3 elements of the glucagon gene 5′-flanking region are mutated into GAL4 binding sites. Luciferase activity is expressed relative to GAL4-Pax6-induced reporter gene activity (*left panel*) or relative to −350GluLuc basal activity (*right panel*). Values are means ± S.E. of three independent experiments, each done in duplicate.

contains a sequence similarity with G3B and homeodomain binding sites including the Pbx core sequence TGAT (from −146 to −149) and is bound by nuclear proteins with Pbx-like immunoreactivity when used as probe in an electrophoretic mobility shift assay (not shown). Thus, this newly identified element is termed G5, in continuation to G1, G2, G3 (22), and G4 (69).

Notably, an oligonucleotide containing the glucagon CRE (from −306 to −280) also competed for the binding of Pbx-Prep1 (Fig. 7*D*), suggesting the presence of at least three Pbx-Prep1 binding sites in the glucagon gene 5′-flanking region. Overall, these results suggest that Pbx-Prep1 heterodimers inhibit glucagon gene transcription through domain B of the G3 element as well as through additional elements (G5, CRE).

Pbx-Prep1 Heterodimers Inhibit Glucagon Gene Transcription Depending on the Transcriptional Activators and Fail to Do So in Glucagon-producing Pancreatic Islet Cells—To examine whether Pbx-Prep1 heterodimers can inhibit glucagon gene transcription activated by transcription factors others than Pax6, cotransfection experiments were performed in JEG-3 cells. The glucagon promoter contains, among others, binding sites for HNF-3β and members of the Ets family of transcription factors (40, 23). The expression of Ets-1 stimulated glucagon promoter activity (Fig. 8, *left panel*), whereas the expression of HNF-3β did not (not shown) as has been reported previously (40). The coexpression of Pbx1a and Prep1 inhibited the Ets-1-induced glucagon promoter activity by about 50% (Fig. 8, *left panel*). However, when glucagon promoter activity was stimulated by Ets-1, Pax6, and HNF3β together, Pbx1a-Prep1 had no effect on glucagon gene transcription (Fig. 8, *right panel*). Thus, although Pbx-Prep1 heterodimers can inhibit glucagon promoter activity when stimulated by transcription factors other than Pax6, these results suggest that the ability of Pbx-Prep1 to inhibit glucagon gene transcription may depend on the nature and combination of glucagon promoter-binding transcription factors.

Glucagon gene transcription in glucagon-producing pancreatic islet cells depends on the synergistic interaction between multiple transcription factors including Pax6 (22, 23, 30, 31, 33). To examine the effect of Pbx-Prep1 on glucagon promoter activity induced by Pax6 in the presence of all glucagon promoter-binding transcription factors that are expressed in glucagon-producing pancreatic islet cells, the Pax6 binding sites within domain A of G3 and within the G1 element were mutated into binding sites of the yeast transcription factor GAL4 (construct −350(mutG1/G3)GluLuc). In the glucagon-producing pancreatic islet cell line αTC2, this mutation decreased glucagon promoter activity to about 1% of wild-type activity (not shown), indicating the functional importance of Pax6 that is expressed in this glucagon-producing islet cell line. When an expression vector encoding Pax6 fused to the DNA-binding domain of GAL4 was transfected together with −350(mutG1/G3)GluLuc into αTC2 cells, the transcriptional activity of the mutant glucagon promoter was raised to a level similar to that of wild type promoter activity (Fig. 9, *left panel*). The expression of Prep1 and Pbx1a had no effect on GAL4-Pax6-induced −350(mutG1/G3)GluLuc activity in αTC2 cells (Fig. 9, *left panel*). However, they did inhibit in JEG-3 cells (not shown). Similarly, Pbx1a-Prep1 had no effect on wild type glucagon promoter activity in αTC2 cells (Fig. 9, *right panel*) or in another glucagon-producing pancreatic islet cell line, InR1-G9 (not shown). In contrast, the expression of Pbx1a-Prep1 inhibited 4xG3T81Luc activity in αTC2 cells (not shown), similar to the inhibition by Pbx1a-Prep1 of Pax6-induced 4xG3T81Luc activity in JEG-3 cells (see above). Thus, these results suggest that Pbx-Prep1 heterodimers are not able to inhibit glucagon gene transcription stimulated by a combination of transcription factors that confers pancreatic islet cell-specific activity to the glucagon gene.

As shown above, Pbx-Prep1 heterodimers can bind to multiple sites within the glucagon promoter, the most proximal one being within the G5 element (from −169 to −140). As shown in Fig. 10, a 5′-deletion of G5 led to enhanced promoter activity in choriocarcinoma JEG-3 cells, whereas it had no effect in glucagon-producing αTC2 cells (Fig. 10) and InR1-G9 cells (22). These results indicate that a Pbx-Prep1 binding site confers transcriptional repression to the glucagon promoter in non-glucagon-producing cells but not in glucagon-producing pancreatic islet cells.

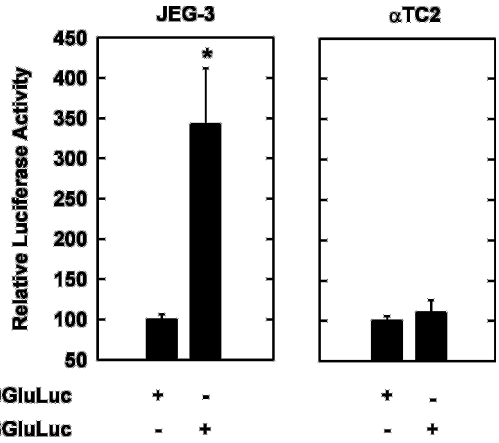

FIG. 10. **5′-Deletion of the G5 element (from −169 to −140), containing the proximal Pbx-Prep1 binding site, relieves glucagon gene transcription in non-glucagon-producing JEG-3 cells but not in glucagon-producing pancreatic islet αTC2 cells.** Plasmids −169GluLuc and −136GluLuc were transfected into JEG-3 or αTC2 cells. *−136GluLuc*, luciferase activity is expressed relative to −169GluLuc activity. Values are means ± S.E. of three independent experiments, each done in duplicate. *, $p < 0.05$ compared with −169GluLuc activity (one-way analysis of variance, Bonferroni's method).

DISCUSSION

How the PBC homeodomain proteins EXD in *Drosophila* and vertebrate Pbx specify developmental pathways and cell-specific gene transcription is incompletely understood (35, 70). Initial genetic and biochemical evidence showed that PBC proteins act as transcriptional co-factors of homeodomain proteins of the Hox cluster that are critical regulators of cell fate and segment identity (3). Through cooperative DNA binding, PBC factors increase Hox target gene specificity and affinity (71–76). Furthermore, the transcriptional activity of certain preformed Hox-Pbx complexes can be modulated by the recently cloned and closely related homeodomain proteins Meis, HTH, and Prep1 (43, 77–80). However, it is becoming increasingly clear that PBC proteins in complex with Meis/HTH/Prep1 also perform Hox-independent functions. A first example was the recent demonstration that HTH and EXD dictate antennal development in *Drosophila* (35, 81). The results of the present study identify a novel target and Hox-independent function of Pbx-Prep1 heterodimers that is proposed to help to establish pancreatic islet α-cell-specific transcription of the glucagon gene.

The proteins binding domain B of the G3 enhancer-like element are identified in the present study as distinct members of the three-amino acid loop extension (TALE) class of homeodomain transcription factors. Based on their binding specificity and their immunoreactivity and in comparison with *in vitro* transcribed/translated proteins, the G3B-binding proteins are heterodimers consisting of Prep1 and the Pbx isoform 1a, 1b, or 2. Pbx-Prep1 binds to additional sites within the glucagon gene 5′-flanking region, one being located within the newly identified G5 element (from −169 to −140) and the other within the glucagon CRE. Notably, a sequence similarity between G3B and the glucagon CRE includes a sequence (5′-TTCATTC-3′) that has previously been shown to bind a nuclear protein complex that can repress CRE function (82). Consistent with the ubiquitous distribution of G3B-BPs (29), Pbx-Prep1 is expressed in almost every tissue in the mouse, although the Pbx isoforms may vary (83). This study shows by immunohistochemistry that Pbx-Prep1 is expressed also in human islets including non-glucagon-producing cells. Prep1 shares with Meis and *Drosophila* HTH two conserved amino-terminal regions, termed HR1 and HR2 (65). HR1 and HR2 bind the N-terminal region of Pbx and are essential for Pbx-Prep1 heterodimerization (65). This association with Pbx increases the affinity of Prep1 for DNA such that heterodimerization is virtually required for DNA binding as has been shown previously at the human urokinase enhancer (65) and holds true also for the G3B binding site in the rat glucagon gene (this study). The Pbx-Prep1 complexes are very stable and form independently of DNA (65, 83). Thus, although Pbx can cooperatively bind with various proteins including Hox proteins (68, 70, 71, 84, 85), Pdx1 (86), and Engrailed (62, 63), Prep1 is the predominant partner of Pbx proteins in adult mouse tissues as demonstrated by immunoprecipitation studies (83). Whereas this suggests potentially widespread functions for Pbx-Prep1 that are likely to play key roles in the regulation of coordinated gene transcription, explicit examples for functionally significant Pbx-Prep1-directed transcription are limited to the human urokinase (87) and rat somatostatin genes (88). The present study provides the glucagon gene as a novel target of Pbx-Prep1 heterodimers.

The expression of Pbx-Prep1 failed to activate transcription through multimerized glucagon G3B binding sites. This is consistent with previous data showing lack of transactivation by Pbx-Prep1 complexes themselves (43). They might rather modulate the activity of other transcription factors. Thus, a mutation, specifically destroying the Pbx-Prep1 binding site (UEF3) in the human urokinase gene enhancer weakens the response of the enhancer to a protein kinase C-activating phorbol ester, indicating that a Pbx-Prep1 heterodimer together with additional proteins allows the cooperation between two flanking AP-1 sites (65, 87, 89). Furthermore, the expression of Pbx-Prep1 had no effect on somatostatin promoter activity in a colon carcinoma cell line but enhanced the transcriptional response to cotransfected Pdx1 (88). In contrast to these stimulatory effects in previous investigations, the present study provides direct evidence that Pbx-Prep1 heterodimers can also repress transcription as Pbx-Prep1 inhibited glucagon promoter activity when stimulated by Pax6. Such a repressive action of Pbx-Prep1 may be more common, since the *Drosophila* homologue of Pbx, EXD, together with HTH, suppresses eye development in *Drosophila*, and among the many nuclear factors required for eye development, the *Drosophila* homologue of Pax6, EYELESS, is a candidate for this suppressive interaction with EXD/HTH (90). Furthermore, Pbx-Prep1 recognizes a sequence (NIP) that appears to negatively modify the efficiency of phorbol ester-responsive elements in the interleukin-3 gene (91, 92). The mechanism of inhibition of transcription by Pbx-Prep1 is unclear. The fact that both proteins are needed and the 5′-deletion analysis suggest that Pbx-Prep1 heterodimerization and DNA-binding are required. Pbx1b-Prep1 was as effective as Pbx1a-Prep1; thus, transcriptional repression does not appear to depend on the unique C-terminal domain in Pbx1a that associates with the corepressors SMRT and NCoR (93). Another potential repression domain has been characterized in Pbx, which lies upstream of the homeodomain and is highly conserved among Pbx proteins (74).

Although the present study shows that Pbx-Prep1 heterodimers can inhibit the glucagon promoter when activated by transcription factors other than Pax6, it also presents evidence that the ability of Pbx-Prep1 to repress glucagon gene transcription may depend on the nature and/or combination of glucagon promoter-binding proteins. Interestingly, the N-terminal repression domain of Pbx proteins has been shown to be functional against some transcription factors but not others (74). In particular, Pbx-Prep1 failed to inhibit GAL4-Pax6-induced mutant glucagon promoter activity and Pax6-depend-

ent wild-type glucagon promoter activity in glucagon-producing pancreatic islet cells. This suggests that the unique synergistic combination of transcription factors, which confers islet α-cell-specific activity to the glucagon gene, is not sensitive to Pbx-Prep1. When taken together, our data thereby suggest that Pbx-Prep1 heterodimers may inhibit glucagon gene transcription preferentially in non-glucagon-producing cells. Cell specificity of gene transcription is well known to be conferred by a combination of positively and negatively acting transcription factors as exemplified in yeast, where MATα2p represses a-specific genes in α cells (94). None of the transcription factors that are known to activate the glucagon gene in pancreatic islet α-cells are restricted in their expression to this cell type (23). They are also expressed in other islet cell types (*e.g.* Pax6, HNF-3β) and in other organs like liver (HNF-3β) (95, 96) and brain (Pax6, Brain-4) (61, 97). Furthermore, many transcription factors in diverse tissues may recognize binding sites in the glucagon promoter due to overlapping binding specificity. Pbx-Prep1 may repress this erroneous activation. Consistent with this view, the 5′-deletion of G5, containing the proximal Pbx-Prep1 binding site, enhanced activity of the glucagon promoter in a non-glucagon-expressing cell line but not in glucagon-expressing pancreatic islet cell lines (this study). Similarly, the 5′-deletion of a Pbx-Prep1 binding site (G3B) enhanced glucagon promoter activity in a plurihormonal intestinal cell line (98). Homeodomain proteins like Brain-4, Cdx2/3, and Isl-1 show a restricted tissue distribution and appear to play a key role in establishing islet α-cell-specific glucagon gene transcription by binding to the proximal promoter element G1 and stimulating transcription synergistically with other transcription factors (24, 25, 27, 28). In contrast, Pbx-Prep1 are distinct members of the homeodomain family of transcription factors (99); our data are consistent with a model according to which Pbx-Prep1 heterodimers bind more distal glucagon promoter elements and help to establish cell-specific glucagon gene transcription by inhibiting promoter activity in non-glucagon-producing cells. Their ubiquitous expression is ideal to subserve this function.

The Pax6 protein contains a paired type homeodomain, which is located about 80 residues downstream of the paired domain (61). The homeodomain and the paired domain of Pax6 are able to interact cooperatively in DNA binding (100). Interestingly, it has been shown recently that full binding of Pax6 to the G3 enhancer-like element requires both the Pax6 paired domain and homeodomain (34). When taken together with previous evidence that G3B can positively modulate G3 activity (36, 37), the identification by the present study of G3B as a homeodomain binding site suggests a dual function of domain B of G3. On the one hand, Pbx-Prep1 homeodomain proteins bind to G3B and repress transcription in non-glucagon-producing cells. On the other hand, Pax6 may contact domain B through its homeodomain, which promotes Pax6 binding to domain A of G3 through its paired domain and thereby enhances G3 activity. This is consistent with recent evidence that sequences outside the paired domain recognition sequence stabilize Pax6 binding to the somatostatin gene (101). Competitive binding at one of the three known Pbx-Prep1 binding sites in the glucagon gene may thus in some cells be part of the repression of the glucagon gene by Pbx-Prep1 homeodomain proteins.

Acknowledgments—We greatly appreciate generous gifts of unique reagents from the following investigators: S. Saule (Lille Cedex, France) (αPax6); M. German (San Francisco, CA) (pBAT14-mPax6); R. Costa (Chicago, IL) (pCMV-HNF-3β); B. Wasylyk (Straßbourg, France) (pSG5c-ets1p68); F. Blasi (Milan, Italy) (pSG5-Prep1, pSG5-Pbx1b); M. Cleary (Stanford, CA) (pSP65-Pbx1a, pSP64-Pbx2, pSP65-Pbx3a, αMeis1); P. Traber (Philadelphia, PA) (αCdx2); and A. Joyner (New York, NY) (αEnhb1). We thank R. Grzeskowiak for critical reading of the manuscript. We also thank C. Spinhoff and D. Scholz and B. Jünemann for typing the manuscript and for expert technical assistance.

REFERENCES

1. Gehring, W. J., Affolter, M., and Bürglin, T. (1994) *Annu. Rev. Biochem.* **63,** 487–526
2. Krumlauf, R. (1994) *Cell* **78,** 191–201
3. McGinnis, W., and Krumlauf, R. (1992) *Cell* **68,** 283–302
4. Slack, J. M. W. (1995) *Development* **121,** 1569–1580
5. Unger, R. H., and Orci, L. (1981) *N. Engl. J. Med.* **304,** 1518–1524
6. Ahlgren, U., Pfaff, S. L., Jessell, T., Edlund, T., and Edlund, H. (1997) *Nature* **385,** 257–260
7. Habener, J. F., and Stoffers, D. A. (1998) *Proc. Assoc. Am. Physicians* **110,** 12–21
8. Harrison, K. A., Thaler, J., Pfaff, S. L., Gu, H., and Kehrl, J. H. (1999) *Nat. Genet.* **23,** 71–75
9. Jonsson, J., Carlsson, L., Edlund, T., and Edlund, H. (1994) *Nature* **371,** 606–609
10. Li, H., Arber, S., Jessell, T. M., and Edlund, H. (1999) *Nat. Genet.* **23,** 67–70
11. Offield, M., Jetton, T., Labosky, P., Ray, M., Stein, R., Magnuson, M., Hogan, B., and Wright, C. (1996) *Development* **122,** 983–955
12. Sosa-Pineda, B., Chowdhury, K., Torres, M., Oliver, G., and Gruss, P. (1997) *Nature* **386,** 399–402
13. St-Onge, L., Sosa-Pineda, B., Chowdhury, K., Mansouri, A., and Gruss, P. (1997) *Nature* **387,** 406–409
14. Sussel, L., Kalamaras, J., Hartigan-O'Connor, D., Meneses, J., Pedersen, R., Rubenstein, J., and German, M. (1998) *Development* **125,** 2213–2221
15. Sander, M., and German, M. S. (1997) *Mol. Med.* **75,** 327–340
16. St-Onge, L., Wehr, R., and Gruss, P. (1999) *Curr. Opin. Genet. Dev.* **9,** 295–300
17. Lefèbvre, P. J. (1996) in *Handbook of Experimental Pharmacology* (Lefèbvre, P. J., ed) 3rd Ed., Vol. 30, pp. 115–132, Springer-Verlag, Berlin
18. Drucker, D. J., Philippe, J., Jepeal, L., and Habener, J. F. (1987) *J. Biol. Chem.* **262,** 15659–15665
19. Efrat, S., Teitelman, G., Anwar, M., Ruggiero, D., and Hanahan, D. (1988) *Neuron* **1,** 605–613
20. Knepel, W. (1993) *Exp. Clin. Endocrinol.* **101,** 39–45
21. Lee, Y. C., Asa, S. L., and Drucker, D. J. (1992) *J. Biol. Chem.* **267,** 10705–10708
22. Philippe, J., Drucker, D. J., Knepel, W., Jepeal, L., Misulovin, Z., and Habener, J. F. (1988) *Mol. Cell. Biol.* **8,** 4877–4888
23. Knepel, W. (2000) in *Molecular Basis of Endocrine Pancreas Development and Function* (Hussain, M. A., Miller, C. P., and Habener, J. F., eds) Kluwer Academic Publishers, Boston, in press
24. Hussain, M. A., Lee, J., Miller, C. P., and Habener, J. F. (1997) *Mol. Cell. Biol.* **17,** 7186–7194
25. Wang, M., and Drucker, D. J. (1995) *J. Biol. Chem.* **270,** 12646–12652
26. German, M. S., Wang, J., Chadwick, R. B., and Rutter, W. J. (1992) *Genes Dev.* **6,** 2165–2176
27. Jin, T., and Drucker, D. J. (1996) *Mol. Cell. Biol.* **16,** 19–28
28. Laser, B., Meda, P., Constant, I., and Philippe, J. (1996) *J. Biol. Chem.* **271,** 28984–28994
29. Knepel, W., Jepeal, L., and Habener, J. F. (1990) *J. Biol. Chem.* **265,** 8725–8735
30. Beimesche, S., Neubauer, A., Herzig, S., Grzeskowiak, R., Diedrich, T., Cierny, I., Scholz, D., Alejel, T., and Knepel, W. (1999) *Mol. Endocrinol.* **13,** 718–728
31. Sander, M., Neubüser, A., Kalamaras, J., Ee, H. C., Martin, G. R., and German, M. S. (1997) *Genes Dev.* **11,** 1662–1673
32. Andersen, F. G., Heller, R. S., Petersen, H. V., Jensen, J., Madsen, O. D., and Serup, P. (1999) *FEBS Lett.* **445,** 306–310
33. Knepel, W., Vallejo, M., Chafitz, J. A., and Habener, J. F. (1991) *Mol. Endocrinol.* **5,** 1457–1466
34. Ritz-Laser, B., Estreicher, A., Klages, N., Saule, S., and Philippe, J. (1999) *J. Biol. Chem.* **274,** 4124–4132
35. Mann, R. S., and Affolter, M. (1998) *Curr. Opin. Genet. Dev.* **8,** 423–429
36. Diedrich, T., Fürstenau, U., and Knepel, W. (1997) *Biol. Chem.* **378,** 89–98
37. Wrege, A., Diedrich, T., Hochhuth, C., and Knepel, W. (1995) *Gene Expr.* **4,** 205–216
38. Diedrich, T. (1996) *Characterization of the Transcription Factor Binding the PISCES Element and the Domain B of the Cell-specific Enhancer Element G3 of the Rat Glucagon Gene.* Ph.D. thesis, University of Göttingen, Göttingen, Germany
39. Schwaninger, M., Lux, G., Blume, R., Oetjen, E., Hidaka, H., and Knepel, W. (1993) *J. Biol. Chem.* **268,** 5168–5177
40. Fürstenau, U., Schwaninger, M., Blume, R., Kennerknecht, I., and Knepel, W. (1997) *Mol. Cell. Biol.* **17,** 1805–1816
41. Pani, L., Qian, X., Clevidence, D., and Costa, R. H. (1992) *Mol. Cell. Biol.* **12,** 552–562
42. Wasylyk, B., Wasylyk, P., Flores, P., Begue, A., Leprince, D., and Stehelin, D. (1990) *Nature* **346,** 191–193
43. Berthelsen, J., Zappavigna, V., Ferreti, E., Mavilio, F., and Blasi, F. (1998) *EMBO J.* **17,** 1434–1445
44. Di Rocco, G., Mavilio, F., and Zappavigna, V. (1997) *EMBO J.* **16,** 3644–3654
45. Nordeen, S. K. (1988) *BioTechniques* **6,** 454–457
46. Monica, K., Galili, N., Nourse, J., Saltman, D., and Cleary, M. (1991) *Mol. Cell. Biol.* **11,** 6149–6157
47. Kohler, P. O., and Bridson, W. E. (1971) *J. Clin. Endocrinol.* **32,** 683–687
48. Powers, A. C., Efrat, S., Mojsov, S., Spector, D., Habener, J. F., and Hanahan, D. (1990) *Diabetes* **39,** 406–414
49. Takaki, R., Ono, J., Nakamura, M., Yokogawa, Y., Kumae, S., Hiraoka, T., Yamaguchi, K., Hamaguchi, K., and Uchida, S. (1986) *In Vitro Cell. Dev. Biol.* **22,** 120–126

50. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489
51. Oetjen, E., Diedrich, T., Eggers, A., Eckert, B., and Knepel, W. (1994) *J. Biol. Chem.* **269,** 27036–27044
52. Lu, Q., Wright, D. D., and Kamps, M. P. (1994) *Mol. Cell. Biol.* **14,** 3938–3948
53. Turque, N., Plaza, S., Radvanyi, F., Carriere, C., and Saule, S. (1994) *Mol. Endocrinol.* **8,** 929–938
54. Chang, C., Jacobs, Y., Nakamura, T., Jenkins, N. A., Copeland, N. G., and Cleary, M. L. (1997) *Mol. Cell. Biol.* **17,** 5679–5687
55. Suh, E., Chen, L., Taylor, J., and Traber, P. G. (1994) *Mol. Cell. Biol.* **14,** 7430–7351
56. Davis, C. A., Holmyard, D. P., Millen, K. J., and Joyner, A. L. (1991) *Development* **111,** 287–298
57. Clevidence, D. E., Overdier, D. G., Tao, W., Qian, X., Pani, L., Lai, E., and Costa, R. H. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 3948–3952
58. Nourse, J., Mellentin, J. D., Galili, N., Wilkinson, J., Stanbridge, E., Smith, S. D., and Cleary, M. L. (1990) *Cell* **60,** 535–545
59. Rauskolb, C., Peifer, M., and Wieschaus, E. (1993) *Cell* **74,** 1101–1112
60. James, R., Erler, T., and Kazenwadel, J. (1994) *J. Biol. Chem.* **269,** 15229–15237
61. Walther, C., and Gruss, P. (1991) *Development* **113,** 1435–1449
62. Peltenburg, L. T. C., and Murre, C. (1996) *EMBO J.* **15,** 3385–3393
63. Peltenburg, L. T. C., and Murre, C. (1997) *Development* **124,** 1089–1098
64. van Dijk, M. A., Peltenburg, L. T. C., and Murre, C. (1995) *Mech. Dev.* **52,** 99–108
65. Berthelsen, J., Zappavigna, V., Mavilio, F., and Blasi, F. (1998) *EMBO J.* **17,** 1423–1433
66. Knoepfler, P. S., Calvo, K. R., Chen, H., Antonarakis, S. E., and Kamps, M. P. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 14553–14558
67. Rieckhof, G. E., Casares, F., Ryoo, H. D., Abu-Shaar, M., and Mann, R. S. (1997) *Cell* **91,** 171–183
68. van Dijk, M. A., and Murre, C. (1994) *Cell* **78,** 617–624
69. Cordier-Bussat, M., Morel, C., and Philippe, J. (1995) *Mol. Cell. Biol.* **15,** 3904–3916
70. Mann, R. S., and Chan, S. (1996) *Trends Genet.* **12,** 258–262
71. Chang, C. P., Brocchieri, L., Shen, W. F., Largman, C., and Cleary, M. L. (1996) *Mol. Cell. Biol.* **16,** 1734–1745
72. Johnson, F. B., Parker, E., and Krasnow, M. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 739–743
73. Knoepfler, P. S., Lu, Q., and Kamps, M. P. (1996) *Nucleic Acids Res.* **24,** 2288–2294
74. Lu, Q., and Kamps, M. P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 470–474
75. Neuteboom, S. T. C., and Murre, C. (1997) *Mol. Cell. Biol.* **17,** 4696–4706
76. Phelan, M. L., and Featherstone, M. S. (1997) *J. Biol. Chem.* **272,** 8635–8643
77. Ferretti, E., Marshall, H., Popperl, H., Maconochie, M., Krumlauf, R., and Blasi, F. (2000) *Development* **127,** 155–166
78. Ryoo, H. D., Marty, T., Casares, F., Affolter, M., and Mann, R. S. (1999) *Development* **126,** 5137–5148
79. Schnabel, C. A., Jacobs, Y., and M. Cleary, L. (2000) *Oncogene* **19,** 608–616
80. Shanmugam, K., Green, N. C., Rambaldi, I., Saragovi, H. U., and Featherstone, M. S. (1999) *Mol. Cell. Biol.* **19,** 7577–7588
81. Casares, F., and Mann, R. S. (1998) *Nature* **392,** 723–726
82. Miller, C. P., Lin, J. C., and Habener, J. F. (1993) *Mol. Cell. Biol.* **13,** 7080–7090
83. Ferretti, E., Schulz, H., Talarico, D., Blasi, F., and Berthelsen, J. (1999) *Mech. Dev.* **83,** 53–64
84. Phelan, M. L., Rambaldi, I., and Featherstone, M. S. (1995) *Mol. Cell. Biol.* **15,** 3989–3997
85. Piper, D. E., Batchelor, A. H., Chang, C., Cleary, M. L., and Wolberger, C. (1999) *Cell* **96,** 587–597
86. Peers, B., Sharma, S., Johnson, T., Kamps, M., and Montminy, M. (1995) *Mol. Cell. Biol.* **15,** 7091–7097
87. De Cesare, D., Palazzolo, M., and Blasi, F. (1996) *Oncogene* **13,** 2551–2562
88. Goudet, G., Delhalle, S., Biemar, F., Martial, J. A., and Peers, B. (1999) *J. Biol. Chem.* **274,** 4067–4073
89. Nerlov, C., Cesare, D. D., Pergola, F., Caracciolo, A., Blasi, F., Johnsen, M., and Verde, P. (1992) *EMBO J.* **11,** 4573–4582
90. Pai, C. Y., Kuo, T. S., Jaw, T. J., Kurant, E., Chen, C. T., Bessarab, D. A., Salzberg, A., and Sun, Y. H. (1998) *Genes Dev.* **12,** 435–446
91. Berthelsen, J., Vandekerkhove, J., and Blasi, F. (1996) *J. Biol. Chem.* **271,** 3822–3830
92. Mathey-Prevot, B., Andrews, N. C., Murphy, H. S., Kreissman, S. G., and Nathan, D. G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5046–5050
93. Asahara, H., Dutta, S., Kao, H. Y., Evans, R. M., and Montminy, M. (1999) *Mol. Cell. Biol.* **19,** 8219–8225
94. Scott, M. P. (2000) *Cell* **100,** 27–40
95. Lai, E., Prezioso, V. R., Smith, E., Litvin, O., Costa, R. H., and Darnell, J. E., Jr. (1990) *Genes Dev.* **4,** 1427–1436
96. Lai, E., Prezioso, V. R., Tao, W., Chen, W. S., and Darnell, J. E., Jr. (1991) *Genes Dev.* **5,** 416–427
97. Hara, Y., Rovescalli, A. C., Kim, Y., and Nirenberg, M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3280–3284
98. Gajic, D., and Drucker, D. J. (1993) *Endocrinology* **132,** 1055–1062
99. Bürglin, T. R. (1997) *Nucleic Acids Res.* **25,** 4173–4180
100. Jun, S., and Desplan, C. (1996) *Development* **122,** 2639–2650
101. Andersen, F. G., Heller, R. S., Petersen, H. V., Jensen, J., Madsen, O. D., and Serup, P. (1999) *FEBS Lett.* **445,** 315–320
102. Pöpperl, H., Bienz, M., Studer, M., Chan, S. K., Aparicio, S., Brenner, S., Mann, R. S., and Krumlauf, R. (1995) *Cell* **81,** 1031–1042