# Analysis of an insulin gene transcription control element

## Positive and negative regulation appears to be mediated by different element sequences

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**Abstract**

Pancreatic $\beta$-cell-type-specific transcription of the insulin gene is controlled by *cis*-acting sequence elements lying within its enhancer region. An essential element required for expression is the insulin control element (ICE). The activity of this element is regulated by both positive- and negative-acting transcription factors. In this study, we have identified the nucleotide sequences within the ICE that are required for repression in noninsulin producing cells. Our results indicate that the *cis*-acting sequences involved in negative control are distinct from those required in activating expression in $\beta$ cells.

*Key words:* Transcription; Gene expression; ICE; Insulin

## 1. Introduction

The formation of differentiated pancreatic islet cell types during development involves the determination of the specific $\alpha$, $\delta$, and PP islet cell lineages from multipotential progenitor $\alpha$-like cells [1]. The specific mechanisms that regulate cell lineage determination are poorly understood. However, studies in cell culture and transgenic mice have demonstrated that transcription of the major differentiated products of $\alpha$ cells, the hormones glucagon ($\alpha$) and insulin ($\beta$), are controlled through the interaction of regulatory proteins (*trans*-acting factors) with specific DNA sequences (*cis*-elements) found within the 5'-flanking region of each gene [2–6]. Restricted expression of the insulin gene appears to be regulated by both positive- and negative-acting transcription factors [7].

Transcription of the insulin gene is unique to $\beta$ cells as a result of the interaction of cellular factors with the insulin enhancer, which lies between nucleotides −340 and −91 relative to the transcription start site [7]. Insulin gene enhancer-directed expression is mediated by multiple *cis*-elements [8–10]. The most important element regulating cell type specific transcription from this region appears to be the insulin control element (ICE; 5'-GCCATCTGC-3') [9–11], which is conserved within the transcription unit of all characterized mammalian insulin genes at $100 \pm 14$ bp upstream from the transcription initiation site [12]. This element alone is capable of directing $\beta$-cell-specific expression [11,13,14]. These observations indicate that the ICE serves a central and general role in regulating $\beta$-cell-specific expression of the insulin gene.

Regulation of ICE-mediated activity is imparted by positive- and negative-acting transcriptional regulators [11]. The *trans*-acting activator of ICE expression is a member of the basic helix-loop-helix family (B-HLH) of transcriptional regulators [15–17]. Analysis of the activities of individual point mutations throughout the ICE region have shown that the CA and TG bases pairs, which defines the binding site for B-HLH proteins (−100 GC<u>CATCTG</u>CT −91), are crucial for activator function [18]. In this study, we have analyzed the DNA sequences required for negative regulation by the ICE. Individual point mutations within the ICE were constructed and their effects on repressor activity analyzed in noninsulin producing cells. We found that the base pair at −97 was necessary for repressor activity, while mutations at −96,

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−93 and −91 had little or no effect on activity. Since the −93 bp mutation prevents stimulation by the ICE B-HLH activator yet has not effect on the repressor present in noninsulin producing cells, these studies indicate that the repressor of ICE-mediated expression is not a member of the B-HLH family of transcription factors.

## 2. Materials and methods

### 2.1. Tissue culture

HeLa and baby hamster kidney (BHK) cells were grown in monolayer in Dulbecco's modified Eagle medium containing 10% (vol/vol) fetal bovine serum and 50 μg each of streptomycin and penicillin per ml. HeLa spinner culture cells were grown in Dulbecco modified spinner medium supplemented with 2.5% (vol/vol) fetal bovine serum, 2.5% (vol/vol) calf serum, 1 mM L-glutamine, and 1 mM nonessential amino acids.

### 2.2. DNA constructs

The mutant −100 expression plasmids were constructed using the Amersham oligonucleotide-directed mutagenesis system (version 2). Single base pair mutations were incorporated into the −100 to −91 bp region of the rat insulin II gene at −97 (A to C), −96 (T to G), −93 (G to T) and −91 (T to G) in the chloramphenicol acetyltransferase (CAT) reporter plasmid −100 CAT [11]. The luciferase reporter expression plasmids were made by subcloning the HindIII/BglII insulin gene fragment obtained from the mutant and wild-type −100 and −90 CAT plasmids into a Firefly luciferase reporter plasmid, pSV0ARPL2L [19].

The −97 base pair mutant used in the in vitro transcription studies was constructed from a rat insulin II gene expression vector containing wild-type sequences from −100 to +187 [11]. The nucleotide sequences of all plasmid constructs were confirmed by DNA sequencing analysis. Plasmids were prepared by banding twice to equilibrium in cesium chloride gradients.

### 2.3. DNA transfection and in vivo transcription assays

Approximately 18 h prior to transfection, 5 × 10⁵ cells HeLa cells or 2 × 10⁵ cells BHK cells were plated onto 100 mm² plates. The DNA constructs were introduced as a calcium phosphate coprecipitates made up of 10 μg of total DNA per plate containing 8 μg of an insulin-luciferase reporter plasmid (or carrier pUC19 DNA) and 2 μg of a recovery marker for transfection efficiency, RSV CAT [20], as described previously [11]. At 4 h after addition of the calcium phosphate-DNA precipitate, the BHK cells were exposed to 20% glycerol for 2 min. Cells were harvested 40 to 48 h after transfection. Cell extracts were prepared as described by De Wet et al. [21]. Luciferase and CAT enzymatic assays were performed as described previously [21,22]. Each experiment was repeated several times with at least two different plasmid preparations.

### 2.4. In vitro transcription assays

The cell-free transcription reactions were conducted with HeLa nuclear extracts prepared from HeLa spinner cultures using the procedure described by Shapiro et al. [23]. Each reaction contained 8 mM HEPES (pH 7.9), 8% v/v glycerol, 7 mM MgCl₂, 2.5 μg template DNA, 1 mg/ml bovine serum albumin, 75 mM KCl, 80 μM EDTA, 80 μM EGTA, 80 μM dithiothreitol, 500 μM each of ATP, UTP, GTP and CTP in a final assay volume of 25 μl. Approximately 100 μg of HeLa extract protein was preincubated with template DNA for 10 min at 25°C followed by the addition of ribonucleotides and the incubation was
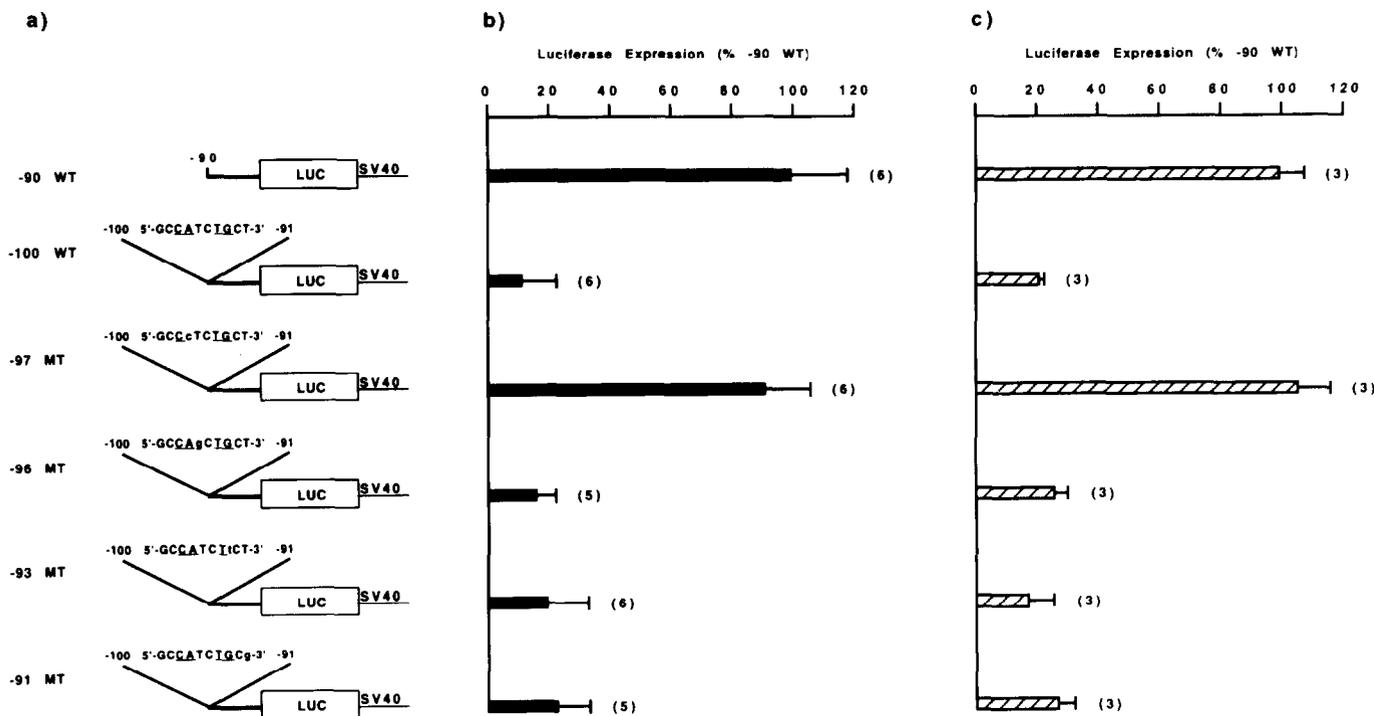


Fig. 1. Expression from ICE mutant constructs. (a) ICE-LUC chimeric gene constructs are shown schematically. The names of the plasmids are listed and site of mutation (MT) within the ICE shown. The ICE-LUC chimeric genes were cotransfected into (b) HeLa and (c) BHK cells with an internal reporter plasmid, RSV CAT. Normalized luciferase values are presented relative to −90WT LUC expression as the mean of several independent exeriments ± standard deviation. The number in parenthesis indicates the number of times each transfection was independently performed. The normalized −90WT LUC luminescence signal was 64,641 and 13,486 in arbitrary light units in HeLa and BHK cells, respectively. The luciferase measurements were made with a Analytical Luminescence Laboratory luminometer (Monolight 2010). Standard deviations for the samples are indicated by horizontal lines in the histograms.
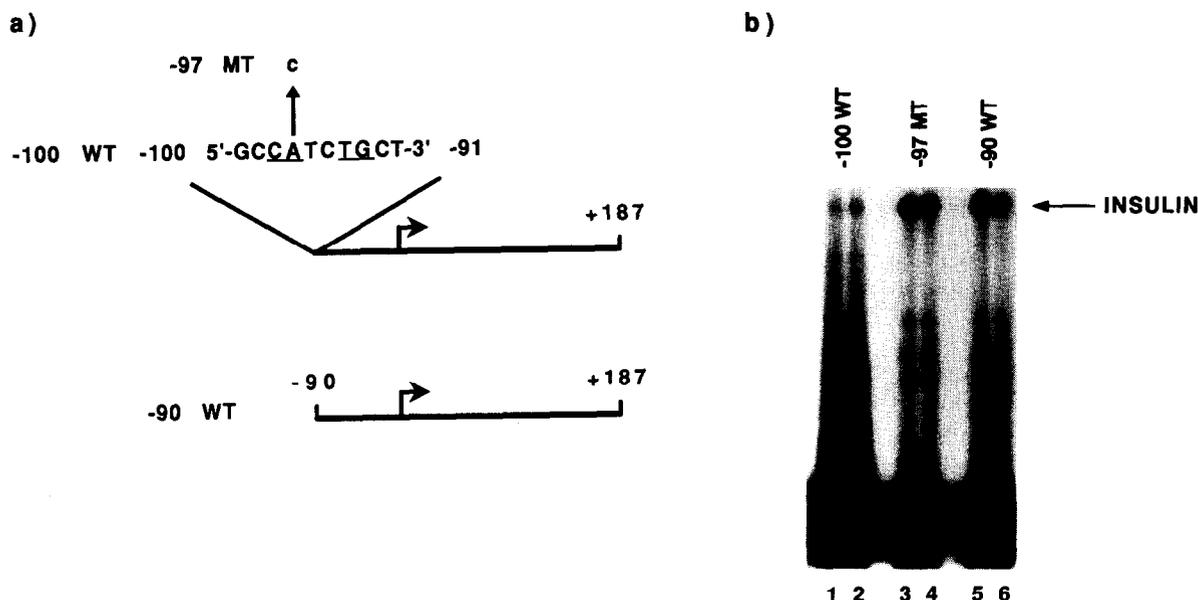
Fig. 2. Analyses of transcripts synthesized in vitro from the −100WT, −97MT and −90WT plasmids. (a) Schematic representation of the −100 and −90 wild-type and −97 mutant constructs. (b) Transcription reactions were conducted as described in section 2 using HeLa nuclear extracts. The insulin specific primer extension product is labeled. The −100WT (lanes 1,2), −97MT (lanes 3,4) and −90WT (lanes 5,6) templates were assayed in duplicate. A representative autoradiogram of the resulting analyses is shown.

continued at 30°C for 1 h. RNA was extracted with phenol/chloroform and analyzed by the primer extension methodology [11]. Insulin-specific transcription was analyzed with a primer complementary to bases +33 to +66 of the noncoding strand of the rat insulin II gene [11]. The primer extension products were visualized by autoradiography.

## 3. Results and discussion

### 3.1. Identification of the −97 base pair as being required for repressor function in vivo

The ICE alone can direct β-cell-type-specific expression when linked to either the insulin promoter or a heterologous minimal promoter [11,13,14]. The absence of ICE-mediated activity in noninsulin producing cells was in part a result of trans-negative regulation [11]. To identify the base pairs within the ICE that are important in repressor function, individual transversion mutations were made at base pairs −97, −96, −93 and −91 in the ICE/insulin promoter expression plasmid, −100WT (Fig. 1). The effect of these mutations on ICE-directed repression were compared to the activity of the insulin promoter expression plasmid, −90WT, in two noninsulin producing cell lines, BHK and HeLa. We reasoned that a mutation at a base pair crucial for repressor interaction would result in an increase in activity from −100WT levels to the level found for the ICE-less promoter expression plasmid.

The results obtained with the insulin/LUC gene expression plasmids described in Fig. 1 were normalized to expression from a cotransfected Rous sarcoma virus enhancer-driven CAT expression plasmid, RSV CAT. The activity of −100WT was approximately tenfold less than

−90WT. Mutations within the ICE at base pairs −96, −93 and −91 did not have any effect on ICE-mediated repression, as expression from these constructs was similar to that obtained for −100WT. In contrast, the plasmid with a mutation at base pair −97 was much more active than −100WT. The activity of this mutant was comparable to that obtained for −90WT. The same expression pattern was found with insulin/CAT constructs (data not shown).

### 3.2. The ICE mutation at −97 base pair relieves ICE-mediated repression in a HeLa-soluble transcription system

ICE-mediated repression has also been observed in in vitro transcription assays [11]. To determine whether the point mutation at −97 also relieved ICE-mediated repression in this system, we assayed the relative template activity of the −100WT, −97MT and −90WT in a HeLa-soluble in vitro transcription extract. Specific in vitro transcription products were measured by primer extension analyses. The −100WT construct was transcribed less efficiently than either the insulin promoter, −90WT, or the ICE mutant −97 expression plasmids (Fig. 2b). Expression from the −97MT plasmid was similar to −90WT. The pattern of activity from these plasmids is identical to that observed in vivo (Fig. 1).

Together with the in vivo transfection studies, these results suggest that the −97 mutation relieves ICE-mediated repression in noninsulin producing cells. We believe that this mutation decreases the interaction of a negative-acting DNA-binding protein with the ICE; however, we have not been able to detect any specific repressor-like

complexes in gel mobility shift assays conducted with wild-type and base pair −97 ICE probes (data not shown). These results also indicate that the repressor is in a functionally distinct class of transcriptional regulators from the ICE activator since the mutation at −93, which disrupts the B-HLH binding site and activation by the ICE [18], has no effect on repression.

## 4. Conclusions

The mechanisms that control pancreatic $\beta$-cell-specific transcription of the insulin gene are not fully understood but appear to involve both positive- and negative-acting regulatory factors. Previous work has demonstrated that selective expression of the insulin gene is conferred, to a large extent, by a single cis-acting element found within its enhancer, which we have termed the ICE. The nucleotide sequences necessary for activation are those required for binding of its B-HLH positive regulator (−98 CANNTG −93; the nucleotides underlined are essential for activation [18]). In this investigation, we conducted a point mutagenesis study to identify nucleotides within the ICE which are responsible for transcriptional repression. Wild-type and mutants in the ICE at base pairs −97, −96, −93 and −91 were constructed and their activity analyzed in noninsulin producing cells. The mutant at base pair −97 was the only one found by both in vivo and in vitro functional tests to be crucial for repressor action. In contrast, mutations at either −97 and −93 eliminated ICE-mediated activation [18]. These results clearly indicate that the nucleotide sequences required for activation are distinct from those necessary for repression.

The ICE activator appears to be induced during islet development prior to insulin gene transcription [24]. Interestingly, MyoD, a key B-HLH transcriptional regulator important in muscle determination is also induced prior to differentiation [25]. The absence of transcription of B-HLH protein activated muscle target genes appears to be a result of the activity of negative-acting regulators [26–28]. By analogy, these results indicate that repression mechanisms function to regulate ICE-mediated transcription. To address this question, we are working towards isolating the factor(s) involved in negative control of ICE-mediated expression.

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