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c-*jun* Inhibits Transcriptional Activation by the Insulin Enhancer, and the Insulin Control Element is the Target of Control

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Selective transcription of the insulin gene in pancreatic β cells is regulated by its enhancer, located between nucleotides −340 and −91 relative to the transcription start site. One of the principal control elements within the enhancer is found between nucleotides −100 and −91 (GCCATCTGCT, referred to as the insulin control element [ICE]) and is regulated by both positive- and negative-acting transcription factors in the helix-loop-helix (HLH) family. It was previously shown that the c-*jun* proto-oncogene can repress insulin gene transcription. We have found that c-*jun* inhibits ICE-stimulated transcription. Inhibition of ICE-directed transcription is mediated by sequences within the carboxy-terminal region of the protein. These c-*jun* sequences span an activation domain and the basic leucine zipper DNA binding-dimerization region of the protein. Both regions of c-*jun* are conserved within the other members of the *jun* family: *junB* and *junD*. These proteins also suppress ICE-mediated transcription. The *jun* proteins do not appear to inhibit insulin gene transcription by binding directly to the ICE. c-*jun* and *junB* also block the *trans*-activation potential of two skeletal muscle-specific HLH proteins, MyoD and myogenin. These results suggests that the *jun* proteins may be common transcription control factors used in skeletal muscle and pancreatic β cells to regulate HLH-mediated activity. We discuss the possible significance of these observations to insulin gene transcription in pancreatic β cells.

Cell-specific transcription appears to be a result of the interaction of multiple families of transcription factors acting in combination to either activate or repress gene expression (reviewed in references 30, 32, 40, 43, 46, and 55). In general, transcriptional regulation is mediated by proteins that bind directly to specific DNA sequences (32, 40). However, heteromeric interactions between transcription factors can also modulate gene expression. For example, Id, a member of the helix-loop-helix (HLH) family of transcription factors, lacks the basic amino acid residues within the DNA-binding region of basic helix-loop-helix (bHLH) proteins (7) and, as a result, inhibits the binding and activation properties of the bHLH family of regulators by forming nonfunctional heteromeric complexes. I-POU appears to have a similar role in the context of the POU domain family of proteins (56). The transcriptional mechanisms underlying the cell-specific expression of pancreatic islet hormones are poorly understood. However, studies in cell culture and transgenic mice have demonstrated that transcription of the major products of differentiated islet α and β cells, the hormones glucagon and insulin, respectively, are controlled by specific DNA sequences found within the 5′-flanking region of each gene (24, 31, 36, 44, 58).

Pancreatic β-cell-type-specific transcription of the insulin gene appears to be due to the recognition by specific positive- and negative-acting transcription factors of its enhancer region, which is located between nucleotides −340 and −91 relative to the insulin gene transcription start site (reviewed in reference 53). There are a number of distinct DNA sequence elements within the insulin gene enhancer that are important for regulating cell type-specific expression (9, 18, 35, 61). Detailed characterization of the insulin gene enhancer region indicates that β-cell-specific expression is mediated predominantly by the element at −100 to −91 bp (18, 35, 61), whose core motif 5′-GCCATCTG-3′ is found within the transcription unit of all characterized insulin genes (54). This element, which we refer to as the insulin control element (ICE), is activated by a *trans*-active DNA binding factor that appears to be uniquely distributed in islet α and β cells (41, 45).

The positive regulator of ICE expression is a member of the bHLH family of transcription factors (17, 26, 51). The ICE activator appears to be a heteromeric bHLH complex, composed of the ubiquitously expressed E2a gene products, E12 and/or E47 (or antigenically related proteins), and an islet α- and β-cell-specific factor (17, 26, 51). The heteromeric structure proposed for the ICE activator is analogous to the composition of the bHLH regulators involved in tissue-specific transcription of neuron- (reviewed in reference 12) and muscle-specific genes (reviewed in references 42 and 59).

The cellular signaling pathways that are required for islet cell differentiation are unknown. Understanding the regulatory network that links the differentiation signals at the cell surface to transcriptional responses in the nucleus represents a major investigative challenge. The objective of our studies has been to identify the *cis*- and *trans*-acting factors within the 5′-flanking region of the insulin gene that serve as the nuclear targets for these pathways. An unfortunate limitation in studying islet cell development is our inability to mimic steps of the differentiation process in cell culture. As a result, the potential involvement of a *trans*-acting factor(s) in regulating cell-specific expression cannot be readily assessed. However, results from a number of studies indicate that the ICE bHLH activator is a key regulator. Furthermore, we assume that the regulatory mechanisms used to control ICE-directed activity will have features similar to

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those described for the myogenic and neuronal bHLH activators. Our observation that Id, an important negative HLH regulator of skeletal muscle cell development (7), also inhibits ICE-mediated activity in pancreatic β cells lends support to this hypothesis (17). The impetus for the studies described below with the *jun* proteins was, in part, a result of their inhibitory effect on the activity mediated by the myogenic bHLH proteins, MyoD and myogenin (8, 37).

There are three members of the *jun* family of proto-oncogenes, c-*jun*, *junB* (48), and *junD* (47). These proteins belong to the family of transcription factors that contain a basic leucine zipper (basic LZ) motif. The LZ motif consists of a heptad repeat of leucine residues that mediate dimerization and a contiguous basic region involved in DNA binding. The expression of these transcription factors is stimulated by growth-promoting agents such as growth factors, tumor promoters, and transforming oncoproteins (reviewed in references 4 and 57). Normally, the *jun* proteins confer growth-responsive transcription to various genes by directly binding to their cognate *cis*-acting element (3, 10). However, the c-*jun* and *junB* proteins also appear to inhibit skeletal muscle differentiation by interacting directly with the muscle bHLH activator proteins, MyoD and myogenin, and/or their coregulators (8, 37). Similar mechanisms have been proposed for c-*jun* in the regulation of estrogen (21) and glucocorticoid receptor function (20, 49, 62).

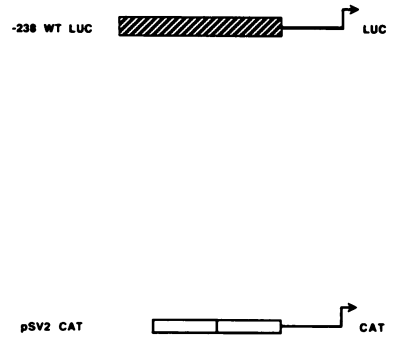
Recent studies have revealed that c-*jun* and *junB* may also be important regulators in pancreatic β cells. Inagaki et al. (34) demonstrated that there was a concomitant decrease in c-*jun* mRNA levels in HIT T-15 β cells upon stimulation of insulin gene transcription by glucose. In addition, they showed that c-*jun* could inhibit insulin enhancer-mediated expression in these cells. The studies by Honeyman et al. (33) also indicate that *junB* may be involved in β-cell physiology, because sera from patients with insulin-dependent type I diabetes mellitus, a disease caused by autoimmune destruction of pancreatic β cells, contain autoimmune antibodies to *junB*. In the studies described below we have shown that c-*jun*, *junB*, and *junD* can repress insulin enhancer activity. The *cis*-acting target of c-*jun* control within this region is the ICE. The basic LZ region and a contiguous activation domain within c-*jun* are important for repression. The amino acid sequences within the homologous regions of *junB* and *junD* are similar. These results suggest that the *jun* proteins may represent common control factors used in skeletal muscle and pancreatic β cells to regulate bHLH-mediated activity.

MATERIALS AND METHODS

Cell culture and transfections. The insulin-producing HIT T-15 2.2.2 (22) and βTC3 (23) cell lines were grown in Dulbecco modified Eagle medium supplemented with 15% (vol/vol) horse serum, 2.5% (vol/vol) fetal bovine serum, and 50 μg each of streptomycin and penicillin per ml. The insulin luciferase (LUC) expression plasmids were introduced into HIT T-15 cells as calcium phosphate coprecipitates made up of 11 μg of total DNA containing 2 μg of the insulin LUC plasmid, 8 μg of the *jun* expression plasmid or carrier DNA (CMV4), and 1 μg of a recovery marker for transfection efficiency, pSV2CAT (29), as detailed by Whelan et al. (61). The 5′-flanking rat insulin II gene expression constructs were introduced into βTC3 cells (10^7 cells per point) by electroporation with a Bethesda Research Laboratories Cellporator as described previously (45). We used 10 μg of the insulin test plasmid, 40 μg of the *jun* expression plasmid or carrier DNA (CMV4), and 10 μg of pSV2CAT per point. Cells were harvested 40 to 48 h after transfection. The amount of extract used in the LUC assay was normalized relative to CAT activity. LUC and CAT enzymatic assays were performed as described by De Wet et al. (19) and Nordeen et al. (39), respectively. The LUC activity is defined as arbitrary relative light units (RLU). The background LUC activity from the expression plasmids lacking rat insulin II gene sequences (e.g., pSV0ARPL2L [14], pOV-50 LUC [51], and OVEC-1 LUC [61]) was approximately 125 RLU. Each experiment was repeated several times with at least two different plasmid preparations.

DNA constructs. The wild-type and ICE mutant rat insulin II gene enhancer/promoter LUC expression plasmids were constructed as previously described (16, 45). The RIPE3 minienhancer constructs contain three copies of the −126 to −86 bp region of the rat insulin II gene inserted in its normal orientation directly upstream of the ovalbumin TATA box in the chloramphenicol acetyltransferase (CAT) expression plasmid, pOVCAT-50 (18). The insulin enhancer and ovalbumin promoter sequences from the wild-type RIPE3, RIPE31b mutant, RIPE3a2 mutant, RIPE3 ICE mutant (referred to as RIPE3a1m in reference 51), and pOV-50 CAT expression constructs (51) were obtained by the polymerase chain reaction and cloned just upstream of the LUC gene in pSVOARPL2L (14). ICE-driven expression was assayed from ICE OVEC LUC. This construct was made by subcloning the ICE-rabbit β-globin promoter sequences from OVEC4 (61) into pSVOARPL2L. The OVEC4 expression plasmid contains three copies of the −102 to −87 bp rat insulin II gene ICE inserted into the rabbit β globin promoter in OVEC-1 (61). *jun* expression plasmids were constructed by subcloning *jun* coding sequences from characterized constructs into the polylinker of the cytomegalovirus (CMV) enhancer-driven expression vector, CMV4 (2). The *jun* mRNA expressed from these plasmids contains at their 5′ end a segment of the alfalfa mosaic virus 4 RNA that encodes a translational enhancer, and at the 3′ end, the *jun* mRNA has transcription termination and polyadenylation signals from the human growth hormone gene. The CMV-*Jun* expression plasmids were constructed from *jun* DNA obtained from the following sources: *JunB*, *JunD*, and c-*jun* coding sequences (37); an N-terminal c-*jun* activation region mutant, *Jun* A63/73 (serines at amino acids 63 and 73 were changed to alanine [52]); a c-*jun* distal activation region mutant, *Jun* F243 (serine at amino acid 243 was changed to phenylalanine [11]); a c-*jun* basic region mutant, *Jun* BS (deletion of amino acids from 260 to 266 [25]); a c-*jun* LZ mutant, *Jun*LZ (leucine at amino acid 297 was changed to phenylalanine [25]); and a c-*jun* basic-leucine region expression construct that encodes amino acids 250 to 334 (8), *JunBLZ*.

Electrophoretic mobility shift assays. Double-stranded oligonucleotides to the ICE (5′-TCTGGCCATCTGCTGATC CT-3′) and the 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (TRE) (5′-ATTAGCATGACTCATTGT-3′) factor binding sites were end labeled with [α-^{32}P]dATP (6,000 Ci/mmol) and the Klenow fragment of *Escherichia coli* DNA polymerase I and used as probe. ICE- and TRE-binding reactions were performed as described previously (60) with purified, bacterially synthesized c-*jun* proteins. The full-length c-*jun* protein was purchased from Promega. The amino-terminal truncated c-*jun* protein (Jun199-334 [1]), that encodes amino acids 199 to 334, was provided by Tom Kerppola. βTC3 cell extracts were prepared under the conditions described by Whelan et al. (60).



Test Plasmid	HIT T-15 Activity	(n)	Fold Repression	βTC-3 Activity	(n)	Fold Repression
None	110,133 +/- 24,234	(4)	—	37,799 +/- 5,104	(3)	—
c-*Jun* [S] (0.5:1)	21,692 +/- 5,322	(3)	5.08	—		—
c-*Jun* [S] (1:1)	14,214 +/- 3,039	(3)	7.75	—		—
c-*Jun* [S] (4:1)	11,225 +/- 2,367	(4)	9.81	2,763 +/- 1,067	(3)	10.5
c-*Jun* [A] (4:1)	105,727 +/- 25,431	(4)	1.04	35,704 +/- 6,011	(3)	0.94
Jun B (4:1)	21,128 +/- 5,231	(4)	5.21	5.815 +/- 1,831	(3)	6.65
Jun D (4:1)	20,031 +/- 4,335	(3)	5.55	—		—
None	121,128 +/- 5,231	(3)	—	46,383 +/- 8,695	(3)	—
c-*Jun* [S] (4:1)	156,018 +/- 26,529	(3)	0.87	45,853 +/- 7,574	(3)	1.01

FIG. 1. The c-*jun*, *junB*, and *junD* proteins inhibit insulin LUC activity. Structure of −238 WT insulin LUC (A) and pSV2 CAT (B) expression plasmids. The boxes correspond to the enhancer regions within each construct. The c-*jun* cDNA was cloned in both the sense (c-*Jun*[S]) and antisense (c-*Jun*[A]) orientation into the plasmid pCMV4. HIT T-15 and βTC3 cells were transiently transfected with either the −238 WT LUC reporter plasmid (panel A) or pSV2 CAT (panel B) and CMV enhancer-driven *JunB*, *JunD*, *Jun*[S], or *Jun*[A] expression plasmids, as specified. The activities of −238 WT LUC and pSV2 CAT are in arbitrary relative light units and cpm, respectively. The numbers in parentheses correspond to the ratio of the *jun* test plasmid DNA to −238 WT LUC DNA used in the transfection. Each value is expressed as the mean and standard deviation. All the data are presented as fold repression relative to either −238 WT LUC (panel A) or pSV2 CAT (panel B). Fold repression is expressed as the ratio of the LUC activity in the absence of *jun* DNA divided by the level of LUC activity in its presence. (n) indicates the number of times each transfection was performed.

After the binding mixtures were incubated at 4°C for 20 min, they were loaded onto a 6% nondenaturing polyacrylamide gel (acrylamide/bisacrylamide ratio, 29:1) and were run in TGE buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA [pH 8.5]). The gel was dried and subjected to autoradiography after electrophoresis.

RESULTS

Repression of insulin gene transcription by *jun*. The *jun* proteins contain regions of high homology, especially within their respective basic LZ domains (38, 47, 48). To determine whether c-*jun*, *junB*, and *junD* regulate insulin gene transcription in β cells, we analyzed the effect of these proteins on the activity of −238 WT LUC, a construct containing 5′-flanking enhancer/promoter sequences from −238 to +2 bp of the rat insulin II gene linked to the LUC reporter gene. (In rodents there are two nonallelelic insulin genes [I and II], which differ in the number of introns and chromosomal location [54]). The −238 WT LUC plasmid was transfected into the insulin-producing β cell lines, HIT T-15 and βTC-3, either alone or with a CMV enhancer-driven *JunB*, *JunD*, c-*jun* sense (c-*Jun*[S]) or a c-*jun* antisense (*Jun*[A]) expression plasmid. Only *JunB*, *JunD*, and *Jun*[S] were able to inhibit −238 WT LUC activity (Fig. 1). Each of these proteins reduced −238 WT LUC activity to a similar low level. Repression of transcription by the *jun* proteins appears to be specific, since these proteins did effect activation mediated by either the simian virus 40 (Fig. 1) or CMV enhancers (data not shown). These results suggest that *JunB*, *JunD*, and c-*jun* can inhibit insulin enhancer-promoter-driven activity from −238 WT LUC in transfected pancreatic β cells.

c-*Jun* regulates ICE-mediated activity. The target site(s) within the 5′-flanking region of the rat insulin II gene required for *jun* repression was determined by analyzing the effect of these proteins on the activity of a series of insulin expression plasmids containing sequences spanning the region from −697 to +2 bp linked to LUC. All of these insulin LUC expression constructs, with the exception of the insulin promoter-driven −91 WT LUC plasmid, are selectively transcribed in pancreatic β cells (16, 61). The HIT T-15 cell transfection assays described in Fig. 2 and 3 were performed in the presence of the c-*jun* expression plasmid, Jun[S]; however, identical results were obtained with the *junB* and *junD* proteins (data not shown). The activities of all of the 5′-flanking insulin gene expression plasmids except the −91 chimera were repressed by c-*jun*. These results indicated that a target of c-*jun*-mediated repression resides within sequences between −135 and −91 bp.

To identify the *cis*-acting element(s) within the −135- to −91-bp region required for c-*jun*-mediated repression, we analyzed the effect of c-*jun* on the expression from constructs driven by rat insulin II gene sequences from −126 to −86 bp. This area of the rat insulin II gene is referred to as the RIPE3 region (18, 51). The wild-type RIPE3 construct contains three copies of the −126 to −86-bp region inserted in its normal orientation directly upstream of the ovalbumin TATA box in a LUC expression vector. There are at least three mutationally sensitive elements within the RIPE3 transcription unit (18, 51): RIPE3b1 element (−115 to −107 bp), RIPE3a2 element (−108 to −99 bp), and the ICE (Fig. 3A). The factors which bind to these elements synergistically activate the RIPE3 minienhancer unit transcription to approximately 75% of that mediated by the intact rat insulin II enhancer (51). The ICE- and RIPE3b1-binding factors appear to be uniquely present in islet α- and β-cell types (28, 41, 45, 51, 60), whereas RIPE3a2-binding activity is more ubiquitously distributed (16, 51).

The effect of c-*jun* on expression of the wild-type RIPE3 plasmid was compared with that of constructs containing binding-site mutations in either the ICE, RIPE3a2, or RIPE3b1 element. The LUC activity from each of these constructs was normalized to the CAT activity from the internal transfection control plasmid, pSV2 CAT. The expression from the wild type, RIPE3a2 mutant, and RIPE3b1

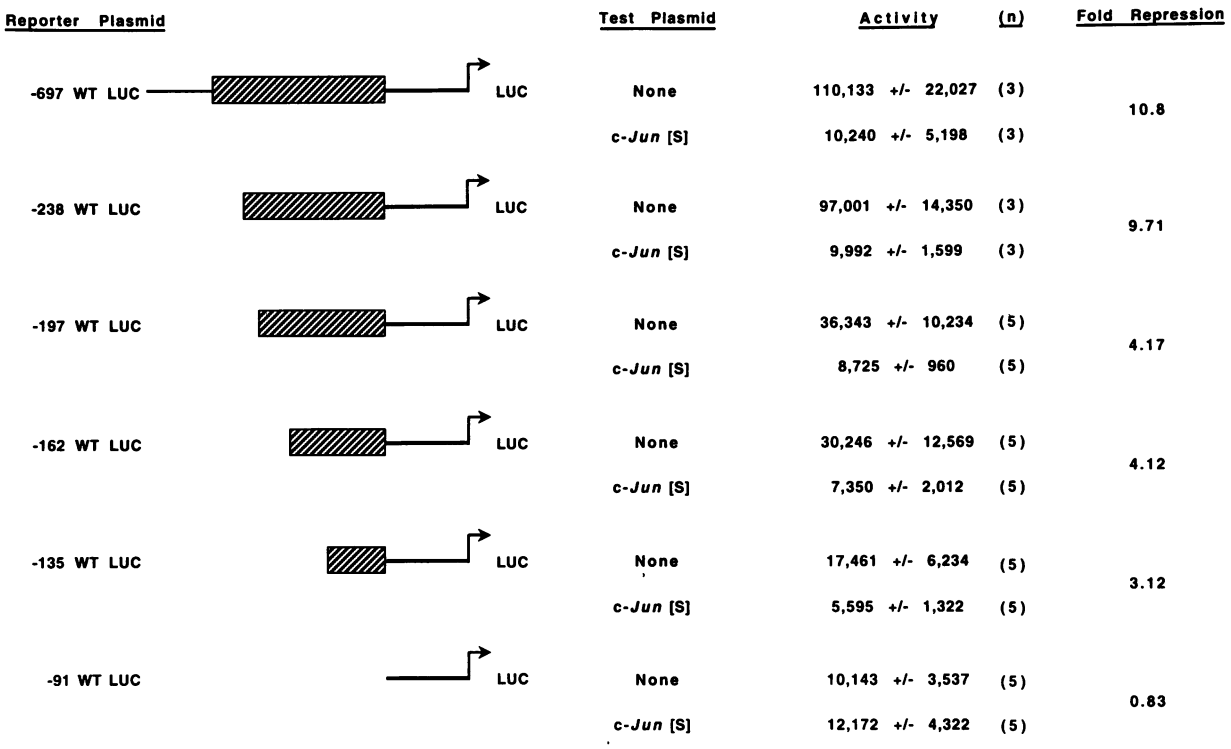


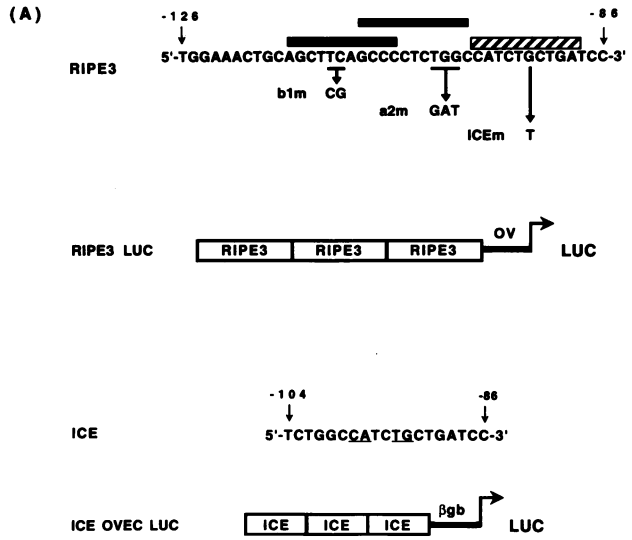
FIG. 2. Effect of c-*jun* on the activity of various insulin 5′-flanking deletion mutants. Maps of rat insulin II deletion mutants. Each mutant is named according to the 5′ endpoint of the deletion; the 3′ end is at +2 bp. The hatched boxes correspond to the rat insulin II gene enhancer region (−340 to −91 bp). The 5′-flanking deletion mutants were introduced into HIT T-15 cells made up of 11 μg of total DNA containing 2 μg of the insulin-LUC construct and either 8 μg of c-*Jun*[S] or CMV4 carrier DNA and 1 μg of pSV2 CAT. The activities of the insulin-LUC constructs are expressed as the mean and standard deviation. Fold repression is expressed as the ratio of the LUC activity in the absence of c-*jun* DNA divided by the level of LUC activity in the presence of c-*jun* DNA. The successive deletion of 5′-flanking sequences from the rat insulin II enhancer region results in reduced activity (16, 61). (n) indicates the number of times each transfection was repeated.

mutant was inhibited by c-*jun* (Fig. 3B). In contrast, a mutation in the ICE relieved c-*jun*-mediated repression (Fig. 3B). These results indicated that c-*jun* inhibited stimulation dependent on the ICE. This was confirmed by analyzing the effect of c-*jun* on the expression of ICE OVEC LUC, a luciferase expression plasmid whose activity is β cell specific and entirely dependent on the ICE (61). As illustrated in Fig. 3B, ICE OVEC LUC activity was also reduced in the presence of c-*jun*.

On deletion of 5′-flanking sequences from the −238 WT LUC construct, a two- to threefold loss of c-*jun*-mediated repression was observed (Fig. 2; compare the fold repression value of −238 WT LUC with those of −197 WT LUC and −135 WT LUC). There was also a concomitant decrease in their basal level of transcription (Fig. 2). However, these constructs are still expressed at significant levels and selectively in β cells (16, 61). These results may indicate that there is an additional site(s) for c-*jun* repression that is distinct from the ICE. To determine whether other *cis*-acting sequences were a target of c-*jun*, we analyzed repression of the −697 and −238 LUC plasmids that had the ICE deleted. c-*jun*-mediated repression was abolished when ICE sequences were deleted from these constructs (Fig. 4). Therefore, we conclude that the ICE is the sole target for c-*jun* repression within the rat insulin II gene enhancer.

c-*jun* does not bind to the ICE. We next asked whether c-*jun* inhibition resulted from direct binding to the ICE. The sequences required for ICE activator binding 5′-GCCAT CTG-3′ (underlined nucleotides are essential for binding and stimulation of the bHLH ICE activator factor [60]) are different from the consensus DNA-binding site for the *jun* proteins, 5′-TGAC/GTCA-3′, which is referred to as the tumor promoter TRE. The c-*jun*, *junB*, and *junD* proteins bind to the TRE either as homodimers or as heterodimers with another basic LZ protein, c-*fos* protein (15, 38, 63). All have very similar TRE-binding properties (15, 38, 63). The binding of an amino-terminal-truncated c-*jun* protein, Jun199–334, and the wild-type c-*jun* protein to ICE and TRE probes were compared in the gel mobility shift assay. The DNA binding-dimerization region of the c-*jun* protein is intact in the Jun199–334 protein (1). Both the wild-type c-*jun* and Jun199–334 proteins bound to the TRE probe (Fig. 5). In contrast, there was no detectable binding of either of these c-*jun* proteins to the ICE probe (Fig. 5). However, we were able to detect the formation of ICE protein-DNA complexes with this probe. We conclude from these results that c-*jun* does not bind directly to the ICE.

Regions of the c-*jun* protein important in mediating repression. To identify the region(s) of the c-*jun* protein involved in mediating repression, we examined the effect of various c-*jun* mutants on repression of ICE-driven expression. The functional organization of the c-*jun* protein is depicted in Fig. 6A. The DNA binding-dimerization region of c-*jun* (referred to as the basic LZ region) is found in the C-terminal portion of the protein, whereas the segments of the protein involved in activation are located N terminal to this domain. To determine which region(s) of the c-*jun* protein was involved in regulating ICE-mediated activity, we examined



(B)

	Fold Repression	
	c-Jun	Jun LZ
RIPE3 LUC	7.14	0.92
b1m	2.86	0.88
a2m	5.65	1.01
ICEm	1.05	1.09
ICE OVEC LUC	2.70	1.08

FIG. 3. ICE-mediated activity is the target for *trans*-repression by c-*jun*. (A) The sequences within the RIPE3 and ICE constructs. The boxes span the residues required in RIPE3b1, RIPEa2, and ICE binding (51). The mutations in the RIPE3 constructs eliminate activation in vivo and factor binding in vitro (51). The RIPE3 LUC expression constructs contain three copies of either wild-type or mutant (m) rat insulin II gene sequences from −126 to −86 bp cloned upstream of the ovalbumin minimal promoter (OV) and LUC coding sequences in pSVOARPL2L. ICE OVEC LUC contains three copies of an oligonucleotide containing rat insulin II gene ICE sequences from −102 to −87 inserted directly upstream of the rabbit β globin (βgb) TATA box (61) and LUC coding sequences. The underlined nucleotides are essential for ICE activator binding. (B) HIT T-15 cells were transfected with either RIPE3, RIPE3b1m, RIPE3a2m, RIPE3 ICEm, or ICE OVEC LUC together with *Jun*[S] or *Jun*LZ. pSV2 CAT was included as an internal control. The normalized basal activities from the RIPE3, b1 mutant, a2 mutant, ICE mutant, and ICE OVEC constructs were 377,686, 21,016, 82,009, 57,671, and 11,032 light units, respectively. The fold repression values are representative of the results obtained from several independent experiments.

the effect of mutants within both the N-terminal activation regions and the C-terminal basic LZ region of c-*jun* on −135 WT LUC activity. All of the mutants were subcloned into the CMV enhancer-driven expression vector, CMV4, such that the c-*jun* mRNA contained the alfalfa mosaic virus 4 RNA translational enhancer at its 5′ end and 3′ polyadenylation signals from the human growth hormone gene. Previous transfection experiments indicate that the mutant plasmid vectors expressed stable proteins that accumulate at comparable levels (8, 11, 37).

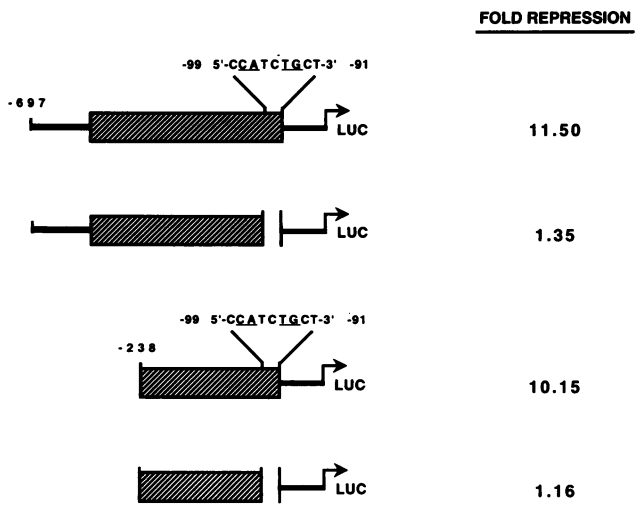


FIG. 4. Effect of c-*jun* on the activity of ICE deletion mutants. The wild type and the ICE deletion mutants were introduced into HIT T-15 cells with either c-*Jun*[S] or CMV4 carrier DNA and an internal control, pSV2 CAT. The boxes correspond to the insulin enhancer region (−340 to −91 bp); the underlined nucleotides are essential for ICE activator binding. Fold repression values represent the averages of three independent experiments ± 25%. The ICE deletion in the −697 and −238 constructs reduces basal activity approximately 10-fold (61).

There appear to be at least two transcriptional activation domains in c-*jun*. The principal domain is near the amino terminus of the protein (5, 6), and a second relatively weak activation region is next to the basic LZ region (6). Phosphorylation at serines 63 and 73 within the amino-terminal activation domain is important in both *trans*-activation and oncogenic cooperation functions of c-*jun* (52). However,

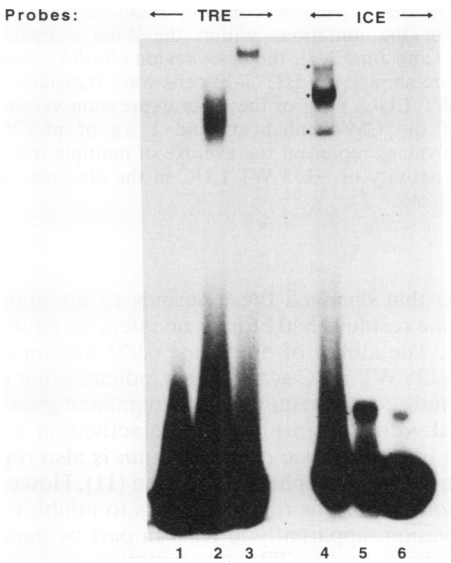


FIG. 5. c-*jun* is unable to bind to the ICE. Electrophoretic mobility shift experiments conducted with TRE (lanes 1 through 3) and ICE (lanes 4 through 6) probes, using 2.2 μg of wild-type c-*jun* protein (lanes 3 and 6), 0.4 μg of the *Jun*199–334 protein (lanes 2 and 5), and 40 μg of βTC3 extract (lane 4). No protein was added to the binding reaction in lane 1. The ICE-specific complexes formed in β extracts are labeled with an asterisk (*) (60).

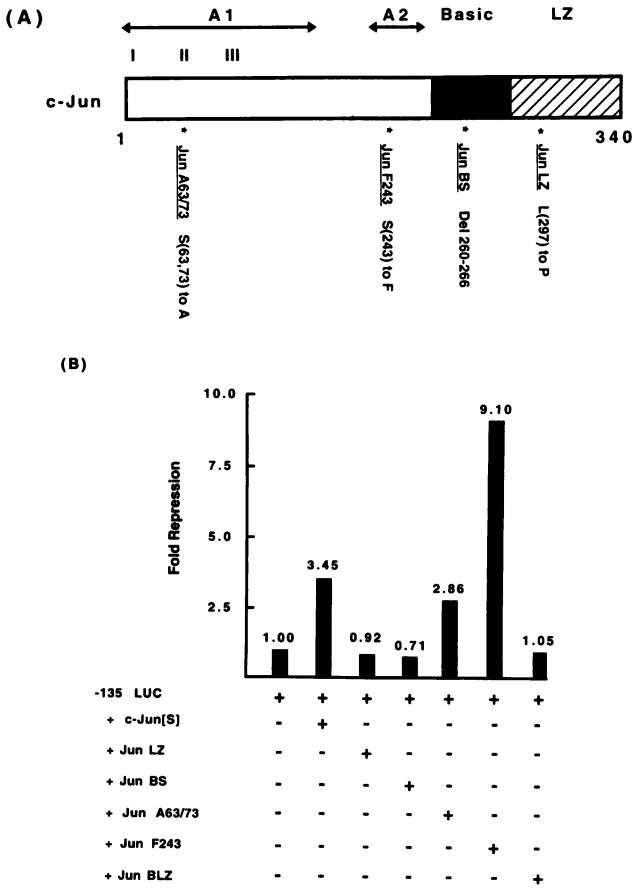


FIG. 6. The carboxy-terminal region of c-*jun* mediates repression of −135 WT LUC activity. (A) Diagrammatic representation of the c-*jun* protein shows the positions of the activation and basic LZ domains. Regions I (amino acids 6 to 12), II (68 to 81), and III (105 to 120) were described by Angel et al. (5), and regions A1 (5 to 196) and A2 (238 to 257) were described by Baichwal and Tjian (6). The locations of the mutations within the c-*jun* activation regions (*JunA63/73* and *JunF243*), the basic region (*JunBS*), and LZ region (*JunLZ*), are shown. (B) HIT T-15 cells were transfected with 2 μg of −135 WT LUC, 8 μg of the c-*jun* expression vector under the control of the CMV enhancer, and 1 μg of pSV2CAT. Fold repression values represent the average of multiple transfections ± 30%. The activity of −135 WT LUC in the absence of c-*jun* was 18,001 ± 4,246.

mutations that changed these serines to nonphosphorylatable alanine residues had little or no effect on c-*jun* inhibition (Fig. 6B). The ability of the serine 63/73 mutant of c-*jun* to repress −135 WT LUC activity also indicates that repression is not an indirect consequence of deregulated growth control associated with transformation. The activation domain just upstream from the basic region of c-*jun* is also regulated by serine (and threonine) phosphorylation (11). However, phosphorylation within this region appears to inhibit c-*jun*-mediated activation, apparently at least in part by decreasing its ability to bind to the TRE. A mutation of serine 243 to phenylalanine reduces phosphorylation within this region and increases the DNA-binding and *trans*-activation properties of the protein (11). This mutant, *JunF243*, also inhibits −135 WT LUC activity (Fig. 6B). The F243 mutation appears to convert the c-*jun* protein into a more effective repressor, which parallels the increased activation potential of this mutant protein (11). The basic LZ region of c-*jun* is also involved in repressing ICE activation. Thus, *JunBS* and *JunLZ*, mutants defective in DNA binding or dimerization, respectively, failed to inhibit −135 WT LUC activity (Fig. 6B). The repression pattern obtained with these c-*jun* mutants has been recapitulated with other ICE reporter constructs. The effect of the *JunLZ* mutation on various RIPE3 LUC constructs and ICE OVEC LUC activity is shown in Fig. 3. These results indicate that sequences important in c-*jun* repression span the carboxy-terminal activation and DNA binding-dimerization regions. In contrast, Bengal et al. (8) demonstrated that MyoD-mediated stimulation was inhibited by expression of only the basic LZ region of c-*jun*. Expression of this region from the plasmid *JunBLZ* (8), which encodes amino acids 250 to 334 of c-*jun*, had no effect on −135 WT LUC activity (Fig. 6B).

The sequences within the carboxy-terminal activation and DNA binding-dimerization regions of c-*jun* are very similar to those found within *junB* and *junD* (38, 47, 48). We believe that the same functional domains within *junB* and *junD* are necessary for repression. However, other basic LZ proteins, such as the cyclic AMP response binding protein and the CAAT enhancer-binding protein, did not inhibit, whereas c-*fos* repressed −135 WT LUC activity much less effectively than did c-*jun* (data not shown). These results suggested that there are specific features of the *jun* proteins that enable them to repress ICE-driven activity.

DISCUSSION

Insulin gene transcription in adults is limited to the β cell of the pancreatic islet. Cell-type-specific transcription appears to be due to the recognition by specific positive- and negative-acting transcription factors of its enhancer region, which is located between nucleotides −340 and −91 relative to the insulin gene transcription start site. The sequences of mammalian insulin genes within this region are closely related. Therefore, it is likely that these genes are controlled by similar transcriptional regulatory mechanisms. One control element that appears to be essential for insulin enhancer activity is the ICE. The positive regulator of ICE expression is a member of the bHLH family of transcription factors. In the present study, we have shown that the *jun* proteins, members of the LZ family, can inhibit *trans*-activation of the ICE.

The ICE activator is a heteromeric bHLH complex composed of the ubiquitously expressed E2a gene products, E12 and/or E47 (or antigenically related proteins), and a factor that appears to be uniquely distributed in pancreatic islet α- and β-cell types (41, 45). We have shown in transfection experiments that c-*jun*, *junB*, and *junD* can selectively inhibit activation through the ICE. At present it is unclear how the *jun* proteins suppress ICE-mediated activity. Inhibition does not appear to result from *jun* blocking ICE activation function by binding directly to the ICE (Fig. 5). Furthermore, c-*jun* also does not appear to suppress bHLH-mediated activation by inducing the cellular concentration of the negative regulator, Id (8).

The carboxy-terminal basic LZ DNA binding-dimerization domain and the contiguous activation domain in c-*jun* were required for efficient repression. The amino acid sequences within this region of c-*jun* are similar within the homologous area of the *junB* and *junD* proteins (47, 48); therefore a common mechanism of action may be used by the *jun* proteins. Bengal et al. (8) have demonstrated that the basic LZ region of c-*jun* is important for the physical interaction

between c-*jun* and MyoD which results in repression. We showed that (over)expression of the c-*jun* basic region and LZ domain alone was not capable of suppressing ICE-stimulated activity (Fig. 6). These experiments were conducted with the *JunBLZ* expression plasmid, a construct containing amino acids 250 to 334 which span the basic LZ region, which inhibited *trans*-activation by MyoD (8). Since the results with the F243 mutant (c-*jun* protein in which serine 243 was changed to phenylalanine [Fig. 6]) indicate that the contiguous activation region of c-*jun* is also important in mediating repression, efficient inhibition may require sequences spanning this region. These results may imply, when compared with the targeted bHLH region of MyoD and myogenin, that there are distinct structural features in the region required for c-*jun*-mediated repression within an ICE activator factor(s). We believe that c-*jun* repression is directed at the activity mediated by the α/β-cell-specific bHLH factor present in this complex, since c-*jun* does not appear to interact or influence *trans*-activation by the ubiquitously expressed E2a gene products, E12 and/or E47 (13, 37). Although our results indicate that c-*jun* does not suppress ICE-mediated activation by binding directly to the ICE, it may influence activation indirectly by interacting with an effector (i.e., coactivator) required for ICE activator function. If c-*jun* inhibits by competing for a limiting transcription factor, it is unlikely to be a general transcription factor, since c-*jun* does not inhibit transcription mediated by either the CMV or simian virus 40 enhancers (Fig. 1).

What is the biological significance of transcriptional repression by the *jun* protein(s) in the β cell? There are several possible roles for these proteins in regulating insulin gene transcription in the β cell. The ICE is an essential control element in both cell-type-specific and inducible transcription of the insulin gene. Thus, regulation of ICE activation by inducible regulators, such as c-*jun*, would provide an effective mechanism to modulate insulin gene transcription. The ICE is one of the essential *cis*-acting sequences of the rat insulin I and II genes that have been mapped within the glucose response region (27, 50). The observed 10-fold decrease in the steady-state mRNA levels of c-*jun* after treatment with glucose (34), the principal transcriptional inducer of insulin gene expression in differentiated cells, suggests that c-*jun* may be a negative regulator of insulin gene transcription in vivo. The demonstration that *junB* and c-*jun* inhibit myogenesis could also indicate that these factors mediate β-cell differentiation. The observation that *junB* is an antigen in patients with insulin-dependent type I diabetes mellitus also implies that this *jun* protein is expressed in islet β cells (33). Clearly, the expression of the *jun* proteins will have to be further analyzed to assess whether they are bona fide mediators of insulin gene transcription.

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