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# c-Jun Inhibits Insulin Control Element-Mediated Transcription by Affecting the Transactivation Potential of the E2A Gene Products

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**Pancreatic β-cell-type-specific transcription of the insulin gene is principally controlled by *trans*-acting factors which influence insulin control element (ICE)-mediated expression. The ICE activator is composed, in part, of the basic helix-loop-helix proteins E12, E47, and E2-5 encoded by the E2A gene. Previous experiments showed that ICE activation in β cells was repressed in vivo by the c-*jun* proto-oncogene (E. Henderson and R. Stein, Mol. Cell. Biol. 14:655–662, 1994). Here we focus on the mechanism by which c-Jun inhibits ICE-mediated activation. c-Jun was shown to specifically repress the transactivation potential of the E2A proteins. Thus, we found that the activity of GAL4:E2A fusion constructs was inhibited by c-Jun. The transrepression capabilities of c-Jun were detected only in pancreatic islet cell lines that contained a functional ICE activator. Repression of GAL4:E2A was mediated by the basic leucine zipper regions of c-Jun, which are also the essential regions of this protein necessary for controlling ICE activator-stimulated expression in vivo. The specific target of c-Jun repression was the transactivation domain (located between amino acids 345 and 408 in E12 and E47) conserved in E12, E47, and E2-5. In contrast, the activation domain unique to the E12 and E47 proteins (located between amino acids 1 and 99) was unresponsive to c-Jun. Our results indicate that c-Jun inhibits insulin gene transcription in β cells by reducing the transactivation potential of the E2A proteins present in the ICE activator complex.**

Transcription of the insulin gene is limited to pancreatic islet β 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 (reviewed in reference 40). This region also contains the *cis*-acting elements that are essential for glucose-stimulated expression of the insulin gene (17, 18, 29, 37). The mechanisms that are involved in regulating insulin enhancer-mediated expression are not fully understood. Insulin enhancer-directed expression appears to be mediated by multiple *cis* elements, which are regulated by both positive and negative *trans*-acting factors (6, 10, 25, 46). The insulin control element (ICE; 5′-GCCATCTGC-3′) is essential for directing transcription from this region (10, 25, 46), which is conserved within the transcription unit of all characterized mammalian insulin genes at 100 ± 14 bp upstream from the transcription initiation site (41). This element alone is capable of directing β-cell-specific transcription (26, 37, 46); in addition, it is essential for glucose-inducible expression (18, 37). These observations indicate that the ICE serves both a central and a general role in regulating expression of the insulin gene.

Regulation of ICE-mediated activity is imparted by positive- and negative-acting transcriptional regulators (9, 10, 20, 25, 45). The ICE activator is a heteromeric complex composed of the insulin activator factor (INSAF) proteins (34), which are uniquely distributed to α and β cells, and the generally expressed E2A-encoded basic helix-loop-helix (B-HLH) proteins, E12, E47, and/or E2-5 (9, 16, 32, 35, 38). The E2A proteins (E12, E47, and E2-5) are all very similar (21, 22, 42). The E12 and E47 proteins differ in their carboxy-terminal B-HLH sequences, whereas the differences between the E2-5 and E47 proteins are within their amino-terminal sequences. ICE activation can be inhibited by overexpression of the negative B-HLH regulator, Id, which appears to bind to and sequester the E2A proteins into transcriptionally nonfunctional complexes (9). The c-*jun* proto-oncogene can also repress ICE activation in β cells (20), although it is unclear how. The direct involvement of c-Jun in regulating insulin gene expression is suggested by the finding that an increase in glucose-stimulated transcription in β cells is accompanied by a concomitant decrease in c-*jun* expression (23). Interestingly, Id (4) and c-Jun (5, 27) also negatively regulate the activity of skeletal muscle B-HLH proteins (i.e., MyoD and myogenin), suggesting that these proteins may be common transcription control factors.

In this study, we have analyzed how c-Jun represses ICE-mediated activation. In contrast to Id, we found that c-Jun does not impair ICE activator binding in vitro. However, using GAL4:E2A fusion products, we demonstrated that c-Jun inhibits the transactivation potential of the E2A-encoded proteins that constitute the ICE activator complex. There are two distinct but conserved activation domains within the E12 and E47 proteins (3, 33), which are found between amino acids 1 and 99 and amino acids 345 and 408. c-Jun was found to specifically repress the activity of the domain found between amino acids 345 and 408. The E2A (345–408) activation domain has previously been shown to function more efficiently in pancreatic β cells (3). We propose that the selective recognition by c-Jun of this activation domain within the ubiquitously expressed E2A-encoded proteins suggests an important function for these proteins in regulating ICE-mediated expression in pancreatic β cells.

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## MATERIALS AND METHODS

**Tissue culture.** The glucagon-producing mouse islet αTC-6 (19), mouse myoblast C2C12, mouse pancreatic acinar 266-6, baby hamster kidney (BHK), and HeLa cell lines were grown in Dulbecco modified Eagle medium containing 10% (vol/vol) fetal bovine serum and 50 μg each of streptomycin and penicillin per ml. The insulin-producing mouse islet βTC-3 (14) and HIT T-15 2.2.2 (13) 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.

**DNA constructs.** The c-*jun* expression plasmids were constructed by subcloning c-*jun* coding sequences from characterized constructs into the polylinker of the cytomegalovirus (CMV) enhancer-driven expression vector CMV4 (1) as described previously (20). The Jun[A] and Jun[S] constructs express the c-*jun* mRNA in the sense and antisense orientations, respectively (20). The serines at amino acids 63 and 73 were changed to alanine in the N-terminal c-Jun activation region mutant, JunA63/73 (39); the amino acids from 260 to 266 were deleted in the basic region mutant, JunBS (15); and the leucine at amino acid 297 was changed to phenylalanine in the leucine zipper (LZ) mutant, JunLZ (15). The c-*jun* mRNAs expressed from these plasmids contain at the 5′ end a segment of the alfalfa mosaic virus 4 RNA that encodes a translational enhancer; at the 3′ end, each c-*jun* mRNA has transcription termination and polyadenylation signals from the human growth hormone gene (1, 20). GAL4:VP16 (36), GAL4:E1a (121–223) (28), GAL4:E2A (259–366) (33), GAL4:E2A (1–426) (33), and $(GAL4)_5$ E1bCAT (28) constructs have previously been described. The GAL4:E2A (1–99) construct was constructed by PCR using a full-length E12 cDNA and the following primers: 5′-GGGGAATTCATGAACCAGCCGCAGAGGA TG-3′ and 5′-GGGGAATTCCCGAGTCCCGGTCCCAGGAAT-3′. The resulting fragment was digested with *Eco*RI and ligated into the *Eco*RI site of the GAL4 expression plasmid pBXG1 (33) to create the in-frame GAL4 fusion protein. The construct was verified by DNA sequencing. The GAL4:E2A (259–366) and GAL4:E2A (1–426) constructs (28) are referred to here as GAL4:E2A (329–436) and GAL4:E2-5 (1–426), respectively.

**DNA transfection and in vivo transcription assays.** The expression constructs were introduced into αTC-6, βTC-3, and 266-6 cells by electroporation as described previously (34). Each point contained 5 μg of $(GAL4)_5$ E1bCAT, 5 μg of GAL4:E2-5 (1–426), 40 μg of CMV Jun[S], CMV Jun[A], or CMV4, and 10 μg of a recovery marker for transfection efficiency, pSV2 LUC (12). HIT T-15, BHK, C2C12, and HeLa cells were cotransfected with calcium phosphate precipitates made up of 11 μg of total DNA containing 2 μg of $(GAL4)_5$ E1bCAT, 2 μg of GAL4:E2-5 (1–426), 8 μg of CMV Jun[S], CMV Jun[A], or CMV4, and 1 μg of pSV2 LUC. Four hours after the addition of the DNA precipitate, HIT, BHK, and C2C12 cells were treated with 20% glycerol for 2 min as detailed by Whelan et al. (46). Cells were harvested 40 to 48 h after transfection. The amount of extract used in the chloramphenicol acetyltransferase (CAT) assay was normalized relative to luciferase activity. Luciferase and CAT enzymatic assays were performed as described by De Wet et al. (12) and Nordeen et al. (31), respectively. Each experiment was repeated several times with at least two different plasmid preparations.

**Electrophoretic mobility shift assays.** Double-stranded oligonucleotides to detect ICE (5′-TCTGGCCATCTGCTGATCCT-3′), USF (5′-TAGGTGTAG GCCACGTGACCGGGTGTTC-3′), and GAL4 (5′-GGCGGAAGACTCTC CTCCG-3′) factor binding were end labeled by using [α-$^{32}$P]dATP and the Klenow fragment of *Escherichia coli* DNA polymerase I. The ICE and USF binding reaction mixtures contained 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 5 mM dithiothreitol, 0.2 mM EDTA, 0.1 μg of poly(dI-dC) per μl, 0.1 μg of single-stranded DNA per μl, 9% (vol/vol) glycerol, 10 fmol of $^{32}$P-labeled double-stranded oligonucleotide (~5,000 dpm/fmol), and 200 mM KCl. These reaction mixtures were incubated at 4°C for 20 min. Binding reaction mixtures (20 μl) with the GAL4 probe contained 50 mM HEPES (pH 7.9), 0.2 mM dithiothreitol, 0.2 mM EDTA, 10 mM $MgCl_2$, 20% (vol/vol) glycerol, and 1 μg of single-stranded DNA. Approximately 10 to 25 μg of HIT T-15 or βTC-3 extract protein was used per assay point. βTC-3 and HIT T-15 whole-cell extracts were prepared as described previously for βTC-1 monolayer cells (45). The bacterially expressed Id protein from pGEX-2T-Id was purified as described previously (24). Full-length c-Jun protein was purchased from Promega. Each reaction mixture was incubated at room temperature for 20 min. Samples were subjected to electrophoretic separation on a 4% nondenaturing polyacrylamide gel at 200 V for 1.5 to 2 h, using the high-ionic-strength polyacrylamide gel electrophoresis conditions described previously (45). The gel was then dried, and labeled DNA-protein complexes were localized by autoradiography.

## RESULTS

**c-Jun does not appear to interact with the factors that compose the ICE activator.** To determine if c-Jun inhibited transcription by binding directly to a protein(s) present in the ICE activator complex, we analyzed whether adding purified c-Jun protein influenced the formation of the activator complex detected in DNA binding assays with β-cell extracts. The effect of c-Jun was compared with that of Id in these assays. The HLH domain in the Id protein enables it to form heterodimers with E12, E47, and E2-5; however, Id is missing a basic region, and these complexes therefore fail to bind DNA (4). Pancreatic β-cell extracts prepared from βTC-3 cells (25 μg) were supplemented with purified, bacterially synthesized c-Jun or Id protein (4 μg), and ICE activator complex formation was analyzed with a $^{32}$P-labeled ICE probe. Neither c-Jun nor Id can bind directly to ICE sequences (9, 20). The inability of the purified c-Jun protein to bind to ICE sequences reflects the distinct difference in the consensus DNA-binding site for the c-Jun protein, which is a member of the basic LZ family of transcription factors (reviewed in references 2 and 43) and the B-HLH proteins of the ICE activator.

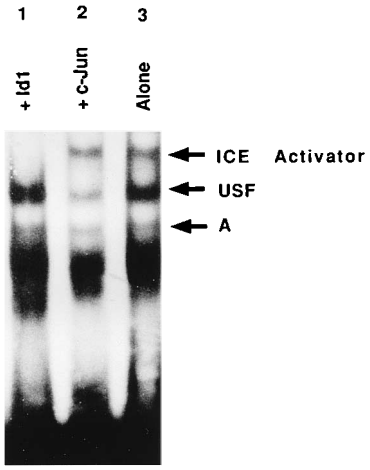
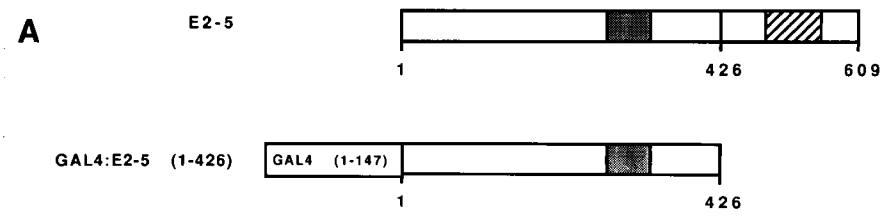



FIG. 1. c-Jun does not effect ICE activator complex formation. Binding reactions were conducted with whole-cell βTC-3 extract (25 μg), $^{32}$P-labeled ICE probe, and purified Id1 (4 μg) or c-Jun (4 μg) protein. The major bands discussed in the text are labeled. Lanes: 1, βTC-3 extract plus Id; 2, βTC-3 extract plus c-Jun; 3, βTC-3 extract alone.

The results from a typical gel shift experiment conducted with c-Jun and Id are shown in Fig. 1. In the absence of c-Jun or Id, three protein-ICE DNA complexes are routinely detected in βTC-3 extracts (45). These complexes are labeled ICE, USF, and A in order of decreasing mobility. The ICE and USF complexes contain the ICE activator and the adenovirus type 2 upstream stimulatory transcription factor, respectively (45). When c-Jun was preincubated with extract and then added to the ICE probe, there was no significant effect on the levels or mobility of the ICE activator. In contrast, Id specifically blocked the formation of this complex. The nonphysiological amounts of c-Jun and Id protein used in these assays (4 μg) clearly illustrate the difference in their abilities to interact with proteins in the ICE activator complex. We believe that Id inhibits ICE activation by associating with the E2A proteins present in the activator complex, a proposal consistent with the mechanism by which this factor regulates myogenic B-HLH-mediated activation (4). In contrast, c-Jun (at least in vitro) does not appear to directly interact with proteins in the ICE activator complex.

**c-Jun inhibits transactivation by the E2A-encoded proteins in islet α and β cells.** We next considered the possibility that c-Jun might interfere with activation mediated by the E2A-encoded proteins. There are three well-characterized, ubiquitously expressed E2A products: E12, E47, and E2-5. Each of these gene products arises from differential mRNA splicing




B

| | GAL4:E2-5 (1-426) | (n) | + c-*Jun* [S] | (n) | Fold Repression Alone / + c-*Jun* [S] | + c-*Jun* [A] | (n) | Fold Repression Alone / + c-*Jun* [A] |
|---|---|---|---|---|---|---|---|---|
| HIT T-15 | 719,785 +/- 78,345 | (4) | 161,118 +/- 14,751 | (4) | 4.47 | 642,515 +/- 78,230 | (3) | 1.12 |
| βTC-3 | 128,323 +/- 22,565 | (3) | 22,135 +/- 2,257 | (3) | 5.80 | 130,886 +/- 18,176 | (3) | 1.02 |
| αTC-6 | 91,064 +/- 15,481 | (3) | 18,947 +/- 3,126 | (3) | 4.81 | 80,866 +/- 16,013 | (3) | 1.13 |
| C2C12 | 161,128 +/- 25,780 | (3) | 136,958 +/- 20,547 | (3) | 1.18 | 185,297 +/- 22,236 | (3) | 0.87 |
| BHK | 177,952 +/- 33,811 | (3) | 211,955 +/- 38,152 | (3) | 0.84 | 155,120 +/- 32,575 | (3) | 1.15 |
| 266-6 | 700,656 +/- 152,157 | (3) | 736,207 +/- 155,214 | (3) | 0.95 | 634,361 +/- 119,725 | (3) | 1.10 |
| HeLa | 1,762,645 +/- 141,316 | (3) | 1,656,886 +/- 127,381 | (3) | 1.06 | 1,728,134 +/- 120,969 | (3) | 1.02 |

FIG. 2. Inhibition of GAL4:E2-5 (1–426) activity by c-Jun. (A) Diagrammatic representation of the E2-5 protein. The shaded box represents the activation domain conserved between the E2A proteins (located in E12 and E47 between amino acids 345 and 411), and the hatched box represents the B-HLH region (located between amino acids 495 and 553). (B) HIT T-15, βTC-3, αTC-6, 266-6, BHK, C2C12, and HeLa cells were transfected with GAL4:E2-5 (1–426), $(GAL4)_5$ E1bCAT, and either Jun[S], Jun[A], or carrier DNA (CMV4). pSV2 LUC was included in each transfection experiment to serve as an internal control. The activity of GAL4:E2-5 (1–426) is in counts per minute. The activities $(GAL4)_5$ E1bCAT alone were 12,030, 2,653, 3,274, 4,324, 2,351, 1,748, and 56,445 cpm in HIT T-15, βTC-3, αTC-6, 266-6, BHK, C2C12, and HeLa cells, respectively. Each value is the mean ± standard deviation. All data are presented as fold repression relative to GAL4:E2-5 (1–426). Fold repression is expressed as the ratio of the CAT activity in the absence of c-*jun* DNA divided by the level of CAT activity in its presence. (n) indicates the number of times each transfection was repeated. The relative transfection efficiency of HIT-15:C2C12:BHK:HeLa cells is approximately 0.1:0.1:0.1:1; that of βTC-3:αTC-6:266-6 cells is approximately 0.20:0.4:1.

events. The E12 and E47 proteins differ only in their C-terminal B-HLH sequences (42). The amino acid sequence of E2-5 is essentially identical to that of E47 except that the first 44 amino acids of E47 are missing (21, 22, 42). Transactivation by the E2A proteins is mediated by sequences contained within their N-terminal regions (3, 21, 33).

The effect of c-Jun on the transactivation ability of a fusion protein containing amino acids 1 to 426 of E2-5 and the GAL4 DNA-binding domain was analyzed in insulin-producing cells (HIT T-15 and βTC-3), a non-insulin-producing pancreatic islet α-cell line (αTC-6), and a variety of non-islet cell lines, including HeLa, C2C12 (myoblasts), 266-6 (acinar), and BHK (kidney). The GAL4:E2-5 (1–426) construct did not contain any C-terminal B-HLH sequences (Fig. 2A). Expression from the GAL4:E2-5 (1–426) and c-*jun* plasmids was mediated by the simian virus 40 and CMV enhancers, respectively. We have previously shown that c-Jun does not significantly alter the activity of either of these enhancers (20). The c-*jun* and GAL4:E2-5 (1–426) expression plasmids were cotransfected along with a CAT reporter plasmid bearing five copies of the GAL4 binding site linked to the E1b promoter [referred to as $(GAL4)_5$ E1bCAT]. CAT activity from $(GAL4)_5$ E1bCAT was normalized to the luciferase activity from a cotransfected simian virus 40 enhancer-driven luciferase expression plasmid, pSV2 LUC. The GAL4:E2-5 (1–426) construct was expressed efficiently in both the insulin- and non-insulin-producing cell lines (Fig. 2B). The stimulatory activity of GAL4:E2-5 (1–426) was inhibited by approximately fivefold by a c-*jun* sense but not by a c-*jun* antisense expression plasmid in HIT T-15, βTC-3, and αTC-6 cells. In contrast, GAL4:E2-5 (1–426) activity was not affected by c-Jun in transfected non-islet cell types. A number of other investigators have also demonstrated that GAL4:E2A-mediated activation is unaffected by c-Jun in non-islet cell types (8, 27). Since the ICE activator is functional in both insulin-producing β cells and the glucagon-producing αTC-6 cells but not non-islet cell types (26, 35, 37, 45), these results suggest that c-Jun inhibits the transactivation potential of the E2A proteins only in cells containing a functional ICE activator.

**The basic LZ region of c-Jun is important in mediating repression from the ICE and GAL4:E2-5 (1–426).** Our studies had demonstrated that the DNA-binding/dimerization region of c-Jun (referred to as the basic LZ region) was essential for repression (20). In contrast, a mutant in the major transcriptional activation domain located within the N-terminal region of c-Jun did not influence repression. To determine whether the N-terminal activation domain and basic LZ region of c-Jun affected transactivation by the E2A-encoded proteins, we examined the effects of mutants within these regions on GAL4:E2-5 (1–426) activity in HIT T-15 cells (Fig. 3). The c-Jun activation domain mutant used in this analysis contains nonphosphorylatable alanine residues in place of the serine residues 63 and 73 (referred to as JunA63/73). Phosphorylation at serine residues 63 and 73 within the amino-terminal activation domain is important in both transactivation and oncogenic cooperation functions of c-Jun (39). However, mutations that changed these serines to nonphosphorylatable alanine residues did not affect c-Jun inhibition of GAL4:E2-5 (1–426) activity. In contrast, JunBS and JunLZ, mutants defective in either DNA binding or LZ-mediated dimerization, failed to inhibit.

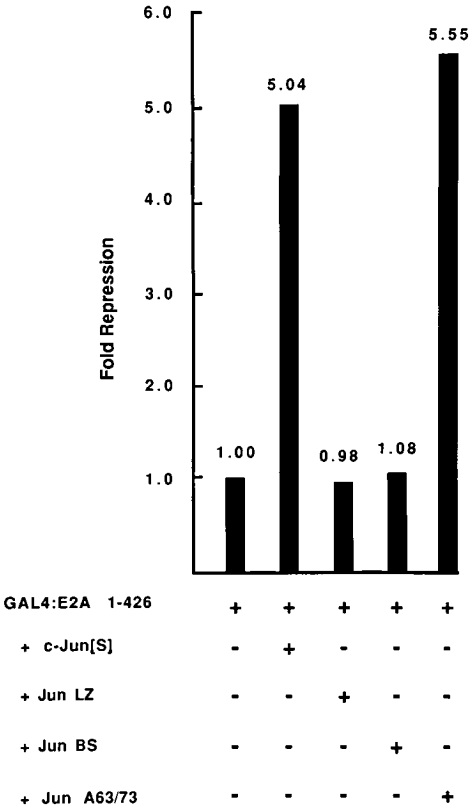



FIG. 3. The carboxy-terminal region of c-Jun mediates repression of GAL4: E2-5 (1–426) activity. HIT T-15 cells were cotransfected with 2 μg of GAL4:E2-5 (1–426), 2 μg of $(GAL4)_5$ E1bCAT, 8 μg of the c-*jun* expression vector under the control of the CMV enhancer, and 1 μg of pSV2 LUC. The locations of the mutations within the c-Jun activation region (JunA63/73), the basic region (JunBS), and the LZ region (JunLZ) are described in Materials and Methods. Fold repression values represent the averages of three transfections ± 25% standard deviation.

The repression pattern obtained for GAL4:E2-5 (1–426) with these c-Jun mutants was identical to that found with ICE reporter constructs (20). These results suggested that repression of ICE activation by c-Jun results from this factor influencing E2A activation.

**c-Jun does not affect the transactivation mediated by the herpesvirus VP16 or adenovirus E1a activator.** To determine whether transactivation mediated by the E2A-encoded proteins was a specific target for c-Jun repression, we analyzed whether c-Jun influenced transcriptional stimulation by GAL4 derivatives of the well-characterized herpesvirus VP16 acidic activator region (GAL4:VP16) and the adenovirus E1a activator [GAL:E1a (121–223)] in HIT T-15 and HeLa cells. We found that c-Jun did not significantly alter either GAL4:VP16- or GAL:E1a-mediated activity in these cells (Table 1), whereas under the same condition, c-Jun had substantially decreased GAL4:E2-5 (1–426) activity in HIT T-15 cells (Fig. 2B).

These results were consistent with the hypothesis that c-Jun specifically inhibited the transcriptional stimulation mediated by the E2A proteins. However, it was important to ascertain whether inhibition was a consequence of an effect on GAL4: E2-5 (1–426) protein expression. We therefore examined whether the levels of the GAL4:E2-5 (1–426)-binding complexes produced in HIT T-15-transfected cells were affected by c-Jun. Gel mobility shift assays were conducted with HIT T-15 extracts prepared from cells transfected with GAL4:E2-5 (1–426) and either Jun[S] or carrier DNA and a GAL4 binding-site probe. Control reactions were also conducted to analyze the binding activity of the ubiquitously expressed USF transcription factor in these extracts. The results from a typical gel shift experiment conducted with the GAL4 and USF probes are shown in Fig. 4. There were no significant differences in GAL4- and USF-binding activities in extracts prepared from either c-*jun*- or carrier DNA-transfected cells. Two protein complexes were detected with the GAL4 probe under these conditions (Fig. 4). Both complexes were supershifted upon addition of the GAL4 antibody (data not shown). Together with the data described above, these results indicate that the transactivation potentials of the E2A proteins are specifically targeted for c-Jun-mediated repression.

**The E2A transactivation domain found between amino acids 345 and 408 is the target of c-Jun-mediated repression.** The E12 and E47 proteins contain two activation domains, one between amino acids 1 and 83 and the other between amino acids 345 and 408 (3, 33). The N-terminal activation domain appears to be equally active in all cell types (3). In contrast, the activation domain defined by amino acids 345 to 408 has recently been shown to be preferentially active in β cells (3). The first 44 amino acids present in both E12 and E47 are missing from E2-5, and as a result, the E2-5 protein does not contain a functional N-terminal activation domain. Thus, it appears from the experiments described above with the E2-5 fusion protein, GAL4:E2-5 (1–426), that the transactivation domain found between amino acids 345 and 408 is a site of c-Jun-mediated repression. To directly identify the activation domain(s) in E2A that plays a key role in this response, we compared the effects in HIT T-15 cells of c-Jun or GAL4 fusion constructs spanning the 345–408 [GAL4:E2A (329–436)] and N-terminal [GAL4:E2A (1–99)] activation domains. Both GAL4: E2A (329–436) and GAL4:E2A (1–99) showed substantial transactivation potential in HIT T-15 cells (Fig. 5). GAL4:E2A (329–436) was significantly more active than GAL4:E2A (1–99), a result consistent with previous studies (3). c-Jun differed substantially in its capacity to repress GAL4:E2A (1–99)- and GAL4:E2A (329–436)-mediated activities. c-Jun was capable

TABLE 1. c-Jun does not influence transcriptional activation mediated by VP16 or E1a[a]

| Transfection | HIT T-15 | | HeLa | |
|---|---|---|---|---|
| | Mean activity ± SD (cpm) ($n$ = 3) | Fold repression | Mean activity ± SD (cpm) ($n$ = 3) | Fold repression |
| GAL4:VP16 | 3,461,949 ± 380,814 | | 1,494,208 ± 186,510 | |
| +c-Jun[S] | 3,531,534 ± 370,811 | 1.01 | 1,673,512 ± 123,513 | 1.12 |
| GAL4:E1a (121–223) | 1,725,989 ± 163,968 | | 849,709 ± 118,959 | |
| +c-Jun[S] | 1,570,649 ± 141,884 | 0.91 | 798,726 ± 103,834 | 0.94 |

[a] HIT T-15 and HeLa cells were cotransfected with $(GAL4)_5$ E1bCAT (2 μg), c-Jun[S] (8 μg), and either GAL4:VP16 or GAL4:E1a (121–223) (2 μg). pSV2 LUC (1 μg) served as an internal control.

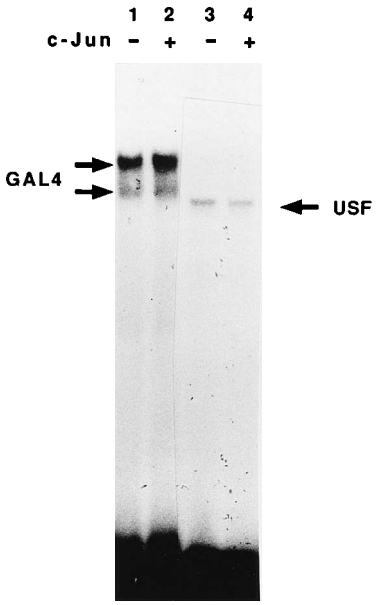



FIG. 4. c-Jun does not affect the formation of the GAL4:E2-5 (1–426) activator complex in HIT T-15 cells. Extracts were prepared from HIT T-15 cells alone or HIT T-15 cells transfected with GAL4:E2-5 (1–426) (2 μg) plus carrier DNA (8 μg) or GAL4:E2-5 (1–426) (2 μg) plus Jun[S] (8 μg). Binding reactions were conducted with $^{32}$P-labeled GAL4 (lanes 1 and 2) and USF (lanes 3 and 4) binding-site probes and 10 μg of extract. Lanes: 1 and 3, GAL4:E2-5 (1–426) alone; 2 and 4, GAL4:E2-5 (1–426) plus Jun[S].

of suppressing only GAL4:E2A (329–436) activation. These results indicate that c-Jun neither directly nor indirectly interacts with factors that regulate the activation potential of the E2A (345–408) domain in α and β cells. Together, these studies strongly suggest that ICE activation is inhibited by c-Jun as a result of the influence of this protein on the E2A proteins present in the ICE activator complex.

## DISCUSSION

The mechanisms that control pancreatic β-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 the ICE (10, 25, 46). The activator of ICE expression is composed of an islet cell-specific factor and the ubiquitously distributed B-HLH proteins, E12, E47, and E2-5 (9, 16, 38). We had previously demonstrated that ICE activation was negatively regulated in β cells by c-Jun and Id. The results of Inagaki et al. (23) and Sharma and Stein (37) support the possibility that c-Jun is a direct mediator of insulin gene transcription by its effects on ICE activation in glucose-treated β cells. The studies described here were undertaken to characterize how c-Jun suppressed ICE activation. Unlike the well-characterized B-HLH repressor Id, we found that c-Jun does not appear to repress ICE-mediated activation in β cells by disrupting the formation of the activator complex. c-Jun also did not affect the mobility of the GAL4:E2-5 (1–426)-binding complex prepared from transfected β-cell extracts. These results indicated that c-Jun does not inhibit activation by directly interacting with the proteins that constitute the ICE activator, INSAF and the E2A proteins. We next considered whether c-Jun might affect the transactivation potential of a factor present in the ICE activator complex. This was tested by comparing the effects of c-Jun on the activities of GAL4 fusion constructs containing the E2A transactivation domains with the effects of other well-characterized activators. These experiments indicated that c-Jun could specifically inhibit transactivation by the E2A proteins. Regulation by c-Jun was detected only in cell types that contained a functional ICE activator. Our results suggest that E2A suppression by c-Jun is specific and also requires the same functional domain (basic LZ region) of c-Jun that mediates its repressive effects on the endogenous ICE activator. We propose that c-Jun is an important regulator of insulin gene transcription as a consequence of its actions on E2A transactivation.

The target of c-Jun control appears to be directed at the

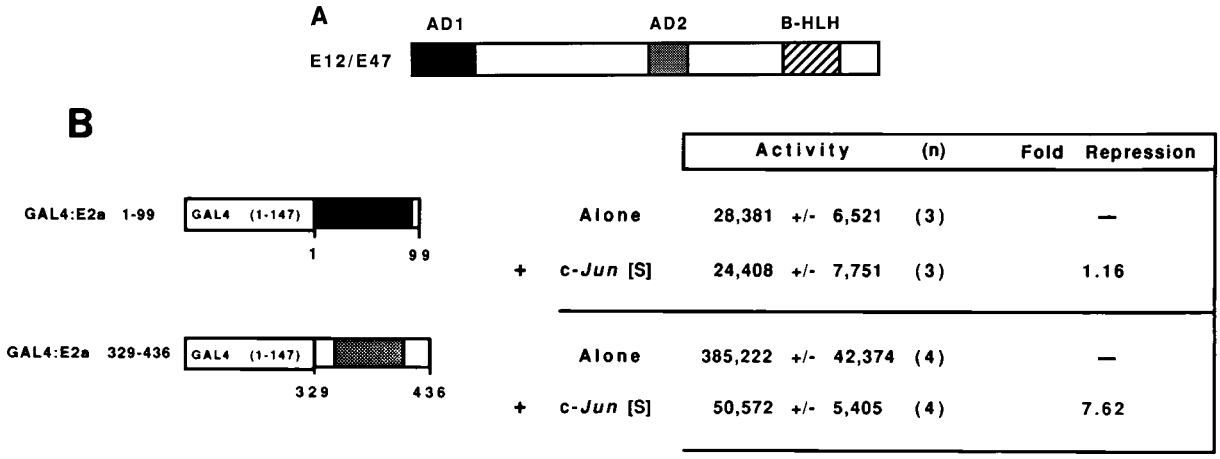



FIG. 5. The E2A activation domain located between amino acids 345 and 408 is the target for c-Jun-mediated repression. (A) Diagrammatic representation of the E12 and E47 proteins shows the positions of the activation domains (AD) and B-HLH domain. The N-terminal activation domain (AD1) is located between amino acids 1 and 83 (3), and the activation domain that functions preferentially in β cells (AD2) is found between amino acids 345 and 411 (3, 33). The B-HLH region is located between amino acids 539 and 597 (42). (B) HIT T-15 cells were cotransfected with $(GAL4)_5$ E1bCAT (2 μg), Jun[S], or carrier DNA (CMV4) (8 μg) and either GAL4:E2A (1–99) or GAL4:E2A (329–436) (2 μg). pSV2 LUC (1 μg) served as an internal control. Each value is the mean ± standard deviation. (n) indicates the number of times each transfection was repeated. The activity of $(GAL4)_5$ E1bCAT alone was 8,150 cpm.

transactivation domain (located between amino acids 345 and 408 in E12 and E47) common to the E2A-encoded proteins. Thus, comparative analysis of GAL4:E2A fusion constructs containing sequences spanning this region [GAL4:E2A (329–436)] and the N-terminal activation domain [GAL4:E2A (1–99)] demonstrated that only GAL4:E2A (329–436) activity was responsive to c-Jun-induced repression (Fig. 5). Interestingly, recent studies have shown that the E2A (345–408) domain in these ubiquitously distributed factors is preferentially more active in islet β cells (3). The majority of the transcriptional activation of E2A is mediated by this region in β cells (Fig. 5 and reference 3). The E2A (345–408) region contains an activation motif which is defined by a stretch of amino acids capable of forming a loop region (located between amino acids 345 and 395 in E12 and E47) adjacent to an amphipathic α helix (found between amino acids 396 and 408) (33). This has been termed the loop-helix motif and is structurally distinct from the acidic blob, proline-rich, and glutamine-rich activation domains (33). Mutations that disrupt the loop-helix domain drastically reduce the activity of this region in β cells (data not shown). These results suggest that c-Jun suppresses ICE activation in β cells by interacting with a factor(s) necessary for E2A (345–408)-mediated stimulation.

The mechanism by which c-Jun represses E2A (345–408) transactivation activity remains to be elucidated. The failure of c-Jun to influence the formation of the ICE activator complex suggests that its effects are not mediated through direct protein-protein interactions. c-Jun appears to repress E2A mediated activation only in islet cell types. Thus, we found that GAL4:E2A activity was repressed in islet cell lines (HIT T-15, βTC-3, and αTC-6) that contain a functional ICE activator but not in a variety of non-islet cells (HeLa, C2C12, 266-6, and BHK) (Fig. 2B). In this regard, E2A activity has also been shown to be unresponsive to c-Jun in other non-islet cell types (8, 27). However, the c-Jun protein in islet cells does not appear to be inherently different from the protein present in non-islet cells. Thus, we found that c-Jun binding and response element-mediated activation were similar in responsive and nonresponsive cells (data not shown). These results indicate that a feature of E2A-mediated activation is targeted by c-Jun in islet cell types. As the E2A (345–408) activation domain is more active in these islet cell lines, it is conceivable that c-Jun induces the expression of an inhibitory factor in HIT T-15, βTC-3, and αTC-6 cells that suppresses islet cell-specific stimulation. Alternatively, since the DNA dimerization region of c-Jun is required for repression, c-Jun may compete for a coregulatory factor that normally interacts and stimulates E2A (345–408) activity in islet cells.

The studies conducted by Bengal et al. (5) and Li et al. (27) have demonstrated that c-Jun can efficiently repress transactivation mediated by the skeletal muscle-specific B-HLH regulators myogenin and MyoD. These muscle cell-specific activators also exist in a heteromeric complex containing the E2A proteins (30, 42). However, c-Jun does not inhibit the transactivating capacity of myogenin and MyoD by influencing E2A activity (8, 27). The activation potential of the muscle-specific B-HLH regulators is the target of c-Jun action (5, 27). In contrast, we believe that transactivation by E2A is the principal site of c-Jun control in islet cells, as GAL4:E2A and ICE activator functions are inhibited to similar low levels (Fig. 2B and reference 20). We have recently isolated a factor, termed INSAF, that appears to be an islet-specific heteromeric partner of E2A in the ICE activator (34), and studies are in progress to determine whether transactivation by INSAF is also repressed by c-Jun. Interestingly, repression by c-Jun is targeted at the B-HLH region of myogenin and MyoD (5, 27). This region of myogenin and MyoD appears to be regulated by a factor that is required for cell-specific activation (7, 11, 44) much like the E2A (345–408) domain in β cells. Experiments are under way to determine whether c-Jun interacts with proteins from β cells that influence the transactivation potential of the E2A (345–408) domain.

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