# Differential Regulation of the Glucagon and Insulin I Gene Promoters by the Basic Helix-Loop-Helix Transcription Factors E47 and BETA2*

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The insulin and glucagon genes are expressed in the beta and alpha cells of the islets of Langerhans, respectively. The factors controlling their cell- and islet-specific expression are poorly known. Insulin-enhancer factor-1 (IEF1) has previously been shown to interact with the E boxes of the rat insulin I and II genes and was proposed to play a critical role in beta cell-specific expression. BETA2, a recently identified basic helix-loop-helix (bHLH) protein, binds with high affinity and transactivates the rat insulin II gene upon dimerization with the ubiquitous bHLH protein E47. We show here that the heterodimer E47/BETA2 also binds and transactivates the rat insulin I and glucagon genes and exhibits the same characteristics as IEF1. In transfection experiments, the E boxes of the insulin I and glucagon genes confer transcriptional activity in both insulin- and glucagon-producing cells, which is increased by overexpression of E47 and BETA2. However, overexpression of E47 inhibits only E box-mediated glucagon gene expression, whereas it activates insulin gene transcription, indicating that the E boxes of the insulin and glucagon genes display gene-specific characteristics. We conclude that the heterodimer E47/BETA2 represents an islet-specific factor that controls both insulin and glucagon gene transcription and that the E47/BETA2 ratio may be important for regulated gene expression.

Glucagon and insulin are two major antagonist hormones secreted by the endocrine pancreas which control glucose homeostasis. Glucagon gene expression in the adult is restricted to the alpha cells of the pancreas, the L-cells of the intestine, and some specific cell types in the central nervous system, whereas insulin gene expression is limited to pancreatic beta cells. The regulation as well as the cell-specific expression of both genes rely on specific interactions between cis-acting DNA sequences of their promoters and trans-acting factors (1, 2).

Four DNA control elements have been defined within the first 300 bp[1] of the rat glucagon gene promoter which function as islet- or cell-specific cis-acting elements (3, 4). G1 is a proximal promoter element located upstream of the TATA box and involved in cell-specific expression (5). G2 and G3 are distal enhancers, whereas G4 is located in the proximal promoter just upstream of G1 (4, 6). The G4 element contains two palindromic consensus sequence CANNTG motifs, one of which (E3) functions as an E box, separated by an intervening sequence. The complete element acts as a mini-enhancer in glucagon-producing cells (6).

E box motifs bind factors which belong to the basic helix-loop-helix (bHLH) family of transcription factors. These factors have been shown to participate in the regulation of several cell- and tissue-specific genes (7–11). The bHLH transcription factor family is divided into three groups according to their structure, DNA-binding ability, and cell distribution. Class A factors, such as USF or E47, are ubiquitous and bind DNA as homo- or heterodimers, whereas class B factors are tissue-specific and preferentially bind as heterodimers with class A factors. Class C factors contain a leucine zipper motif at the carboxyl-terminal end of the bHLH domain and are further divided into ubiquitous and tissue-specific subgroups.

Functional studies on rat insulin I and II gene expression have identified several binding sites for trans-acting factors within the first 400 bp of the 5′-flanking sequence which confer beta cell-specific expression including two distinct types of DNA motifs, A boxes, which correspond to the minimal sequence TAAT, and E boxes, corresponding to the consensus CANNTG (12, 13).

The two E boxes E1 and E2 of the rat insulin I gene have been reported to play a major role in beta cell-specific expression and regulation by glucose; they interact with insulin enhancer factor 1 (IEF1), a protein complex composed of the ubiquitous bHLH protein E12/E47 and an islet cell-specific factor (14–16). IEF1 is also present in glucagon-producing cells, and previous results from our group suggested that IEF1 may also interact with the E3 element of the glucagon gene and thus play a role not only in insulin gene expression but rather in islet hormone gene expression (6). Recent work on the E1 box of the insulin II gene (previously called RIPE3a), which is also critical for beta cell-specific expression, has led to the characterization of BETA2, a class B bHLH transcription factor which is capable of binding with high affinity as a heterodimer to E1 and activating insulin gene transcription (17). Since E boxes with the same core motif are also found in the rat insulin I and glucagon genes, we hypothesized that the E47/BETA2 heterodimer may affect their expression also and thus function as an activator of islet hormone gene expression. We tested this hypothesis by investigating the interactions of E47 and BETA2 with E3 and E1 of the glucagon and insulin I genes, respectively. Our data indicate that the heterodimer E47/BETA2 binds to and transactivates the E boxes of the glucagon and insulin I genes and represents IEF1. The E boxes of both genes

1 The abbreviations used are: bp, base pair(s); bHLH, basic helix-loop-helix; IEF, insulin enhancer factor; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay.

function in both glucagon- and insulin-producing cells, but not in BHK-21 cells, indicating that IEF1 is necessary but not sufficient for the E box-mediated transcriptional activity. However, the E boxes of the insulin and glucagon genes are not interchangeable and display clear specificity. Indeed, E47 overexpression inhibits glucagon gene transcription through specific interactions with E3, whereas insulin gene transcription is activated, suggesting that the E47/BETA2 ratio might be critical for the regulation of these genes.

## EXPERIMENTAL PROCEDURES

*Oligonucleotides and Plasmids*—The oligonucleotides IEB1 (gatccG-CCATCTGCCA, where lowercase letters are attached ends and uppercase letters reflect the DNA sequence of the gene promoter fragment) and E130 (gatccTGAGGAGCAGATGAGCAGAGAGTa) containing the E1 box of the rat insulin I gene and the E3 box of the rat glucagon gene, respectively (see Fig. 1) (6), were synthesized on a gene assembler (Amersham Pharmacia Biotech). Reporter plasmids containing glucagon promoter sequences were constructed by placing *Bam*HI-compatible ends on two oligonucleotide copies of the wild type (G4-CAT) or mutated (G4−108N-CAT, and G4−130N-CAT) G4 sequences (corresponding to −100/−140 bp of the glucagon promoter) and inserting them into a *Bam*HI site upstream of the glucagon minimal promoter (−31 to +58 bp) linked to the CAT reporter gene (6). The same strategy was used to insert two copies of the E130 oligonucleotide containing the functional E3 box (found within G4). Reporter plasmids containing insulin I gene promoter sequences were prepared by linking two copies of the E1 (gatcCGCCATCTGCCA) or E2 (gatccGCCATCTGGCa) boxes (formerly IEB1 and IEB2, respectively) to the CAT reporter gene (18), and were generously provided by Dr. M. Walker (Weizmann Institute of Science, Rehovot, Israel).

The E47 expression vector (pCi-Neo-E47) was constructed by inserting the full-length E47 cDNA (19) (generously provided by Dr. Z.-S. Ye, The Rockefeller University, New York, NY) into the *Xba*I site of pCi-Neo (Promega), an expression vector with a CMV promoter. A 1.3-kilobase pair *Hin*dIII-*Bam*HI fragment containing the complete coding sequence of BETA2 was excised from the full-length cDNA (17) (generously provided by Dr. M. J. Tsai, Baylor College of Medicine, Houston, TX), blunted and subcloned into the *Sma*I site of pCi-Neo to create the BETA2 expression vector (pCi-Neo-BETA2).

*Electrophoretic Mobility Shift Assays (EMSAs)*—Double-stranded oligonucleotide probes corresponding to the glucagon E3 (E130) and insulin E1 (IEB1) boxes (6) were labeled to a specific activity of ~$10^8$ cpm/μg in the presence of [$^{32}$P]dCTP with the Klenow fragment of DNA polymerase I. Binding reactions were performed in 25 mM Hepes, pH 7.9, 150 mM KCl, 10% glycerol, 5 mM dithiothreitol, 300 ng of poly(dI·dC), 300 ng of poly(dA·dT), and 20,000−40,000 cpm of oligonucleotide probe on ice for 20 min before loading onto a 5% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide 40:0.8) containing 40 mM Tris-glycine (see Figs. 2A–C, 3, and 7). Reactions were also performed at room temperature in 12 mM Hepes, pH 7.9, 12% glycerol, 60 mM KCl, 5 mM MgCl$_2$, 0.12 mM EDTA, 0.3 mM dithiothreitol, 20,000−40,000 cpm of oligonucleotide probe, 200 ng of single-stranded DNA, and 1 μg of poly(dI·dC) and then applied to 5% polyacrylamide gels containing 0.25× TBE (see Figs. 1 and 2, D and E). Antibodies against BETA2 were kindly provided by M. J. Tsai (Baylor College of Medicine, Houston, TX) and antibodies against E47 by M. Walker (Weizmann Institute of Science, Rehovot, Israel) and C. Murre (University of California, San Diego, CA). The USF antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

*Nuclear Extracts*—Nuclear extracts from BHK-21 and InR1G9 cells were prepared according to Schreiber *et al.* (20).

*In Vitro Transcription/Translations*—BETA2 and E47 proteins were synthesized *in vitro* with a coupled transcription/translation kit (Promega) according to the manufacturer's instructions, using T7 polymerase and linearized pCi-Neo-BETA2 and pCi-Neo-E47 DNA templates. Simultaneous reactions were carried out in the presence of unlabeled or [$^{35}$S]methionine. Synthesis and translation efficiency were assessed by analysis of labeled samples on a 12% SDS-polyacrylamide gel electrophoresis.

*Cell Cultures and Transient Transfections*—Glucagon-producing InR1G9 (21), insulin-producing HIT-T15 (22), and Syrian baby hamster kidney BHK-21 cells were cultured in RPMI 1640 medium containing 5% fetal calf serum and 5% newborn calf serum. InR1G9 cells were transfected by the DEAE-dextran method (23), HIT-T15 cells with Transfectam (Promega) according to the instructions of the manufac-
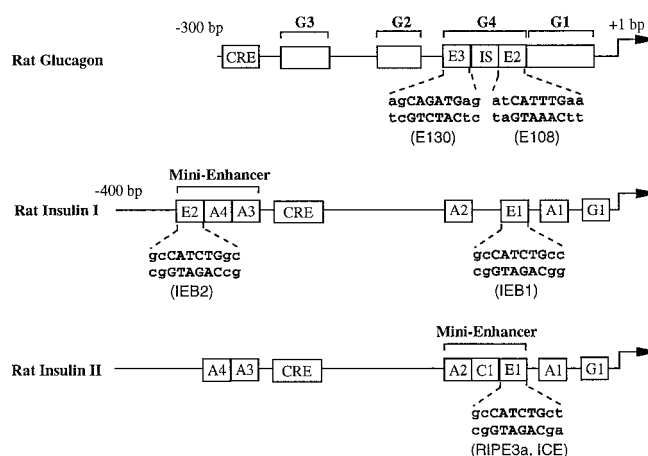


FIG. 1. **Cis-acting elements within the rat glucagon gene and insulin I and II genes promoters.** Defined DNA control elements are indicated by boxes corresponding to E, A, and G motifs, respectively, according to the new nomenclature (40). The sequences of the glucagon and insulin I and II genes E boxes are indicated below their respective positions and their previous names are given in *parentheses*.

turer, and BHK-21 cells by the calcium phosphate method. pRSVCAT and poCAT (24) were used as positive and negative controls, respectively. Cotransfection experiments were performed with reporter plasmids (different sequences of the glucagon or insulin gene promoters linked to the reporter gene CAT) and expression plasmids (pCi-Neo, pCi-Neo-BETA2, and/or pCi-Neo-E47) in a 10/1 ratio for Figs. 5, 6A, and 7, C and D and in the ratios indicated in the legends for Fig. 6, B–D.

*Chloramphenicol Acetyltransferase Assay*—Cell extract was prepared 48 h after transfection and assayed in duplicate for CAT activity as described previously (4). Each transfection was repeated at least six times. Acetylated and nonacetylated forms were quantified using a PhosphorImager (Molecular Dynamics) after separation by thin layer chromatography. Protein concentrations were measured by a Bio-Rad assay kit.

*Data Analysis*—Data are presented as mean ± S.E. and analyzed by analysis of variance and Student's t test when applicable. The threshold for statistical significance was $p < 0.05$.

## RESULTS

*IEF1 Is a Heterodimer of E47 and BETA2 and Binds to the E Boxes of the Insulin and Glucagon Gene Promoters*—We (6) recently characterized a composite element of the glucagon gene promoter, G4, which contains at least three binding sites, the most distal one (E3) acting as a functional E box (Fig. 1). We suggested that the IEF1 complex, previously shown to represent a critical determinant of the beta cell-specific expression of the insulin gene, could interact with E3 of the glucagon gene and thus be involved in the islet-specific expression of the glucagon gene. It has recently been proposed (17) that the likely equivalent of IEF1 which interacts with E1 (RIPE3a) of the rat insulin II gene is a heterodimer composed of E47, an ubiquitous bHLH protein, and BETA2, a class B bHLH protein present in the islets of Langerhans, the intestine, and the brain. We thus investigated whether these two proteins were indeed capable of binding to E3 of the rat glucagon gene and to E1 of the rat insulin I gene. Nuclear extracts prepared from the glucagon-producing cell line InR1G9 were incubated with oligonucleotides containing either E1 (IEB1) (Fig. 2A) of the rat insulin I gene or E3 (E130) (Fig. 2B) of the rat glucagon gene and analyzed by EMSAs. As reported previously (6) and shown in Fig. 2, the islet-specific IEF1 complex and several nonspecific complexes are observed with both oligonucleotides. The addition of either anti-E47 or anti-BETA2 antibodies supershifted or disrupted the IEF1 complex, whereas anti-USF antibodies had no effect (data not shown). Of note, migration of IEF1 with IEB1 was identical to that observed with E130; its intensity with E130, however, was only 1/10th of that seen with
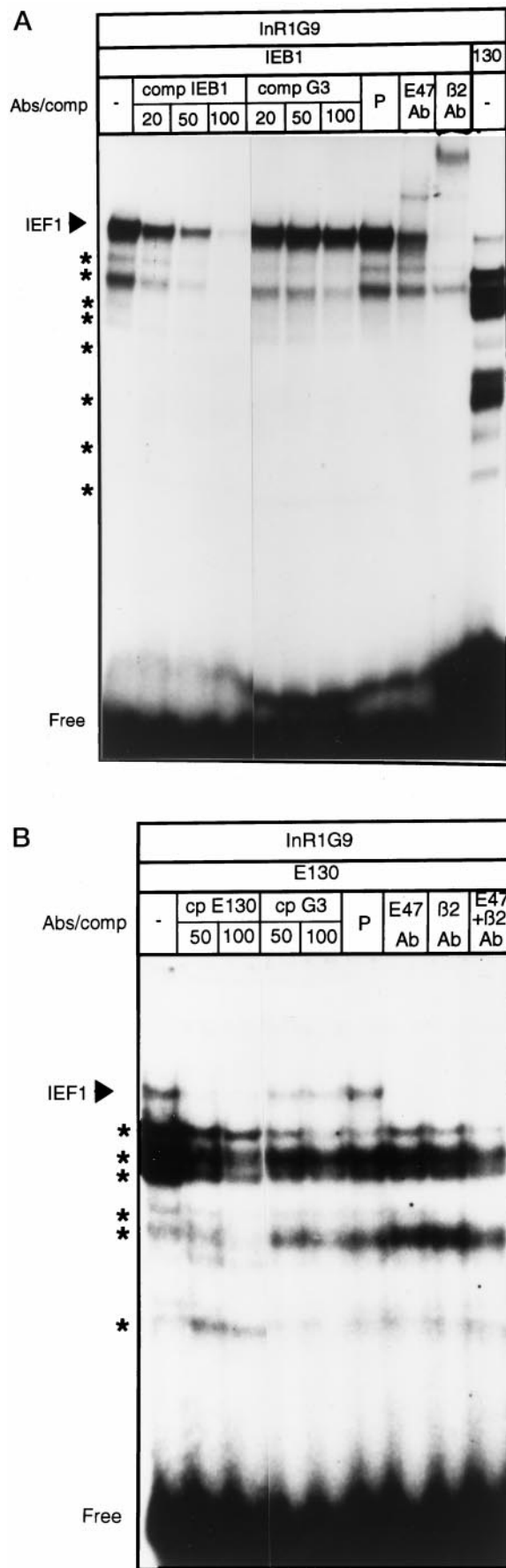
**A**

InR1G9

IEB1 | 130

| Abs/comp | - | comp IEB1 | | | comp G3 | | | P | E47 Ab | ß2 Ab | - |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 20 | 50 | 100 | 20 | 50 | 100 | | | | |

IEF1 ►
\*
\*
\*
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\*
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\*
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Free

**B**

InR1G9

E130

| Abs/comp | - | cp E130 | | cp G3 | | P | E47 Ab | ß2 Ab | E47 +ß2 Ab |
|---|---|---|---|---|---|---|---|---|---|
| | | 50 | 100 | 50 | 100 | | | | |

IEF1 ►
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\*
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\*
\*

\*

Free

FIG. 2. **E47 and BETA2 are present in glucagon producing cells and form the IEF1 complex.** EMSAs were performed with 10 μg of nuclear extracts from glucagon-producing InR1G9 and ³²P-labeled

IEB1, illustrating the lower affinity of IEF1 for E3 compared with E1 (Fig. 2*A*).

To better evaluate the interactions between E47/BETA2 and E1 and E3, we used nuclear extracts from BHK-21 cells transfected with expression vectors containing either E47, BETA2, or both cDNAs and *in vitro* synthesized E47 and BETA2 (Fig. 3). Overexpression of E47 in BHK-21 cells resulted in the appearance of a complex corresponding to E47 homodimers with both IEB1 (Fig. 3*A*) and E130 (Fig. 3*C*). This complex was disrupted by the addition of anti-E47 antibodies and displaced by an excess of cold oligonucleotide competitor (Fig. 3*A*). E47 homodimers were also observed when *in vitro* synthesized E47 was incubated with either IEB1 (Fig. 3*B*) or E130 (Fig. 3*D*). No specific complex was detected, however, with nuclear extracts from BHK-21 cells overexpressing BETA2 on either IEB1 (Fig. 3*A*) or E130, and similar results were obtained with *in vitro* synthesized BETA2 (Fig. 3*B*). The same observation was reported previously with E1 (RIPE3a) of the rat insulin II gene (17), indicating that BETA2 may not be able to form homodimers or at least not be able to bind to the different E boxes of the insulin and glucagon genes as homodimers.

Using nuclear extracts from BHK-21 cells transfected with both E47 and BETA2 cDNAs (Fig. 3, *A* and *C*) and *in vitro* synthesized E47 and BETA2 proteins (Fig. 3, *B* and *D*), we were able to reconstitute IEF1 on both IEB1 and E130. IEF1 comigrated with the complex formed by the *in vitro* synthesized E47 and BETA2 proteins (Fig. 3*B*) and was supershifted or displaced by the addition of anti-E47 and anti-BETA2 antibodies (Fig. 3, *B–D*). Bands migrating close to IEF1 were observed with untransfected BHK-21 cells as well as cells transfected with the E47 or BETA2 cDNAs (Fig. 3*A*). These bands, although not always present, migrated slightly faster when gels were run for longer time periods and were not supershifted or disrupted by the addition of anti-E47 or BETA2 antibodies. Of note, the intensity of the protein-DNA complexes obtained with *in vitro* synthesized E47 and BETA2 were lower compared with those obtained from transfected BHK-21 or InR1G9 cells. We conclude that IEF1 is a heterodimer of E47 and BETA2, which not only interacts with the rat insulin II gene promoter but also with both E1 of the insulin I gene and E3 of the glucagon gene.

To evaluate the respective affinity of E47 homodimers and E47/BETA2 heterodimers for IEB1, we used different ratios of *in vitro* synthesized E47 and BETA2 proteins (10/1, 5/1, 1/1) in the EMSA (Fig. 4). We observed preferential formation of E47/BETA2 and evaluated that E47/BETA2 had at least a 10-fold higher affinity for IEB1 compared with E47 homodimers. Our results thus indicate that when E47 is sufficiently abundant it can form homodimers and bind to E1 and E3, but with a lower affinity compared with the E47/BETA2 heterodimer. BETA2 by contrast is unable to bind as a homodimer.

*E47 Represses whereas E47/BETA2 Activates Glucagon Gene Transcription*—To investigate the functional roles of BE-TA2 and E47 on glucagon gene expression, we first performed cotransfection experiments in BHK-21 cells using the glucagon G4 element (see sequence of Fig. 5) linked to the first 31 bp of the glucagon gene promoter and 58 bp of the first exon as well as to the CAT reporter gene (G4-CAT) and expression vectors

IEB1 (*A*) and E130 (*B*) oligonucleotides (corresponding to the rat insulin I gene E1 box and the glucagon gene E3 box, respectively) as described under "Experimental Procedures." Competition (*comp* or *cp*) was performed with either 20, 50, or 100 ng of unlabeled IEB1 or of an oligonucleotide containing the G3 control element of the rat glucagon gene (4) (*A*) and 50 or 100 ng of E130 or of G3 (*B*). The islet-specific IEF1 complex is indicated on the side with a *bold arrowhead*, and \* corresponds to nonspecific complexes. *Ab*(s) indicates the addition of the indicated antibodies to the binding reaction with InR1G9 nuclear extracts. *Comp* designates competitor oligonucleotide.
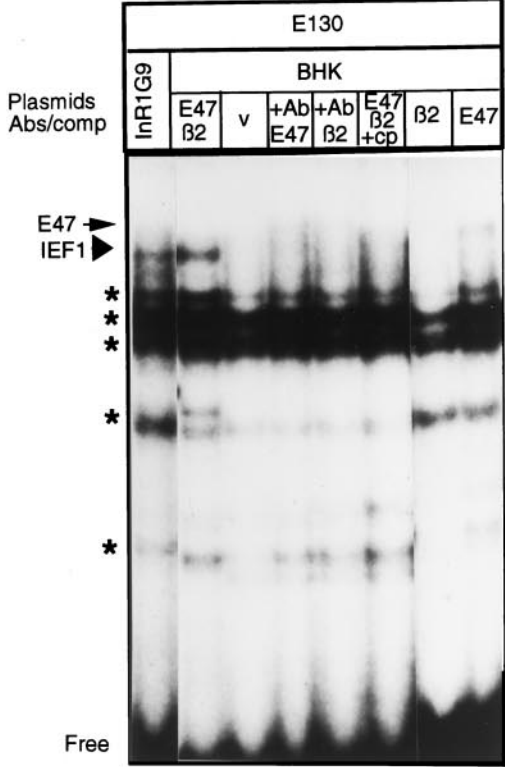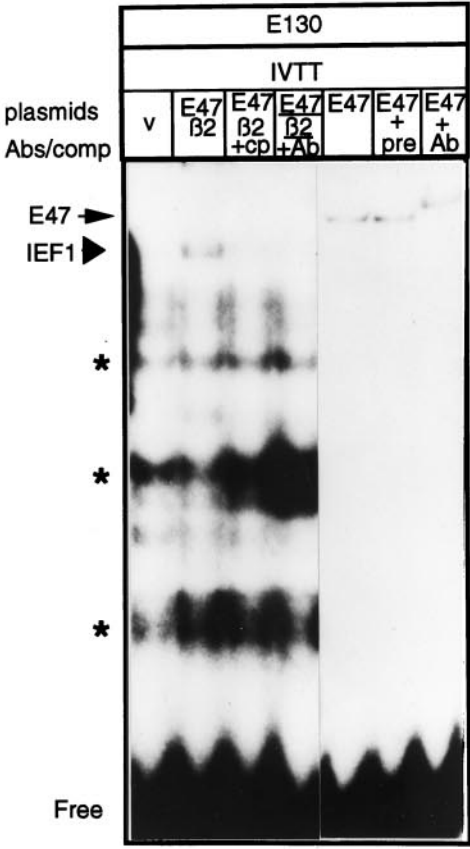
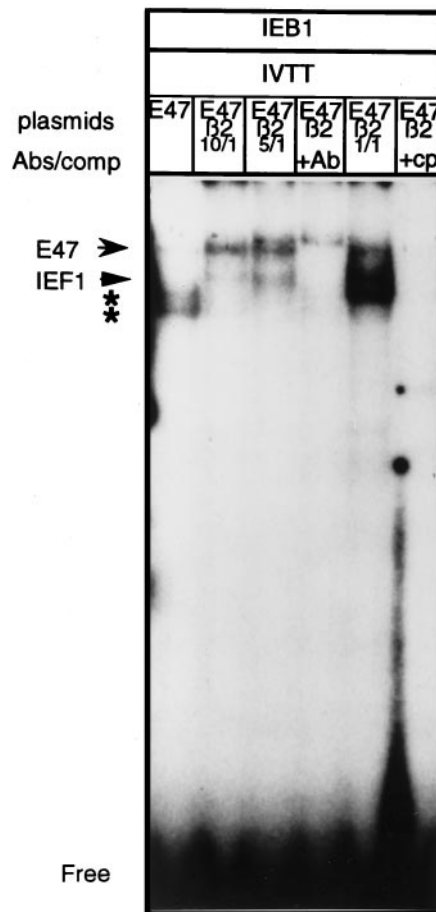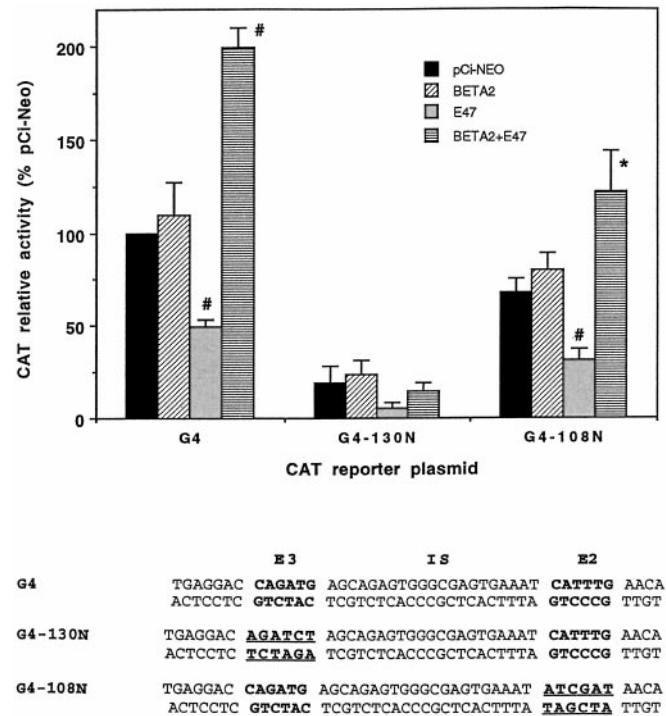*Glucagon and Insulin Gene Expression*
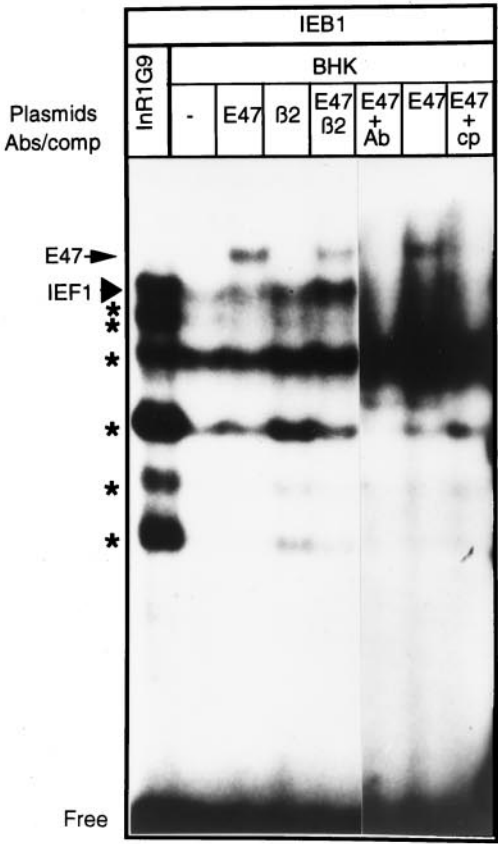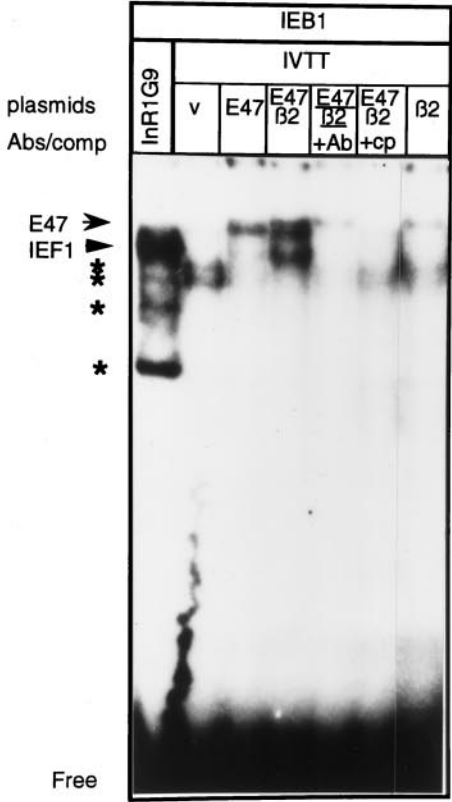
Fig. 4. **Binding affinity of E47 and E47/BETA2 for IEB1.** EMSAs were performed with *in vitro* transcription/translation (*IVTT*) products with the indicated expression plasmids and $^{32}$P-labeled IEB1 oligonucleotides as described under "Experimental Procedures." The ratio of E47 and BETA2 proteins as assessed by [$^{35}$S]methionine labeling is indicated. *Ab*(s) indicates the addition of antibodies to the *underlined* proteins; *cp*, specific competition with 50 ng of unlabeled IEB1.E47 homodimers and E47/BETA2 heterodimers (IEF1) are indicated on the side by *thin* and *bold arrowheads*, respectively and * corresponds to nonspecific complexes.



Fig. 5. **Effect of E47 and BETA2 on the transcriptional activity of the glucagon G4 element in InR1G9 cells.** Three $\mu$g of a CAT reporter plasmid containing two copies of the wild type (G4-CAT) or mutated (G4–130N-CAT and G4–108N-CAT) glucagon G4 element were cotransfected in glucagon-producing InR1G9 cells with 0.3 $\mu$g of a control expression vector with no insert (*pCi-NEO*), or E47 and/or BETA2 expression plasmids, respectively. In the experiments with both E47 and BETA2 cDNA-containing expression plasmids, 0.15 $\mu$g of each plasmid was transfected to keep the total amount of transfected DNA at 3.3 $\mu$g. Wild type and mutated G4 sequences are indicated below the diagram. E2 and E3 designate E boxes and IS, the intervening sequence. CAT activities were normalized to that of the wild type G4 reporter plasmid. Data are presented as mean ± S.E. of seven or four experiments for wild type and mutant G4 constructs, respectively. # and * indicate a significant effect of the expression plasmids on the CAT activity compared with the pCi-NEO vector ($p < 0.005$ and $p < 0.05$, respectively).

containing either the E47, BETA2, or both cDNAs. The G4 element was unable to direct significant expression of the CAT reporter gene in these non-islet cells, and CAT activity was not affected by cotransfection of any of the cDNAs (data not shown). Similar results were reported using the E1 (RIPE3a) element of the insulin II gene (17). Taken together with the fact that nuclear extracts from BHK-21 cells transfected with either the E47 or/and BETA2 cDNAs can form specific complexes with both E1 and E3 boxes, these results indicate that IEF1 by itself is not sufficient for transactivating the insulin and glucagon gene promoter in heterologous cells and that it likely requires additional cell-specific factors such as PDX1 (formally Idx1/IPF1/STF1) or RIPE3b to be functional (17, 18).

Cotransfection experiments performed in InR1G9 cells revealed that overexpression of BETA2 alone had no effect on G4-directed CAT activity (G4-CAT), whereas overexpression of E47 inhibited CAT activity by more than 50% (Fig. 5). In

contrast, overexpression of both E47 and BETA2 led to a 2-fold increase in transcriptional activity compared with G4-CAT alone and a 4-fold increase compared with the activity obtained with overexpression of E47. Similar results were obtained when the first 292 bp of the glucagon promoter were used to direct the CAT reporter gene (data not shown). Our data thus indicate that overexpression of E47 in glucagon-producing cells results in opposite regulatory effects on glucagon gene transcription compared with overexpression of both E47 and BETA2.

Because the G4 element contains an additional E-like motif (E2) separated from E3 by a short intervening sequence (Fig. 5), we wanted to ensure that the transcriptional effects of E47 and E47/BETA2 were mediated through specific interactions with E3. We thus investigated the effects of specifically mutated oligonucleotides representing G4, disrupting either E3 or E2 on transcriptional activity (Fig. 5). Disruption of E2 (G4–108N-CAT) led to a slight decrease in basal activity compared with the wild type sequence, but the modulatory effects of E47

Fig. 3. **Binding properties of E47.** EMSAs were performed with 10 $\mu$g of nuclear extracts from InR1G9 cells or BHK cells transfected with the indicated expression plasmids or 1–2 $\mu$l of *in vitro* transcription/translation (*IVTT*) products and $^{32}$P-labeled IEB1 (*A* and *B*) and E130 (*C* and *D*) oligonucleotides as described under "Experimental Procedures." *Ab*(s) indicates the addition of antibody to the *underlined* protein (antibodies to the *underlined* protein were added in *B–D*); *comp* or *cp*, specific competition with 50 ng of unlabeled oligonucleotides. IEF1 complexes and E47 homodimers are indicated on the side with *bold* and *thin arrowheads*, respectively, and * corresponds to nonspecific complexes. For IVTT products, mock reactions with the empty vector (*v*) were performed.

*Glucagon and Insulin Gene Expression*

**A**

**B**

**C**

**D**
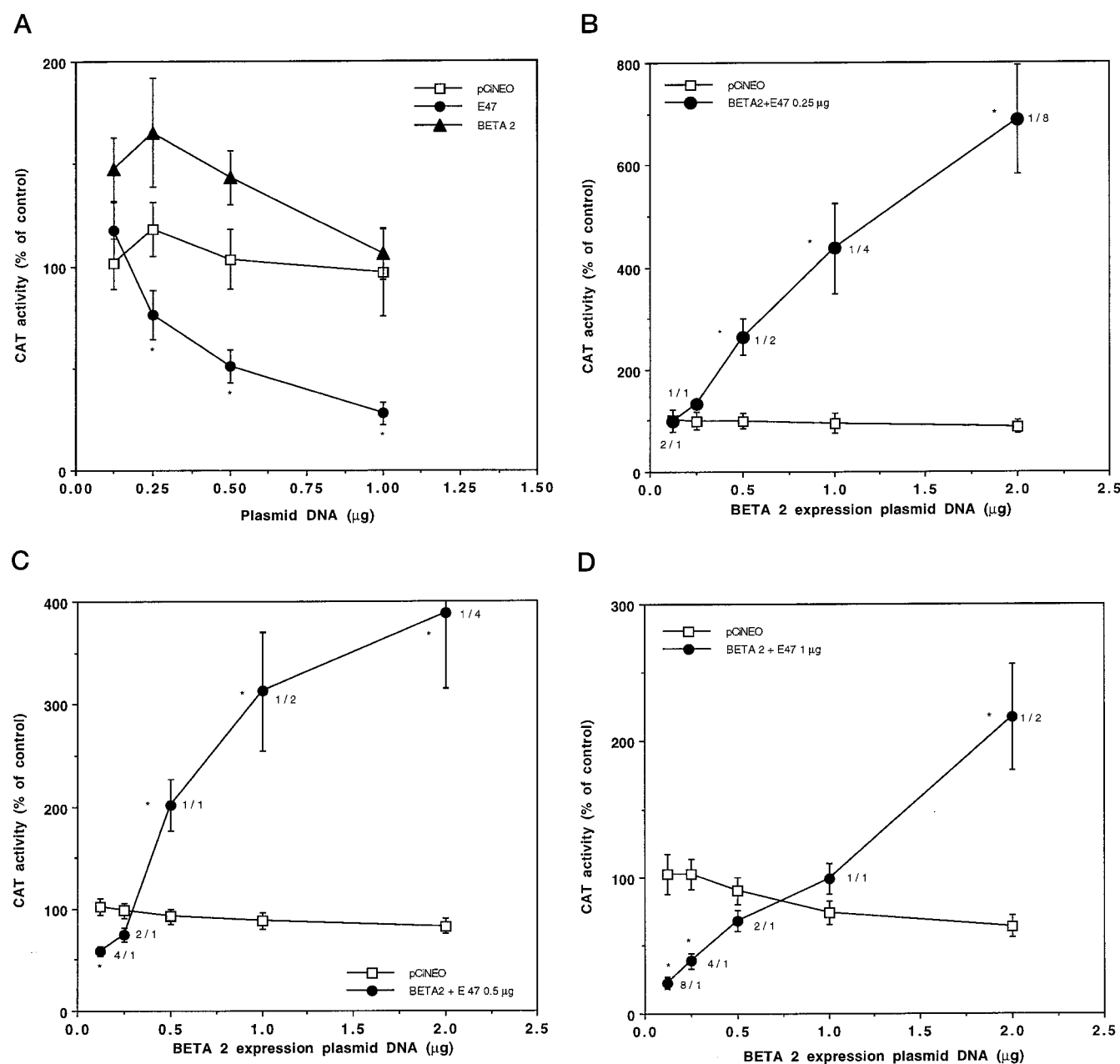
FIG. 6. **Dose-response analysis of E47 and BETA2 effects on transcriptional activity of the glucagon G4 element.** *A*, increasing amounts (0.125 to 1 $\mu$g) of pCi-NEO, E47, or BETA2 expression vectors were cotransfected in InR1G9 cells with 3 $\mu$g of the G4-CAT reporter plasmid. For each condition, total amount of transfected DNA were kept constant at 4 $\mu$g by adding appropriate amounts of pCi-NEO. *B*, increasing amounts of pCi-NEO (0.375 to 2.25 $\mu$g) or BETA2 expression vectors (0.125 to 2 $\mu$g) along with a constant amount of E47 expression vector (0.25 $\mu$g) were cotransfected in InR1G9 cells with 3 $\mu$g of the G4 reporter plasmid (the respective E47/BETA 2 ratios are indicated). *C*, increasing amounts of pCi-NEO (0.625 to 2.5 $\mu$g) or BETA2 expression vectors (0.125 to 2 $\mu$g) along with a constant amount of E47 expression vectors (0.5 $\mu$g) were cotransfected in InR1G9 cells as in *B* (the respective E47/BETA2 ratios are indicated). *D*, increasing amounts of pCi-NEO (1.125 to 3 $\mu$g) or BETA2 expression vectors (0.125 to 2 $\mu$g) along with a constant amount of E47 expression vector (1 $\mu$g) were cotransfected in InR1G9 cells as in *B* (the respective E47/BETA2 ratios are indicated). For each condition, total amount of transfected DNA was kept constant at 6 $\mu$g by adding the appropriate amounts of pCi-NEO (*B–D*). All CAT activities were normalized to that of the reporter plasmid alone. Data are presented as mean $\pm$ S.E. of four experiments. The * indicates a significant effect of the expression plasmids on CAT activity compared with the same amount of pCi-Neo vector ($p < 0.05$).

and E47/BETA2 overexpression were similar to that observed with G4-CAT. A mutation of the E3 motif (G4−130N-CAT), on the other hand, resulted in a marked decrease in basal activity and a loss of any significant modulatory effects of E47 and E47/BETA2. Although E47 overexpression was still able to slightly depress activity, it did not result in any significant difference compared with vector alone. These data confirm that E47/BETA2 exert their effects on glucagon gene transcription through a specific interaction with E3.

To further characterize the effects of E47 and BETA2 on

glucagon gene transcription, we performed a dose-response analysis of their transcriptional potential on the G4 element. Transfection of InR1G9 cells with G4-CAT and increasing amounts of expression plasmids containing the E47 cDNA resulted in a dose-dependent inhibition of transcriptional activity to a maximum of about 70% (Fig. 6*A*). Similar results were obtained when the reporter gene was linked to the first 292 bp of the glucagon gene promoter (data not shown). Cotransfection of G4-CAT with increasing amounts of the BETA2 expression plasmid, by contrast, did not significantly affect CAT activity,

although we noted a small increase at the lowest amounts of transfected BETA2 cDNA (Fig. 6*A*). When a constant amount of E47 expression plasmid (either 0.25, 0.5, or 1 $\mu g$) was cotransfected with increasing amounts of BETA2 cDNA, we observed a dose-dependent increase in transcription compared with cotransfections with the expression vector alone (Fig. 6, *B–D*). The dose-dependent increase in transcription induced by BETA2 was modulated by the amount of E47 expression plasmid; the increase was maximal with 0.25 $\mu g$ of E47 cDNA cotransfected, whereas it was minimal with 1 $\mu g$. Of note, the E47/BETA2 ratio was of major importance in determining the effects on transcriptional activity. A ratio $\leq 1$ resulted in activation of transcription by more than 2-fold, whereas a ratio >1 resulted in a minor increase, no change, or an inhibition of transcription (Fig. 6, *B* and *C*). We conclude that E47 and E47/BETA2 have antagonistic regulatory effects on glucagon gene transcription, with E47 acting as an inhibitor and the heterodimer E47/BETA2 as an activator of transcription.

*Differential Regulatory Effects of E47 and BETA2 on the E Boxes of the Rat Insulin I and Glucagon Genes*—The inhibitory effects of E47 on glucagon gene transcription are opposite to what has been previously reported for the rat insulin I and II genes. Overexpression of E47 in insulin-producing cell lines results in an activation of reporter genes driven by the E1 box of the rat insulin II gene (17). In addition, transfection of antisense oligonucleotides to the E47 mRNA in HIT cells leads to a decrease of insulin mRNA levels (25). To further investigate the different regulatory effects of E47 and BETA2 on glucagon and insulin gene expression, we cotransfected reporter gene constructs driven by the different E boxes of the rat insulin I and glucagon genes along with expression plasmids coding for E47 or BETA2 into glucagon-producing InR1G9 cells and the insulin-producing cell line HIT-T15. The transfected reporter genes were driven by either two copies of G4 (G4-CAT) or E3 (E130-CAT) of the glucagon gene or by two copies of either E1 (IEB1-CAT) or E2 (IEB2-CAT) of the rat insulin I gene. All of these constructs resulted in a significant basal activity in both InR1G9 and HIT-T15 cells (Fig. 7, *A* and *B*), indicating that the E boxes of the insulin and glucagon gene promoter function in both cell lines, a finding in agreement with the presence of IEF1 in these cells. Of note, IEB1-CAT displayed the greatest basal activities in both InR1G9 and HIT-T15 cells compared with G4-CAT or E130-CAT; our transcriptional data thus correspond qualitatively to the degree of IEF1 binding affinity to IEB1 relative to E130 (Fig. 2*A*).

Overexpression of E47, BETA2, and E47/BETA2 in InR1G9 cells led to similar qualitative effects on G4-CAT and E130-CAT (Figs. 5 and 7*C*) confirming that these protein complexes act specifically through E3. These effects were clearly different in HIT-T15 cells inasmuch as basal activities of both reporter constructs were not significantly affected by any of the cotransfected cDNAs. Our results underline the differences between insulin- and glucagon-producing cells and suggest that additional factors present in InR1G9 but not in HIT-T15 cells may play a role in the IEF1-mediated regulatory effects on E3. Alternatively, the basal levels of specific bHLH or of additional proteins such as Id may be different in these cells and allow for distinct effects of overexpressed E47 and/or BETA2 on E3-mediated transcription.

In contrast to the transcriptional repression of G4-CAT and E130-CAT by overexpressed E47, a 2–4-fold increase in CAT activity is observed in cotransfections of the E47 cDNA expression plasmid with IEB1-CAT or IEB2-CAT into both InR1G9 (Fig. 7*C*) and HIT-T15 (Fig. 7*D*) cells. Overexpression of BETA2 resulted in a smaller increase, 1.5–2-fold, whereas E47

and BETA2 together led to a 3–7-fold transcriptional activation of IEB1-CAT or IEB2-CAT in both cell types.

The marked contrast in the effects of E47 overexpression on E3-containing constructs and the differential quantitative effects on IEB1-CAT and IEB2-CAT in InR1G9 cells suggests that the E box motif and its surrounding base pairs play a critical role in the modulatory effects of E47 on insulin and glucagon gene expression. Interestingly, overexpression of E47 in InR1G9 cells led to an increase in the IEF1 complex binding to IEB1, whereas no change was noted for E130, strengthening the hypothesis that sequences of the E box motif play a role in the E47-mediated effects (Fig. 8). No quantitative change in IEF1 intensity was observed with nuclear extracts from InR1G9 cells transfected with the BETA2 cDNA. The correlation between the degree of IEF1 binding affinity for a defined E box and transcriptional activation noted above is well illustrated by the observation that overexpression of E47 and BETA2 in InR1G9 results in a markedly enhanced IEF1 complex on IEB1 compared with E130 (Fig. 8).

## DISCUSSION

Our results indicate that the heterodimer E47/BETA2 is able to bind and transactivate the E boxes of the rat glucagon and insulin I genes. Taken together with similar results from Naya *et al.* (17) on the E1 box of the insulin II gene, E47/BETA2 can be considered to represent IEF1. Originally thought to be critical for beta cell-specific expression of the insulin gene (14, 26, 27), IEF1 was later shown to be present not only in beta cells but also in alpha cells of the islets of Langerhans (6, 28). Furthermore, BETA2 is detected in the mouse intestinal enteroendocrine S-cell line (STC-1) which is expressed in the glucagon gene, the hamster intestine, the mouse corticotroph pituitary cell line (AtT20), and the brain (17). IEF1 may thus have a much wider role than previously proposed for the cell-specific expression of the insulin gene. In fact, our data suggest that IEF1 may exert a critical role in the control of glucagon gene expression. IEF1 binds to E3 of the composite G4 element and transactivates the glucagon gene promoter only in the presence of an intact E3 box. In addition, basal transcriptional activity of the glucagon gene is decreased by 75% when the E3 motif is disrupted (6) (Fig. 4). Finally, we have found BETA2 transcripts by reverse transcriptase-polymerase chain reaction analysis in rat primary islet non- beta cells (essentially alpha and delta cells) in roughly the same abundance as in primary beta cells, suggesting that BETA2 may also be operative in normal alpha cells (data not shown). The fact that BETA2 is present in the STC-1 cell line, the intestine, and the brain, which all express the glucagon gene, further suggests a role for IEF1 in glucagon gene expression. IEF1 may thus represent an important factor in the control of islet hormone gene expression, at least for the insulin and glucagon genes.

The bHLH family of transcription factors not only regulates the activity of many genes but is also critical in specifying cell fate as reported for muscle differentiation (8, 29, 30), neurogenesis (31), hematopoesis (9, 32), and sex determination (33). Although the presence of BETA2 in tissues of different embryological origins does not facilitate the attribution of an obvious role in cell-type determination, BETA2 may still have different functions in the ontogenesis of distinct tissues. Its presence in the intestine and the islets of Langerhans opens the possibility that it may be involved in the differentiation of endoderm-derived tissues.

The combinatorial character of bHLH factors is certainly of major importance for their regulatory capacity. Increased synthesis of one member of the family relative to the others may shift the homodimer/heterodimer equilibrium and result in changes in gene transcription. In this regard, overexpression of

*Glucagon and Insulin Gene Expression*
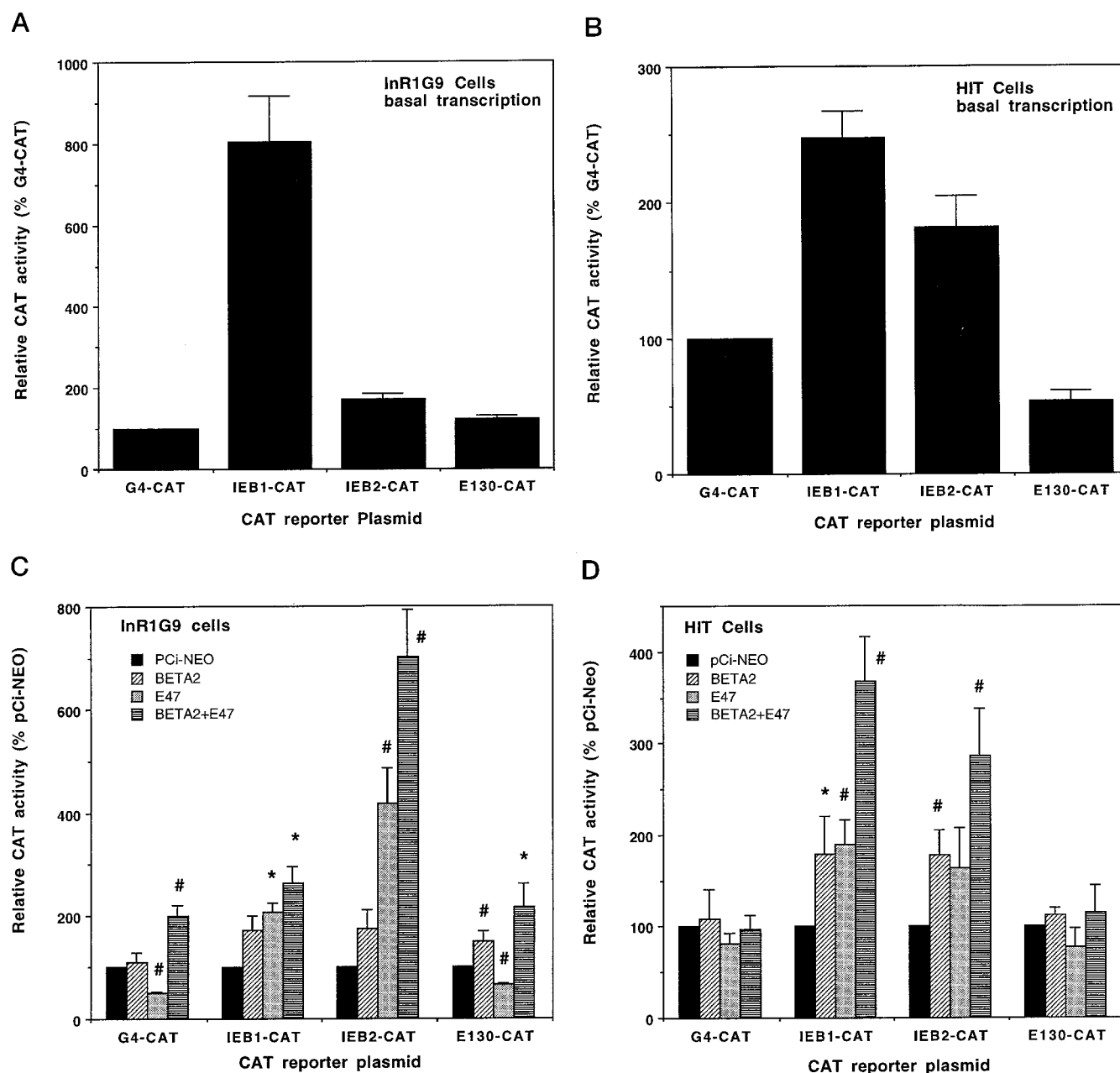
FIG. 7. **Comparative effect of E47 and BETA2 on the transcriptional activity of different E box sequences in glucagon- and insulin-producing cells.** CAT reporter plasmid constructs containing two copies of the glucagon gene element G4 (*G4-CAT*), two copies of the insulin I gene E1 box (*IEB1-CAT*), two copies of the insulin I gene E2 box (*IEB2-CAT*), or two copies of the glucagon gene E3 box (*E130-CAT*), respectively, were transfected into InR1G9 (*A*) and HIT (*B*) cells to evaluate their basal activity. The basal activity of each reporter construct in InR1G9 and HIT cells expressed relatively to G4-CAT is indicated in *A* and *B*, respectively. The control vector pCi-NEO as well as E47, BETA2, and E47 + BETA2 expression plasmids were cotransfected in InR1G9 (*C*) and HIT-T15 (*D*) cells with the same DNA constructs as above. CAT activities were normalized to that of the respective reporter plasmid cotransfected with the control pCi-NEO vector. Data are presented as mean ± S.E. of four to nine experiments. # and * indicate a significant effect of the expression plasmids on the CAT activity compared with the pCi-NEO vector ($p < 0.01$ and $p < 0.05$, respectively).

E47 or BETA2 in islet cell lines has revealed an interesting modulating potential on E box-mediated transcription by such changes in bHLH equilibrium. E47 inhibits E3-mediated transcriptional activity in InR1G9 cells while it stimulates E1 and E2-dependent transcription in both InR1G9 and HIT-T15 cells. The opposite transcriptional effects of E47 on the glucagon E3 and the insulin E1 and E2 boxes may either be explained by the cell environment, by E box sequence specificity, or by both. Since E47 can exert both negative and positive transcriptional effects in InR1G9 cells, it is likely that E box sequence specificity plays a major role. Sequence comparison between the glucagon gene E3 element and the E1 and E2 box of the insulin

I and II genes, respectively, reveals that their core motif is identical. However, the E3 box is inverted 3′ to 5′ compared with the insulin gene E1 and E2 boxes and surrounding nucleotides, which have been shown to be critical for both binding specificity and affinity are different. Of major relevance, E3 has one flanking T residue which may decrease binding of bHLH proteins such as E47 (34, 35). A comparable role of flanking nucleotides was also observed in the transcriptional modulation of tissue-specific genes by MyoD (30). These characteristics may well explain the difference in the IEF1 complex observed with nuclear extracts from InR1G9 cells or from cells transfected with the E47 cDNA on E130 compared with IEB1, as
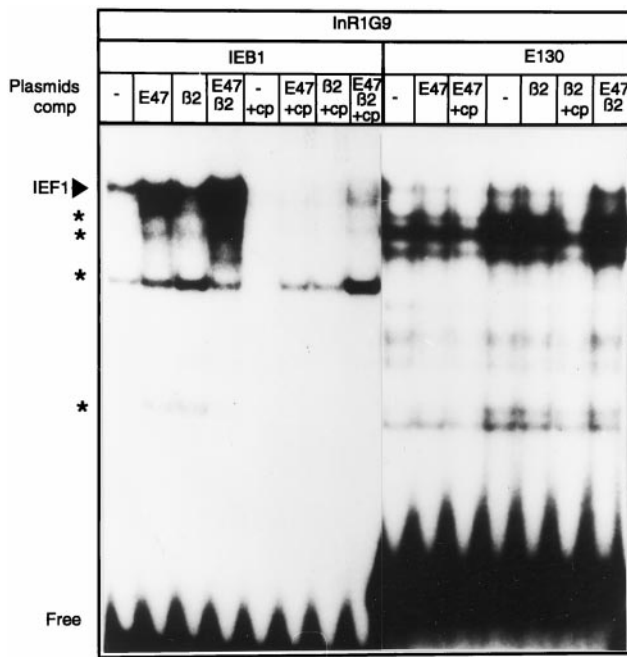
Fig. 8. **Overexpression of E47 and BETA2 in InR1G9 cells modifies IEF1 binding activity on the insulin and glucagon E box sequences.** EMSAs were performed with 10 μg of nuclear extracts from InR1G9 cells transfected with the indicated expression plasmids and $^{32}$P-labeled IEB1 and E130 oligonucleotides as described under "Experimental Procedures." The IEF1 complex is indicated on the side with a *bold arrowhead*, and * corresponds to nonspecific complexes. *Comp* or *cp* indicate the addition of specific unlabeled oligonucleotides.

well as the opposite effects of E47 on E3 *versus* E1- and E2-mediated transcription.

Although E47/BETA2 heterodimers have a higher affinity compared with E47 homodimers for the E boxes of the glucagon and insulin genes and thus must be functionally favored in native cells, major changes in the abundance of one or more bHLH proteins may result in the selection of E47 homodimers to act on E boxes. Due to the combinatorial characteristics of HLH proteins, the changes may not be specific for E47 or BETA2 since formation and binding of E47 homodimers to E boxes may be secondary to heterodimerization with bHLH or HLH proteins such as Id or to indirect effects due to shifting equilibria within the extended bHLH/HLH pool. In this direction, we have detected similar levels of E47 mRNA in InR1G9 and HIT-T15 cells but an 8–10-fold higher abundance of Id-1 mRNA levels in InR1G9 compared with HIT-T15 cells.[2] The higher levels of Id proteins in InR1G9 cells may indirectly result, depending on their relative affinity for E47 and BETA2, in the preferential formation of either E47 homodimers or E47/BETA2 heterodimers by capturing selectively one of the partners.

Posttranscriptional modifications of E47, such as the degree of phosphorylation, may also affect the ratio of E47 homo- to heterodimers (36). The opposite effects of E47 on glucagon and insulin gene transcription may have relevance for gene regulation by physiological stimuli. Indeed, insulin and glucagon secretion are often inversely related and it may be hypothesized that factors involved in the control of glucagon and insulin secretion may also play a role in gene expression and potentially mediate their effects through the E box motif. In that regard, it is of interest to note that E2 of the rat insulin I gene has been suggested to mediate, at least in part, the glucagon

effects on insulin gene expression. These studies indicate that IEF1 increases in intensity with increasing glucose concentrations, and that overexpression of E47 can ablate the transcriptional response to glucose (37).

The transactivating potential of E47/BETA2 in our transfection experiments was found to be variable, from zero in BHK-21 cells to 7-fold for IEB2-CAT in InR1G9 cells. The absence of effects of E47 homodimers or E47/BETA2 heterodimers on E box-mediated transcription in non-islet cells has been noted previously (17) and may result from the fact that these complexes require additional DNA binding proteins to develop their full potential and exert synergistic effects on transcription. Such functional interactions have been suggested between E47/BETA2 and the 3b-binding complex on RIPE3 of the rat insulin II gene and between E47 and PDX-1 (formally STF-1/IDX-1/IPF-1) on the minienhancer of the rat insulin I gene (17, 38). The characteristics of the cell lines used are likely to be of major importance in the resultant levels of basal and stimulated transcriptional activity of specific plasmids. For instance, IEB1-CAT and IEB2-CAT transfections into two different insulin-producing cell lines, RIN2A and HIT-T15 cells, resulted in 25 and 49% (IEB1-CAT) and 37 and 87% (IEB2-CAT), respectively, of the activity generated by a plasmid containing the enhancer (−346 to −103 bp) of the rat insulin I gene (39). These differences observed in cell lines exhibiting the same phenotype alert us to interpret with caution the variations found in the E box-mediated transcription in InR1G9 cells compared with HIT-T15 cells. Indeed, even though our results show major differences in basal transcriptional activity of IEB1-CAT and IEB2-CAT and in E47 and E47/BETA2-mediated regulation of transcription between InR1G9 cells and HIT-T15 cell lines, some of these differences may not be attributed to cell phenotypes *per se* but to random cell characteristics. In conclusion, our findings indicate that E47 and BETA2 may play a major role in islet cell-specific glucagon and insulin gene expression and that their respective levels may affect regulated expression.

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**Differential Regulation of the Glucagon and
Insulin I Gene Promoters by the Basic
Helix-Loop-Helix Transcription Factors
E47 and BETA2**

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and Jacques Philippe