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# Pancreatic β-Cell-Type-Specific Transcription of the Insulin Gene Is Mediated by Basic Helix-Loop-Helix DNA-Binding Proteins

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**The pancreatic β-cell-specific expression of the insulin gene is mediated, at least in part, by the interaction of unique *trans*-acting β-cell factors with a *cis*-acting DNA element found within the insulin enhancer (5′-GC CATCTG-3′; referred to as the insulin control element [ICE]) present in the rat insulin II gene between positions −100 and −91. This sequence element contains the consensus binding site for a group of DNA-binding transcription factors called basic helix-loop-helix proteins (B-HLH). As a consequence of the similarity of the ICE with the DNA sequence motif associated with the *cis*-acting elements of the B-HLH class of binding proteins (CANNTG), the ability of this class of proteins to regulate cell-type-specific expression of the insulin gene was addressed. Cotransfection experiments indicated that overexpression of Id, a negative regulator of B-HLH protein function, inhibits ICE-mediated activity. Antibody to the E12/E47 B-HLH proteins attenuated the formation, in vitro, of a previously described (J. Whelan, S. R. Cordle, E. Henderson, P. A. Weil, and R. Stein, Mol. Cell. Biol. 10:1564–1572, 1990) β-cell-specific activator factor(s)-ICE DNA complex. Both of these B-HLH proteins (E12 and E47) bound efficiently and specifically to the ICE sequences. The role of B-HLH proteins in mediating pancreatic β-cell-specific transcription of the insulin gene is discussed.**

The insulin gene accounts for a large fraction of the total gene expression in pancreatic β cells, yet it is expressed at virtually undetectable levels in all other cell types (10). Pancreatic β-cell-specific expression is controlled at the transcriptional level by 5′-flanking insulin gene enhancer sequences (12, 15, 18, 20, 37, 41). Cell-specific enhancer expression is mediated predominantly by the insulin control element (ICE) (22–24, 40, 41), whose core sequence motif, 5′-GCCATCTG-3′, is found within the transcription unit of all the insulin genes characterized so far (9). This element is regulated by both positive- and negative-acting cellular transcription factors (41). Recent studies have demonstrated that positive activation, mediated by the ICE, is regulated through the binding of factors unique to β cells (31, 40).

The ICE contains the core sequence CANNTG, found in heavy-chain immunoglobulin and muscle creatine kinase enhancer elements (5, 17, 27, 28). A common amino acid sequence motif is present in the proteins that bind to and positively regulate the expression of the heavy-chain immunoglobulin (i.e., E12 and E47) and the muscle creatine kinase (i.e., MyoD) enhancers (29). This motif consists of a helix-loop-helix domain (HLH), which is composed of two segments capable of forming amphipathic α helices connected by a nonconserved loop region that is important in protein-protein interactions, and a basic region which is involved in DNA-protein interactions and is amino terminal to the HLH domain. The combined motif is referred to as the B-HLH region (13, 29, 30). This motif is common to a number of transcription factors involved in cell type determination, including the muscle determination proteins MyoD (14, 26, 34), Myf-5 (3), and myogenin (4, 42), proteins of the *Drosophila* achaete-scute complex important in neural determination (1, 6, 36), and genes essential for cell type determination in *Drosophila* cells, such as daughterless (7, 8, 11), hairy (32), twist (34), and Enhancer of split (25).

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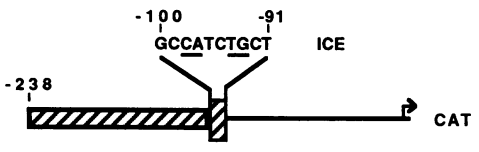
**B-HLH proteins regulate ICE activity in vivo.** To determine whether the B-HLH class of proteins was also important in regulating ICE activity in the β cell, we tested whether Id, a negative regulator of B-HLH protein function (2), affected ICE-dependent transcriptional activation. Two insulin-producing cell lines, HIT T-15 2.2.2 (HIT [15]) and β TC-1 (16), were cotransfected with wild-type or mutant ICE-driven insulin enhancer expression plasmids and either an Id sense (E:Id[S]) or an Id antisense (E:Id[A]) expression plasmid. We reasoned that if B-HLH proteins interacted with the ICE, (over)expression of Id[S] would reduce ICE-dependent transcriptional activation. The results of an experiment which tests this hypothesis are shown in Fig. 1. A threefold reduction in wild-type insulin enhancer-stimulated activity is observed in β cells transfected with Id[S] relative to cells transfected with Id[A]. Id[S] inhibits the activity of the endogenous factors (i.e., MyoD and E12) required for muscle creatine kinase enhancer expression to a similar extent (see Fig. 6B in reference 2). Deletion of the ICE target sequences severely decreased the negative effect of Id[S] on insulin enhancer function (Fig. 1, lower diagram). The basal activity of the ICE mutant is reduced approximately threefold relative to the wild-type enhancer-promoter expression plasmid in transfected HIT and β TC-1 cells (41). This indicated that the ICE site was the site of Id[S]-mediated repression.

To more precisely determine that the ICE was actually the target of Id repression, we analyzed the effect of Id on expression of a single-element ICE reporter plasmid. The construct (ICE OVEC) contains three copies of the ICE inserted directly upstream of the rabbit β-globin TATA box and coding sequences. We previously showed that expression of ICE OVEC is β-cell specific and entirely dependent upon the ICE (41). The level of expression of ICE OVEC in HIT cells transfected with either Id[S] or Id[A] was compared. Only Id[S] was able to inhibit ICE-driven β-cell transcription (Fig. 2B, compare lanes 2 and 3 with lane 1). The residual activity was comparable to that in cells trans-




| Conditions | FOLD REPRESSION HIT | (n) | β TC-1 | (n) |
|---|---|---|---|---|
| + Id[S] | 2.75 +/- 0.25 | (5) | 3.20 +/- 0.30 | (5) |
| + Id[A] | 0.75 +/- 0.20 | (5) | 0.90 +/- 0.12 | (5) |
| + Id[S] | 1.05 +/- 0.12 | (4) | 1.15 +/- 0.10 | (4) |
| + Id[A] | 0.95 +/- 0.10 | (4) | 1.03 +/- 0.05 | (4) |

FIG. 1. The Id protein product inhibits insulin enhancer activity. The structures of the rat insulin II gene enhancer-promoter expression–chloramphenicol acetyltransferase (CAT) expression plasmids used are shown to the left. These expression plasmids were prepared as described previously (41). Symbols: ▨, insulin enhancer sequences; ▬, promoter sequences (these regions are not drawn to scale). The rat insulin II promoter sequences in these plasmids comprise residues −90 to +8. The insulin enhancer-promoter expression plasmids were introduced into HIT or β TC-1 cells as calcium phosphate coprecipitates made up of 2.5 μg of the insulin-CAT plasmid and 7.5 μg of either E:Id[S], E:Id[A], or carrier DNA (pUC19) under conditions described previously (41). E:Id[S] and E:Id[A] contain the mouse Id cDNA cloned into EMSV-scribe expression vector in either the sense (S) or antisense (A) orientation, respectively (2). Cells were harvested for CAT assays 40 to 48 h after transfection. CAT enzyme assays were performed as described previously (19). Fold repression by Id is expressed as the ratio of the CAT enzyme activity in the absence of E:Id divided by the level of CAT activity in the presence of E:Id. All data are presented as means and standard errors of the mean; n indicates the number of times each transfection was performed.

fected with the cloning vector alone, OVEC-1 (Fig. 2B, compare lanes 2 and 4 with lane 1). Together with the insulin enhancer data described above, these results suggest that Id either directly or indirectly inhibits the activity of B-HLH proteins found in pancreatic β cells that are necessary for ICE-stimulated activity.

**The proteins which compose the pancreatic β-cell-specific insulin activator factor-ICE complex detected in vitro are members of the B-HLH structural class.** It has recently been shown by others that Id functions by disrupting (competing for) protein-protein interactions formed between gene-spe-

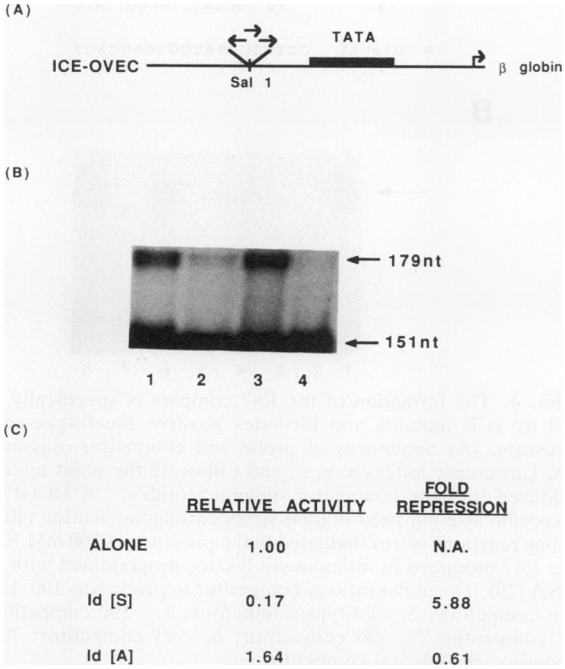



FIG. 2. Repression of ICE-driven activity by Id protein products. (A) Schematic representation of the ICE OVEC reporter gene construct used. Three copies of an oligonucleotide containing rat insulin II gene ICE sequences from −102 to −87 were cloned into the *SalI* site of the OVEC-1 expression vector (41). The orientations of the ICE inserts are indicated by the arrows. (B) Autoradiogram of RNase protection analyses. HIT cells were transfected with 5 μg of plasmid ICE OVEC; 5 μg of either E:Id[S], E:Id[A], or pUC19; and an internal control plasmid, OVEC-REF (0.25 μg). OVEC-1 (5 μg) was cotransfected with pUC19 (5 μg) and OVEC-REF (0.25 μg). Protected RNase protection fragments of 179, 179, and 151 nucleotides (nt) are expected for the correctly initiated ICE OVEC, OVEC-1, and OVEC-REF transcripts, respectively (41). The transcripts from ICE OVEC and OVEC-1 differ from OVEC-REF because of a 28-nucleotide deletion of the globin sequences between −10 and +19 in the OVEC-REF construct (see references 39 and 41 for details). Lanes: 1, ICE OVEC alone; 2, ICE OVEC plus E:Id[S]; 3, ICE OVEC plus E:Id[A]; 4, OVEC-1 alone. (C) Fold repression of ICE-driven transcription by Id. Relative amounts of correctly initiated transcripts were determined by densitometric scanning of the autoradiogram and were normalized relative to the OVEC-REF internal control signal. The fold repression was calculated as the ratio of densitometric units of signal produced from ICE OVEC in the absence of E:Id divided by the units of signal produced in the presence of E:Id.

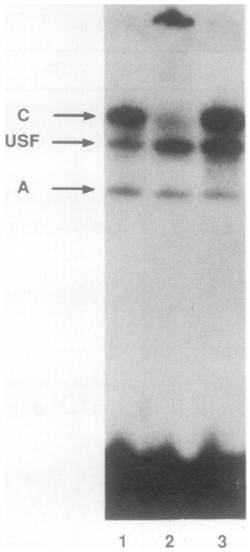



FIG. 3. Anti-E12/E47 antibody inhibits formation of the β-cell-specific factor–ICE complex. Binding and gel electrophoresis were conducted with a $^{32}$P-labeled rat insulin ICE probe containing residues −104 to −85 with β TC-1 extracts as described previously (40). The anti-E12/E47 antibody (2 μl) and control preimmune serum (2 μl) were preincubated with the β TC-1 extracts for 20 min at room temperature before initiation of the DNA-binding reactions. Binding reactions were conducted at 50 mM KCl. The antibody to E12/E47 was generated against the C-terminal 430 amino acids of E12 and recognizes epitopes on both E12 and E47 (30). The major bands discussed in the text are labeled. Lanes: 1, control; 2, plus anti-E12/E47 antibody; 3, plus preimmune serum.

cific and general transcription factors (2). Consequently, Id appears to prevent formation of the MyoD-E12 heterodimer in muscle cells, thus inhibiting or preventing creatine kinase gene transcription. Id appears to interact preferentially with the E12 protein in this complex (2). To examine the potential involvement of the E2A gene products E12 and E47 on ICE-driven activity, we examined whether an antibody raised to the E12/E47 proteins affected the formation of ICE-protein complexes assayed in the gel mobility shift assay. Standard binding reactions were conducted by using a β TC-1 extract and a $^{32}$P-labeled ICE element probe. The binding reactions were conducted in the presence of 50 mM KCl. Three protein-DNA complexes are routinely detected under these conditions (40). These complexes are labeled in Fig. 3 as A, USF, and C in order of decreasing mobility. The C and USF complexes contain the ICE *trans*-activator factor(s) and the adenovirus type 2 upstream transcription factor, respectively (40). The C complex is detected only in β-cell extracts (40). USF binds to ICE sequences only when the binding assay is conducted at low KCl concentrations (i.e., 50 versus 200 mM [40]). When anti-E12/E47 antibody is preincubated with extract and then added to the ICE probe, most of the C complex is supershifted (Fig. 3, compare lanes 1 and 2). This is a specific reaction since the amounts of the A and USF complexes are not reduced by either the immune serum or the preimmune serum (Fig. 3, compare lanes 1, 2, and 3). The increased level of the USF and C complexes detected in the presence of serum appears to be nonspecific since both preimmune and immune sera have the same effect. It thus appears that the ICE *trans*-activator complex contains either E12, E47, or a protein antigenically related to these proteins.

**B-HLH proteins E12 and E47 bind specifically to ICE sequences.** To directly test whether E12 or E47 could bind to the ICE (as either homo- or heterodimers), we made E12 and E47 proteins in a reticulocyte lysate system. The ICE-binding properties of these in vitro-translated proteins were analyzed in the gel retardation assay. The specificity of binding of these proteins to ICE sequences was determined in competition assays in which both wild-type and mutant alleles of the ICE were used as competitor DNAs. We previously determined the effects of point mutations upon ICE activator function in vivo and on binding, to ICE sequences in vitro (40). Point mutations at positions −97 and −93 drastically reduced activation mediated by this element, whereas mutations at positions −100, −96, and −91 had little or no effect on expression levels (Fig. 4A). The pattern of competition with the various alleles of the ICE for the ICE-E47 complex is shown in Fig. 4. Mutant forms of the ICE which fail to activate ICE element transcription in vivo were not efficient competitors for the homodimer E47 complex, whereas silent mutants of the ICE competed as effec-

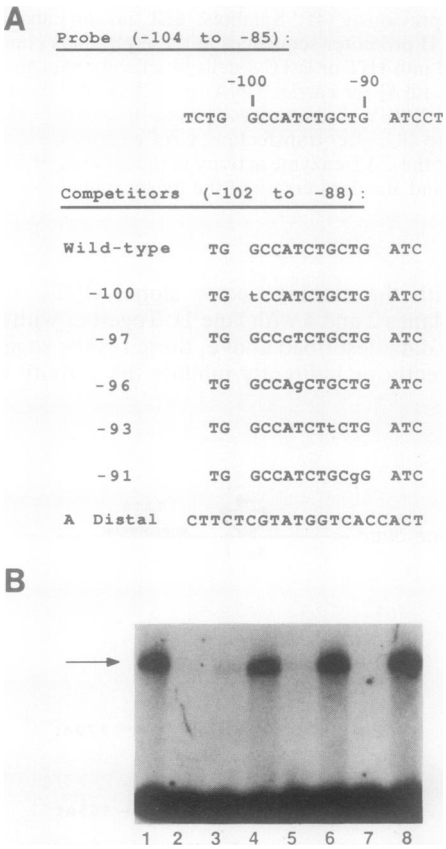



FIG. 4. The formation of the E47 complex is specifically abolished by ICE mutants and mediates positive β-cell-type-specific expression. (A) Sequences of probe and competitor oligonucleotides. Lowercase letters a, g, c, and t indicate the point mutations introduced into the competitor oligonucleotides. "A Distal" is a nonspecific size-matched double-stranded oligonucleotide (40). (B) Binding reactions were conducted in the presence of 200 mM KCl by using E47 produced in reticulocyte lysates programmed with E47S mRNA (29). The molar ratio of competitor to probe was 100. Lanes: 1, no competitor; 2, wild-type competitor; 3, −100 competitor; 4, −97 competitor; 5, −96 competitor; 6, −93 competitor; 7, −91 competitor; 8, A Distal competitor.

tively as wild-type ICE sequences (Fig. 4B, compare lanes 2, 3, 5, and 7 with lanes 4 and 6). Analogous patterns of competition were obtained with E12 homodimers and E12/E47 heterodimers (data not shown). These data indicate that both E12 and E47 are capable of binding to ICE sequences with the same properties as those described for the endogenous insulin activator factor(s)-ICE DNA complex (40).

**Conclusions.** Control of cell-specific insulin gene expression is mediated through the *cis*-acting ICE sequences and involves *trans*-acting factors that are uniquely active in pancreatic β cells. In this study it is shown that β-cell expression is controlled by a factor(s) in the B-HLH protein family. This conclusion is based in part on our observation that ICE activity in β cells is negatively regulated by Id, a distinct member of the B-HLH family which lacks the basic region (2). Id has been proposed to function during muscle cell differentiation as a negative regulator of MyoD function (2). Whether Id is also involved as a negative regulator of ICE-mediated activity during pancreatic β-cell development is under investigation.

The insulin activator factor(s) complex that we have described is detectable only in β-cell nuclear extracts. This complex was shown to contain either E12, E47, or an antigenically related protein(s). The presence of these factors in this complex is supported by recent reports (33, 38) which described the cloning of an E12/E47-related binding protein from a β-cell λgt11 cDNA library by using ICE element screening. Since homo- and heterodimers of E12 and E47 bind specifically to ICE sequences, it is possible that the ICE-activator factor complex is composed exclusively of these factors. The apparent β-cell-specific distribution and activity of such an insulin activator complex might then be the result of a modification of these factors that is unique to this cell type. However, we have found that overexpression of an E47-related cDNA, ITF-1 (21), stimulates ICE element activity with a non-cell-type-specific activation pattern (33a). Alternatively, the ICE-activator complex may be composed of a heterodimer containing a β-cell-specific B-HLH factor and either E12, E47, or an antigenically related protein, as is apparently the situation in myoblasts (13, 29, 30). Experiments are in progress to examine these possibilities in greater detail.

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