Sterol Regulatory Element-binding Proteins Activate Insulin Gene Promoter Directly and Indirectly through Synergy with BETA2/E47*

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Insulin gene expression is regulated by pancreatic β cell-specific factors, PDX-1 and BETA2/E47. Here we have demonstrated that the insulin promoter is a novel target for SREBPs established as lipid-synthetic transcription factors. Promoter analyses of rat insulin I gene in non-β cells revealed that nuclear SREBP-1c activates the insulin promoter through three novel SREBP-binding sites (SREs), two of which overlap with E-boxes, binding sites for BETA2/E47. SREBP-1c activation of the insulin promoter was markedly enhanced by co-expression of BETA2/E47. This synergistic activation by SREBP-1c/BETA2/E47 was not mediated through SREs but through the E-boxes on which BETA2/E47 physically interacts with SREBP-1c, suggesting a novel function of SREBP as a co-activator. These two *cis*-DNA regions, E1 and E2, with an appropriate distance separating them, were mandatory for the synergism, which implicates formation of SREBP-1c·BETA2·E47 complex in a DNA looping structure for efficient recruitment of CREB-binding protein/p300. However, in the presence of PDX1, the synergistic action of SREBP-1c with BETA2/E47 was canceled. SREBP-1c-mediated activation of the insulin promoter and expression became overt in β cell lines and isolated islets when endogenous PDX-1 expression was low. This cryptic SREBP-1c action might play a compensatory role in insulin expression in diabetes with β cell lipotoxicity.

Insulin gene expression is restricted to pancreatic β cells and is induced by glucose (1). Extensive studies on the insulin promoter have unveiled β cell-specific transcription factors responsible for glucose-inducible transcription, such as PDX-1, BETA2/E47, and MafA, and have located their respective binding sites, A3, E1, and C1 (2–7). It has been well documented that PDX-1 plays a major physiological role in glucose-regulated expression of the insulin gene (2, 8, 9) in addition to its important role in the development and differentiation of pancreatic islets. BETA2, a β cell-specific basic helix-loop-helix (bHLH)[2] protein is

also an important regulator of the insulin gene (3) and of pancreatic development (10). The pancreas of BETA2-null mice fail to develop mature islets, and animals become diabetic (10). BETA2 heterodimerizes with E47, a ubiquitous bHLH (3), the gene disruption of which causes no apparent phenotype in β cells (11). The rat insulin promoter I, the most studied insulin gene promoter, contains E2- and A3/4-boxes. BETA2/E47 binds to E2-box and PDX-1 binds to A3/4. PDX-1, BETA2/E47, and HMGI(Y) physically interact (12) and show synergistic activation of insulin gene expression by facilitating recruitment of coactivator CBP/p300 (13, 14).

Sterol regulatory element-binding proteins (SREBPs) are members of the basic helix-loop-helix leucine zipper family of transcription factors that regulate fatty acid and cholesterol synthesis (reviewed in Refs. 15 and 16). SREBPs are initially bound to the rough endoplasmic reticulum membrane. By virtue of the SREBP cleavage-activating protein/Insig system, SREBP is cleaved in a sterol-dependent manner to liberate the amino-terminal portion containing a basic helix-loop-helix leucine zipper domain (nuclear SREBP), which enters the nucleus where it can bind to specific sterol response elements (SREs) in the promoters of target genes. Three isoforms of SREBP, -1a, -1c, and -2, are known. Although SREBP-2 plays a crucial role in the regulation of cholesterol synthesis, SREBP-1c controls the gene expression of lipogenic enzymes (reviewed in Refs. 17 and 18). Nuclear SREBP-2 has a high affinity with classic sterol regulatory elements, which are usually found in the promoters of cholesterogenic genes and the LDL receptor gene. Nuclear SREBP-1 also has broad binding capacity to SRE-like sequences, including E-boxes that are occasionally found in the promoters of lipogenic genes (19).

SREBP-1c has been well established as controlling the nutritional regulation of lipogenic genes in the liver (20). However, the role of SREBP-1c in pancreatic β cells is yet to be elucidated. Because an excess of fatty acids or triglycerides could impair the functions of β cells, a potential pathophysiologic consequence of diabetes, often referred to as lipotoxicity, estimation of SREBP-1c in β cell function is an important issue to be addressed. It has been reported that insulin-resistant obese animals have a high expression of SREBP-1c in β cells (21). A β cell line in which nuclear SREBP-1c is overexpressed showed impaired insulin secretion (22–26). Overall, these data support the potential involvement of SREBP-1c in the lipotoxicity theory of β cells. However, the effect of SREBP on insulin gene expression, especially in the context with interaction with β cell-specific transcription factors, has never been investigated. In the current study, we performed promoter analysis on rat insulin promoter I to investigate the potential effects of SREBPs on insulin gene expression in both non-β and β cell lines.

[2] The abbreviations used are: bHLH, basic helix-loop-helix; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; HEK, human embryonic kidney; Luc, luciferase; SREBP, sterol regulatory element-binding protein; CMV, cytomegalovirus; GST, glutathione S-transferase; ChIP, chromatin immunoprecipitation; β-gal, β-galactosidase; TA, transactivation domain; HIT, hamster insulinoma tumor cells.

MATERIALS AND METHODS

Standard molecular biology techniques were used. We obtained cholesterol and 25-hydroxycholesterol from Sigma, Redivue [α-^{32}P]dCTP (6000 Ci/mmol) from Amersham Biosciences, and restriction enzymes from Takara Bio Inc. Plasmid DNAs for transfection were prepared with EndoFree Plasmid Midi kits (Qiagen).

Expression Plasmids—Expression vectors encoding rat PDX-1, rat BETA2, and rat E47 were generated by PCR amplification and insertion of the cDNAs into the cytomegalovirus (CMV)-driven vector (pCMV7) (27). The following primers were used: PDX-1-(1–284), 5′-primer 5′-ATGAACAGTGAGGAGCAGT-3′, 3′-primer 5′-TCACCGGGGGTT-CCTGCGG-3′; BETA2-(1–357), 5′-primer 5′-ATGACCAAATCAT-ACAGCGAGAG-3′, 3′-primer 5′-CTAATCGTGAAAGATGGCAT-3′; E47-(1–647), 5′-primer 5′-ATGAACCAGTCTCAGAGAATG-3′, 3′-primer 5′-TCACAGGTGCCCGGCTGGGTTGT-3′; BETA2ΔAD-2-(1–299), 5′-CTAAAAGGCATAATTTTTTTCAAACTC-3′; BETA-2ΔAD1AD2-(1–170), 5′-CTACTGTACAAAGGAGACGAG-3′; BET-A2ΔbHLH-(169–357), 5′-GTACAGACACTCTG-3′; E47ΔAD1-(178–647), 5′-CCTGGTCTTCCTTCCTCG-3′; E47ΔAD1AD2-(510–647), 5′-GAAGACAAGAAGGACCTG-3′; E47ΔbHLH-(1–537), 5′-TCACAGGTCCTTCTCCTC-3′. CMV expression plasmids encoding human nuclear SREBPs (pCMV-SREBP-1a, -1c, and -2) (28), Tyr→Arg mutated versions (pCMV-SREBP-1aM, -1cM, and -2M) (19) and pCMV-ΔTA-SREBP-1c, which lacks the amino-terminal transactivation domain (1–90) of SREBP-1c (20), were previously described.

Reporter Plasmids—The reporter plasmid Ins715-Luc contains a fragment of the rat insulin I gene promoter from −715 to +31 bp cloned into the SmaI site of pGL2 basic vector (Promega) containing the coding sequences of firefly luciferase cDNA. Other constructs were produced by PCR using this construct as a DNA template, and the PCR products were inserted into pGL2 basic vector. The primers used for PCR were as follows: 5′-primer Ins715-Luc, 5′-TCTCAACTCCTTGAAAATAGC-TACCT-3′; Ins646-Luc, 5′-GCTGTGCTACTGAGGCCTGATG-3′; Ins265-Luc, 5′-GGTACCTGATTGTGCTGTGAACTGCT-3′; Ins118-Luc, 5′-GGTACCTCTCGCCATCTGCCTACC-3′; Ins104-Luc, 5′-GG-TACCTACCCCTCCTAGAGCCCTT-3′; SRE2 (m)-Luc, 5′-GGTACC-TGTGAACTGCTTCATAAAGCCATCTGGCCC-3′; E2 (m)-Luc, 5′-GGTACCTGTGAACTGCTTCATCAGGCCATCAAGCCCCTTGT-TAAT-3′; del SRE2-Luc, 5′-GCCATCTGGCCCCTTGTTAAT-3′, 3′-primer 5′-AAGCTTGTAGCTGGTCACTTAGGGTT-3′. Restriction sites KpnI and HindIII were added to each 5′-primer and 3′-primer, respectively.

The site-directed mutagenesis constructs, SRE1(m)-Luc and E1(m)-Luc were produced by PCR with the following primers: SRE1 (m)-5′, 5′-GCTAGCCATCTGCCTACCTACCC-3′; SRE1 (m)-3′, 5′-GCTA-GCGGGGCTGAAGCTGTAATT-3′; E1 (m)-5′, 5′-GGTACCAGGC-CTACCTACCCCTC-3′; E1 (m)-3′, 5′-AGGCCTATGGCGAGA-GGGGCTGAA-3′. The constructs lacking the intervening sequences between E1 and E2 regions were produced as follows: The fragment was amplified by 5′-primer, 5′-CCCTCTCGCCATCTG-3′ and 3′-primer, 5′-AAGCTTGTAGCTGGTCACTTAGGGTT-3′ using Ins265-Luc as a template and was inserted to the pGL2 basic vector. Restriction sites NheI and HindIII were added to each primer. 50-bp- and 86-bp-deleted fragments were amplified by 5′-primer 5′-GGTACCTGATTGTGCT-GTGAACTGCT-3′ and 3′-primer, 5′-GGGTAATTAGATTAT-TAA-3′ (del 86) or 3′-primer, 5′-GCGCTCATTGGACGTCA-3′ (del 50). These fragments were inserted into the above vector. Restriction sites KpnI and NheI were added to each primer. Rat insulin II gene promoter InsII251-Luc was previously described (29).

Transfection and Luciferase Assays—HEK293 cells (3.5 × 10^4 cells/well), HepG2 cells (4.2 × 10^4 cells/well), and HIT cells (3.5 × 10^5 cells/well) were plated on 12-well plates. Each expression plasmid (0–0.5 μg), luciferase reporter plasmid (0.5 μg), and pSV-β-gal (0.5 μg) were co-transfected using SuperFect transfection reagent (Qiagen) for HEK293 and HepG2 cells and using JetPEI^{TM}-Man (Polyplus-transfection) for HIT cells. The total amount of DNA in each transfection was adjusted to 1.5–2.0 μg/well. The luciferase activity in transfectants was measured by MicroLumat Plus (Berthold) and normalized to the β-galactosidase activity as measured by standard kit (Promega).

Gel Mobility Shift Assays—Gel shift assays were performed as previously described (30). Recombinant SREBPs (SREBP-1c and -2) (Fig. 1*E*) and nuclear extracts from HEK293 cells transfected with pCMV-SREBP-1c, pCMV-SREBP-1cM, or pCMV-ΔTA-SREBP-1c-Myc (Fig. 6*B*) were used. The DNA probes prepared by annealing both strands were as follows: SRE1/E1-box, 5′-TCAGCCCCTCTCGCCATCTGC-CTACCTA-3′; SRE2, 5′-GAACTGCTTCATCAGGCCATC-3′; E2-box, 5′-GGCCCATCTGGCCCCTTGTTAAC-3′; and SRE2/E2-box, 5′-GGACTGCTTCATCAGGCCATCTGGCCCCTTGTTAATAAT-CTAATTACCCTAGGTCTAAC-3′.

The ^{32}P-labeled probe and proteins were incubated in 15 μl of binding buffer (10 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.05 mM EDTA, 2.5 mM MgCl$_2$, 8.5% glycerol, 1 mM dithiothreitol, 0.5 mg/ml poly(dI-dC), 0.1% Triton X-100, and 1 mg/ml nonfat milk) for 30 min on ice. Myc or SREBP antibodies were preincubated with proteins for 60 min before adding the labeled probes. The DNA·protein complex was resolved on a 4.6% polyacrylamide gel. Gels were dried and exposed to the Bioimaging Analyzer System station software (Fuji Photo Film).

In Vitro Protein-Protein Interaction Assay—[^{35}S]Methionine-labeled proteins, luciferase, SREBP-1c, ΔTA-SREBP1c, BETA2, and E47 were prepared using the *in vitro* translation transcription system (Promega). Labeled protein (3 μl) was mixed with 1 μl of Myc antibody bound to 5 μl of protein G-Sepharose beads (Amersham Biosciences) in 200 μl of interaction buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) and incubated for 1 h at 4 °C. The beads were washed three times with interaction buffer. The bound proteins were eluted with 30 μl of sample buffer (1% SDS, 100 mM dithiothreitol, 50 mM Tris-HCl, pH 7.5, 0.1% Bromothymol Blue) and heated 95 °C for 5 min. The bound proteins (15 μl) were subjected to SDS-PAGE and autoradiography.

GST Binding Assay—GST fusion proteins, GST alone, GST-SREBP-1c (nuclear form-(24–460)), GST-SREBP-1c (bHLH-(286–364)) (31) were expressed in *Escherichia coli* and purified according to the manufacturer's recommendations (Promega). [^{35}S]Methionine-labeled E47 (bHLH-(498-634)) was prepared using the *in vitro* translation transcription system (Promega). GST precipitation assays were modified as previously reported (12, 32).

Preparation of Nuclear Extracts—Nuclear extracts from HEK293, HIT, and INS1 cells were performed as described by Hua *et al.* (33). Two hours prior to cell collection, ALLN (*N*-acetyl-Leu-Leu-norleucinal-aldehyde, calpain inhibitor I) (Calbiochem) (25 μg/μl) was added to the cells. After collection, the cells were washed with phosphate-buffered saline and suspended in buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA). Pellets were passed through a 26-gauge needle eight times and centrifuged at 1,600 × *g* for 5 min. The supernatant was centrifuged at 55,000 rpm for 20 min, and the membrane-containing pellets were suspended in membrane buffer (50 mM Tris-HCl, pH 8.0, 2 mM CaCl$_2$, 80 mM NaCl, 1% Triton X-100). The pellet containing the nuclei was suspended in buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 0.4 M NaCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM

FIGURE 1. **Insulin gene promoters are novel SREBP targets.** *A*, schematic representation of the rat insulin I and II genes and human insulin gene promoters with the sequences and location of novel SRE elements (*SRE1*, *SRE2*, and *SRE3*) and known E-box motifs. SRE1 and SRE2 elements partially overlap with the E1- and E2-boxes. SRE3 has an inverted consensus SRE sequence. *B*, the comparison of relative rat insulin promoter I activities. HEK293 cells were co-transfected with Ins715-Luc (0.5 μg) as a reporter gene, pSV-β-gal (0.5 μg) as a reference, and the indicated amount of CMV-driven expression plasmids PDX-1, BETA2, SREBP-1a, and/or empty vector (pCMV7). The firefly luciferase activity normalized to β-gal activity (mean $\pm$ S.E.) was expressed. *RLU*, relative light unit; *OD*, optical density at 415 nm for β-galactosidase. *C*, transcriptional activities of nuclear SREBP-1a, -1c, -2, and respective Tyr→Arg mutants on Ins715-Luc in HepG2 cells. *D*, deletion studies in SREBP-1c activation of the rat insulin I promoter. Shown is the schematic representation of rat insulin gene I luciferase deletion constructs used in this study. Various reporter genes (Ins-715, -646-, -265-, -118-, and -104-bp-Luc), pSV-β-gal, control vector (pCMV7), or SREBP-1c expression plasmid (0.5 μg) were transfected in HepG2 cells. The relative Luc activity was expressed as a fold change to that of the control vector (pCMV7). *E*, gel shift assay for SREBP binding to SRE1 and SRE2 of the rat insulin I gene promoter. Double-stranded DNA fragments corresponding to the E1 and E2 regions (SRE1/E1-box, SRE2, and E2-box probe) were labeled with [α-^{32}P]dCTP and incubated in the reaction mixture with or without recombinant SREBP-1c and SREBP-2. Specificity of SREBPs binding to the E1 or E2 region probes was confirmed by a supershift after the addition of SREBP-1 or SREBP-2 antibodies (*Ab*) (*lanes 4*, *5*, *10*, *15*). In competition assays of the E2 region, a 1000-fold molar excess of an unlabeled SRE2 element or E2-box probe was added (*lanes 8*, *9*, *13*, *14*).
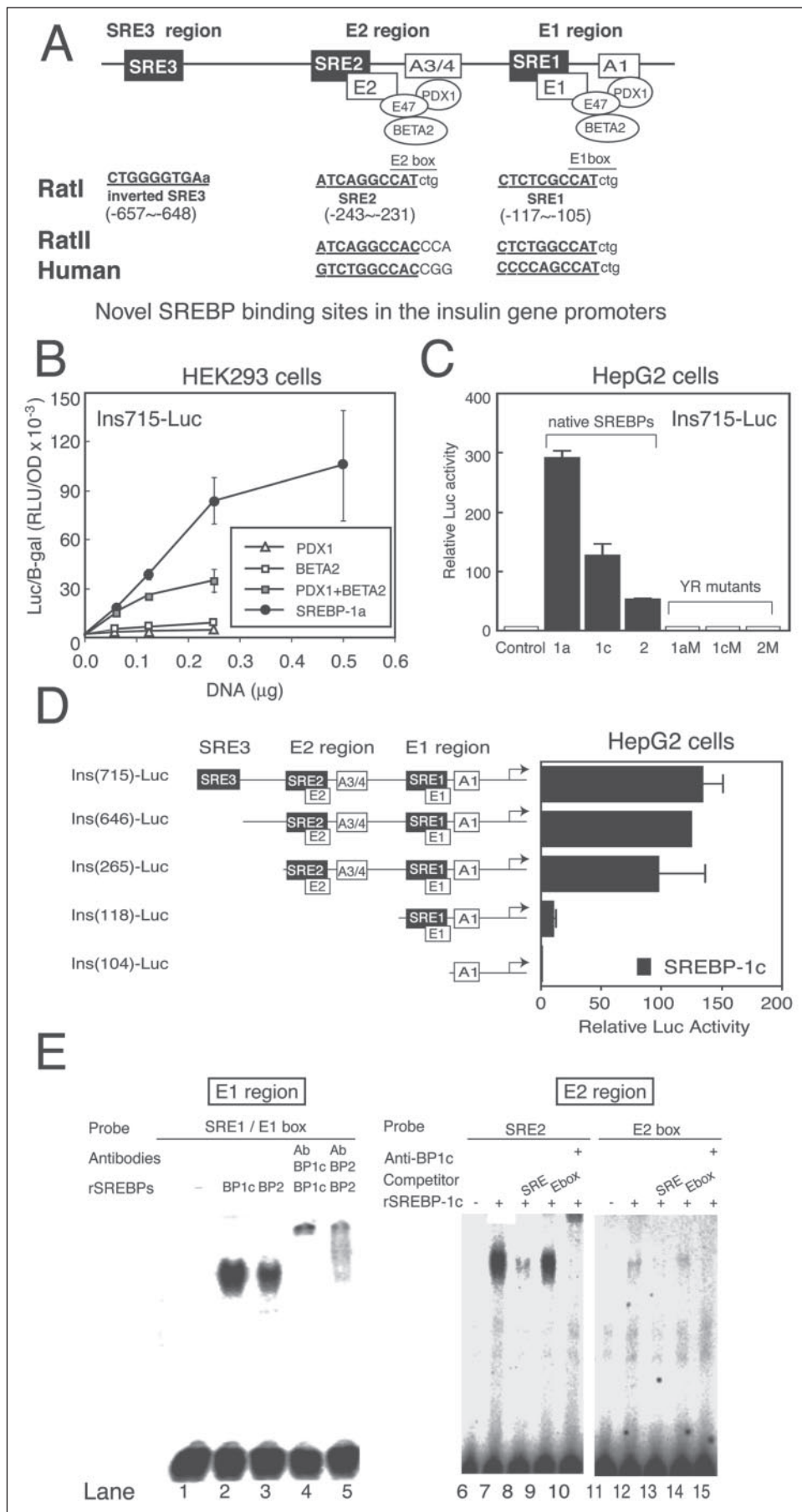
FIGURE 2. **Synergistic activation of the insulin promoter by SREBP-1c with BETA2, E47.** *A*, the comparison of relative insulin promoter activities (Ins265-Luc) by indicated combinations of expression plasmids (0.125 μg each) in HepG2 cells. *B* and *C*, in the presence of the indicated expression plasmids (0.125 μg each), SREBP-1c (0–0.5 μg, *B*) or BETA2 (0–0.125 μg, *C*) was co-transfected with Ins265-Luc and pSV-β-gal in HepG2 cells. The relative Luc activity (mean ± S.E.) normalized to β-gal activity was expressed as a fold change to that of the control vector (pCMV7).
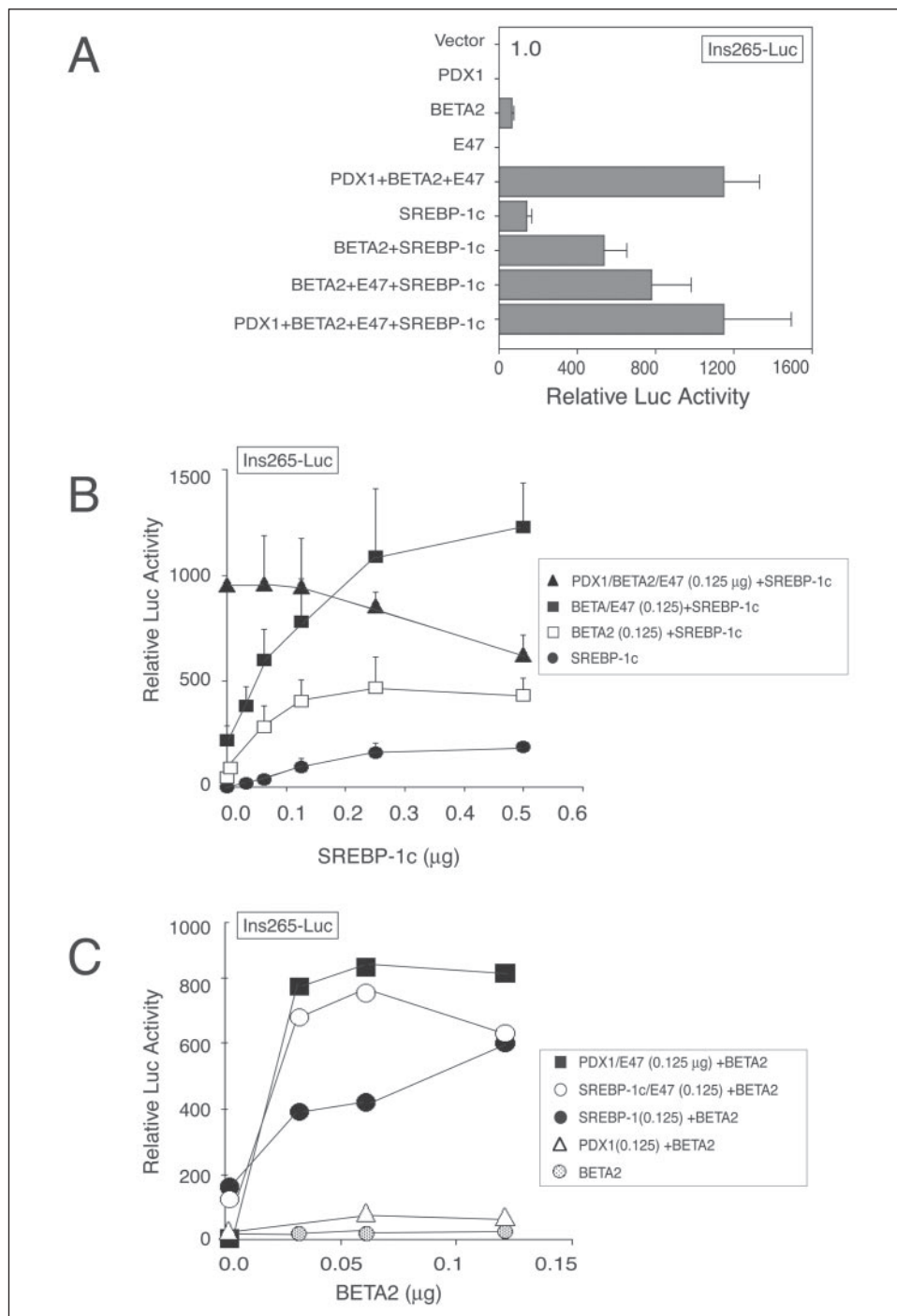
EGTA), rotated at 4 °C for 30 min, and centrifuged at 10,000 × *g* for 20 min. The protein from the nuclear extract is in the supernatant. All buffers used contain a mixture of protease inhibitors (25 μg/μl ALLN, 10 μg/μl leupeptin, 2.1 μg/μl aprotinin, 5 μg/μl pepstatin, and 5 μg/μl phenylmethylsulfonyl fluoride).

Isolation of Rat Islet and Mouse Pancreas—Pancreatic islets of Langerhans were isolated from 18-h-fasting male Sprague-Dawley rats (8 weeks old) and C57Bl6 mice (12 weeks old) by perfusion of the pancreatic duct and *in situ* collagenase digestion (34). The islets were subsequently purified by Ficoll density gradient and by hand picking. Immediately after isolation, the islets were cultured overnight in RPMI 1640 medium containing 5.5 mM glucose with or without T0901317 (3 μM). Total RNA was prepared using TRIzol reagent (Invitrogen).

Immunoblot Analysis—Aliquots of proteins were subjected to 10% SDS-PAGE transferred to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences) and incubated with rabbit anti-mouse SREBP-1 or goat anti-PDX-1 (N-18) antibodies (Santa Cruz Biotechnology). Secondary antibodies were horseradish peroxidase-conjugated donkey anti-rabbit or donkey anti-goat (Santa Cruz Biotechnology), and detection of immunoreactive bands was performed using the ECL kit (Amersham Biosciences).

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed as previously published with some modification (35, 36). A total of 3.0×10^7 INS-1 cells (from two confluent 10-cm plates) was cross-linked with formaldehyde (final concentration 1%) after washing with phosphate-buffered saline and incubation for 15 min at room tem-
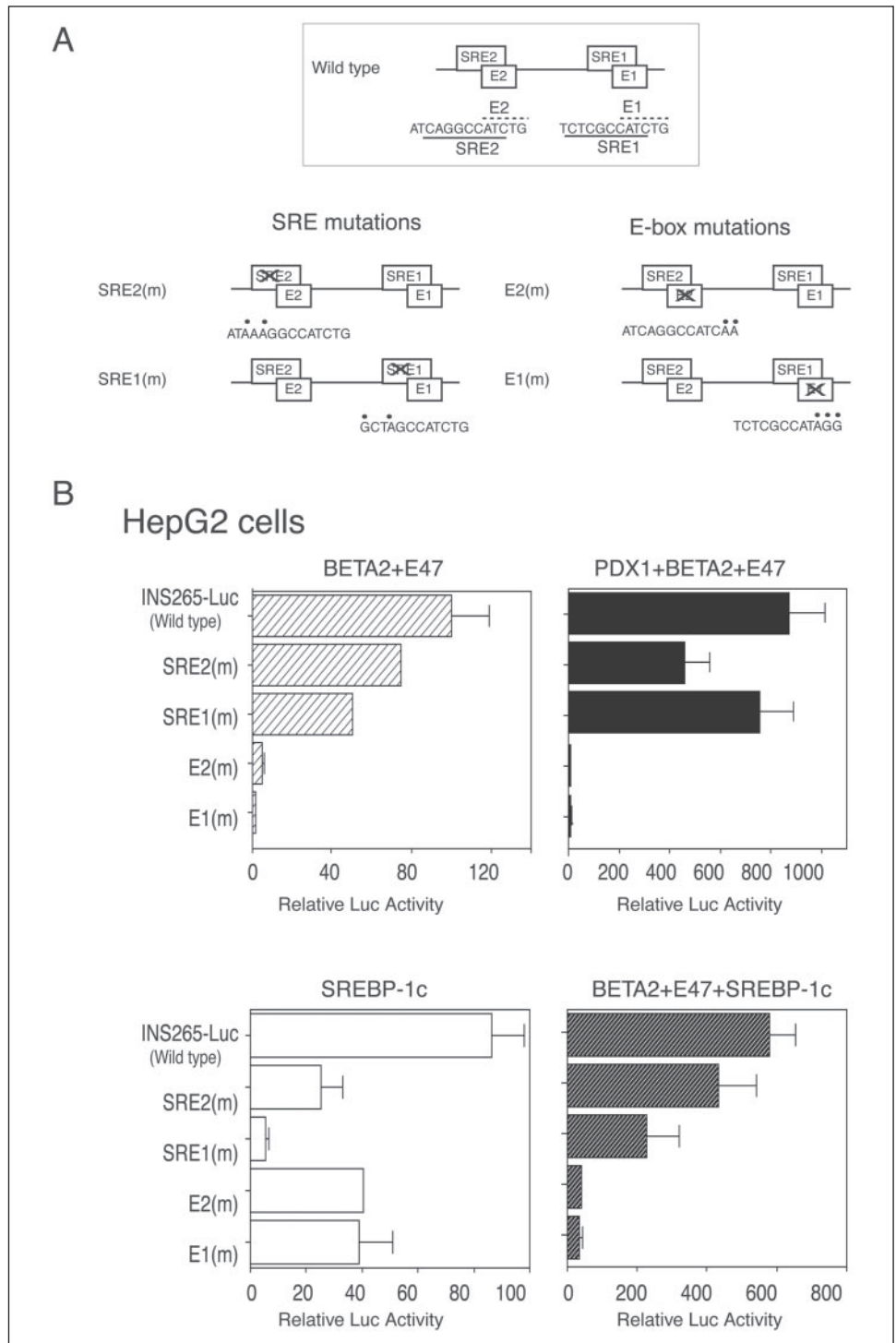
FIGURE 3. **Effects of mutations in SREs and E-boxes on activation of the insulin promoter by SREBP-1c, BETA2/E47, and PDX-1.** *A*, schematic presentation of various luciferase reporters containing native (*Ins265-Luc*) and mutant SREs (*SRE1* and *SRE2*) and E-boxes (*E1* and *E2*) in E1 and E2 regions in the rat insulin I gene. The indicated mutant plasmids were constructed by site-directed mutagenesis of Ins265-Luc (see "Materials and Methods"). *Dots* indicate mutant bases. *B*, native and various mutant reporter plasmids were co-transfected into HepG2 cells with pSV-β-gal and expression plasmids (*BETA2+E47*, *PDX-1+BETA2+E47*, *SREBP-1c*, and *BETA2+E47+SREBP-1c*) (0.125 μg each). The activity (mean ± S.E.) normalized to β-gal activity was expressed as a fold change to that of the control vector (pCMV7).

perature. The reaction was stopped by adding glycine at a final concentration of 0.125 M. After 5 min, the cells were scraped and washed two times with cold phosphate-buffered saline with protease inhibitors (10 μg/ml leupeptin, 2 μg/ml aprotinin, 12.5 μg/ml ALLN, 2.5 μg/μl pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). The pellets were resuspended in 0.5 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, plus protease inhibitors) and subjected to sonication at setting 10 for six 10-s pulses plus setting 30 for five 10-s pulses to share the chromatin to 1000-bp fragments. The samples were diluted 4-fold with immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.0, plus

protease inhibitors). To reduce the nonspecific binding, the samples were incubated with 60 μl of sonicated salmon sperm DNA/protein A-Sepharose slurry (Amersham Biosciences) on a rotating platform at 4 °C for 2 h. After centrifugation, the supernatant was incubated with 10 μl of rabbit anti-mouse SREBP-1 antibody and rabbit normal IgG as a negative control and rotated overnight at 4 °C. 0.9×10^7 INS-1 cells for each antibody were used. Immune complexes were collected by adding 20 μl of salmon sperm DNA/protein G-Sepharose slurry (Amersham Biosciences) for 4 h with rotation. Samples were subsequently washed using 1.0 ml of wash buffer A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1), wash buffer B (0.1% SDS, 1%
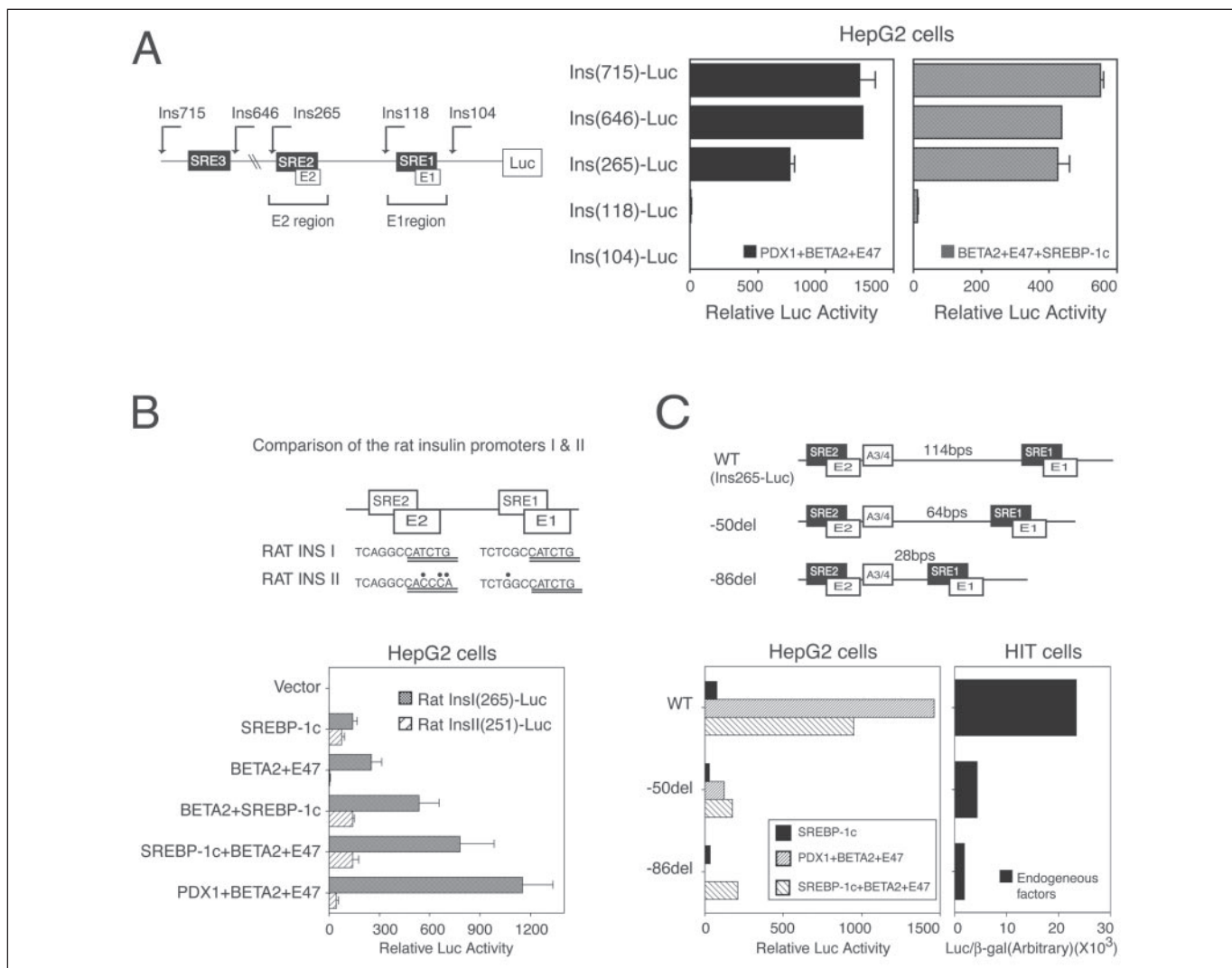
FIGURE 4. **Both the E1 and E2 regions with an appropriate distance are crucial for the synergistic activation of rat insulin I promoter.** *A*, deletion studies in the synergistic activation of the rat insulin I gene promoter in HepG2 cells. The indicated promoter plasmids, pSV-β-gal, and expression plasmids (*PDX-1+BETA2+E47* and *BETA2+E47+SREBP-1c*) (0.125 μg each) were transfected. The relative Luc activity (mean $\pm$ S.E.) normalized to β-gal activity was expressed as a fold change to that of the control vector (pCMV7) (*A, B,* and *C, left*). *B*, schematic representation of the comparison of the rat insulin promoters I and II in the E1 and E2 regions. The synergistic activities of Ins265-luc by the indicated expression plasmids (0.125 μg each) were compared with that of the rat insulin II luciferase promoter InsII-(251)-luc in HepG2 cells. *Dots* indicate different bases. *C*, schematic representation of Ins265-luc and mutant constructs with shorter intervening sequences (64 and 28 bp) between the E1 and E2 regions. Wild type (*WT,* Ins265-Luc) or deleted reporter plasmids (-50del-Luc, -86del-Luc) were transfected into HepG2 cells with pSV-β-gal in the presence of expression plasmids (*SREBP-1c, PDX-1+BETA2+E47,* or *BETA2+E47+SREBP-1c*) (0.125 μg each) (*left panel*). In HIT cells, each reporter plasmid (0.5 μg) and pSV-β-gal were co-transfected (*right panel*). The activities by β cell-specific endogenous factors were measured.

Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.1), wash buffer C (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and wash buffer D (1 mM EDTA, 10 mM Tris-HCl, pH 8.0), and eluted by 30-min incubations with 0.5 ml of elution buffer (1% SDS, 50 mM NaHCO$_3$, 10 mM dithiothreitol). NaCl was added to the elution at a final concentration of 0.3 M, and the samples were incubated at 65 °C for 6 h to reverse the formaldehyde-induced cross-linking. DNA and protein were ethanol-precipitated overnight at -20 °C and dissolved in 100 μl of Tris EDTA buffer (pH 7.4). Digestion was performed by the addition 25 μl of proteinase K buffer (50 mM Tris-HCl, pH 7.5, 25 mM EDTA, 1.25% SDS) with 2 μg/μl proteinase K (2 μl), and samples were placed at 55 °C for 1 h. Chromatin DNA was extracted with phenol/CHCl$_3$ followed by ethanol precipitation. Samples were dissolved in 100 μl of Tris EDTA buffer (pH 7.4). 3-μl aliquots were used for PCR analysis. To amplify the rat insulin I promoter region containing SRE1 and SRE2, the following primer sets were used: 5′-TGCTTCATCAGGCCATCTGG-3′ for sense and 5′-G-GTAGGCAGATGGCGAGAGGG-3′ for antisense. Primers sets for rat fatty acid synthase promoter regions containing SRE were used as a positive control: 5′-GACGCTCATTGGCCTGG-3′ for sense and 5′-T-CTGGAGGCAGACGACAAG-3′ for antisense. The PCR conditions were 5 min at 94 °C and 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C. Following amplification, PCR products were electrophoresed in a 3% agarose gel and visualized by ethidium bromide staining.

Statistics—Statistical significance was assessed with the Student's *t* test for unpaired data.

RESULTS

Identification of Insulin Gene Promoters as SREBP Targets—As schematized in Fig. 1*A*, pancreatic β cell-specific *cis*-elements for the insulin promoter included E-boxes (E1 and E2) as binding sites for bHLH protein heterodimers BETA2/E47, and A-boxes (A1 and A3/4) for a homeobox PDX-1. In the process of searching for glucose/insulin-responsive elements in promoters of nutritionally regulated genes in rela-
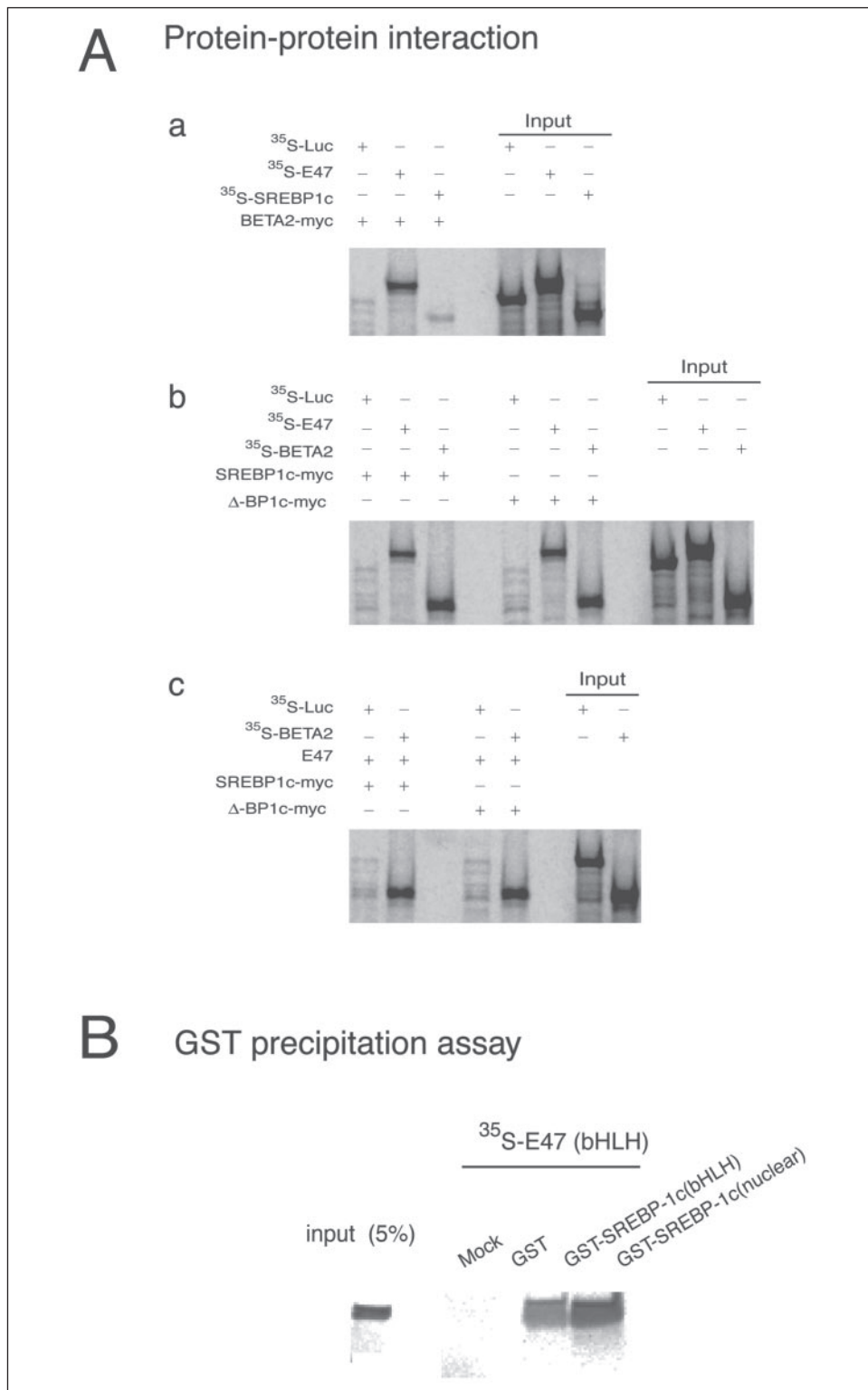
FIGURE 5. **SREBP protein physically binds to E47 and BETA2.** *A, in vitro*-translated, ^{35}S-labeled luciferase (*a–c*), E47 (*a* and *b*), SREBP-1c (*a*), and/or BETA2 (*c*) protein, and/or unlabeled E47 (*c*) were incubated with Myc epitope-tagged BETA2 (BETA2-Myc, *a*), SREBP-1c (SREBP1c-Myc, *b* and *c*), or TA-deleted SREBP-1c (ΔTA-BP1c-Myc, *b* and *c*). The mixtures were immunoprecipitated with anti-Myc antibody. 10% of the ^{35}S-labeled proteins used for the assay were resolved by SDS-PAGE as input. *B, in vitro*-translated ^{35}S-labeled E47 (*bHLH*-(498–634)) was incubated with 1 μg of GST alone, GST-SREBP-1c (nuclear form (24–460)), or GST-SREBP-1c (*bHLH*-(286–364)) fusion proteins immobilized on glutathione-Sepharose beads, and bound proteins were resolved on 15% gel by SDS-PAGE followed by autoradiography. 5% of the ^{35}S-labeled E47 *bHLH*-(498–634) protein was loaded as input.

tion to SREBP regulation, we found that the rat insulin I gene promoter possesses three potential binding sites for nuclear SREBPs putatively designated SRE1, SRE2, and SRE3 (Fig. 1*A*). Intriguingly, SRE1 and SRE2 were located adjacent to the E1- and E2-boxes with partial overlapping sequences, respectively. The cluster of A1, E1, and SRE1 was tentatively designated as the E1 region and the distal cluster of A3/4, E2, and SRE2 as the E2 region (Fig. 1*A*). SRE3 was located upstream and in a reverse orientation. These new potential SREBP binding elements are highly conserved in rat II insulin and human insulin gene promoters (37). Luciferase assays in non-β HEK293 and HepG2 cells (data not shown) demonstrated that overexpression of the active form (nuclear) of SREBP-1a markedly and dose-dependently induced the activity of the rat insulin I promoter containing these regions (Ins715-Luc) (Fig. 1*B*). Strikingly, the activation of Ins715-Luc by nuclear SREBP-1a was even higher than by co-expression of PDX-1 and BETA2, both of which are crucial for β cell-specific and robust expression of the insulin gene (12,

38). Among the nuclear SREBP isoforms (referred to as SREBPs, hereafter), SREBP-1a had the highest activity (Fig. 1*C*). SREBP-1 has been shown to bind both SRE and some E-boxes, low stringent consensus sequences for bHLH proteins (39). Tyr→Arg mutants of SREBPs in which the tyrosine residue in the basic region of SREBP are replaced by arginine conserved in regular bHLH proteins have been shown to abolish SREBP binding to SRE elements but retain binding to E-boxes (39). All SREBP Tyr→Arg mutants (SREBP-1aM, -1cM, and -2M) completely lost activity, supporting evidence that SREBP activation of the insulin promoter is mediated through binding to the SRE elements and not to the E-boxes (Fig. 1*C*). Sequential deletion studies demonstrated that SRE1, SRE2, and SRE3 elements all contribute to the SREBP activation of the insulin promoter (Fig. 1*D*).

To evaluate the direct binding of SREBPs to these putative SRE elements, gel mobility shift assays were performed. Both recombinant SREBP-1c and recombinant SREBP-2 bind to both SRE1/E1 box (Fig. 1*E*, *left*) and SRE2/E2 box (data not shown). The specificity of the binding was confirmed by their supershifts after the addition of SREBP-1c or SREBP-2 antibodies. The signals from DNA probes specific to SRE2 and E2-box and competition with unlabeled probes indicate that SREBP-1 binds to SRE2 and not to E2-box (Fig. 1*E*, *right*). The binding of SREBP to the SRE3 element was also confirmed, although with a slightly weaker signal than to SRE2 and SRE1 (data not shown). These data demonstrated that SREBPs activate the rat insulin I gene promoter through binding to the SRE1, SRE2, and SRE3 elements. SREBPs were also found to bind to other insulin SREs in rat insulin I and II and human insulin promoters (data not shown). Taken together, it was concluded that the insulin gene is a new SREBP target gene.

Synergistic Activation of Insulin Gene by SREBP-1 with β Cell-specific Factors—Insulin gene expression is highly induced by a tertiary complex of PDX-1·BETA2·E47 riding over E-box and A-box. Considering the overlapping location of the SREs and the E-boxes, it is possible that potential interactions between SREBPs and BETA2/E47 could modify the insulin promoter activity. The effects of co-expression of SREBP-1c with these pancreatic β cell-specific factors were evaluated using a luciferase construct containing the E1 and E2 regions (Ins265-Luc) in HepG2 cells (Fig. 2*A*). Activation of Ins265-Luc by SREBP-1c was enhanced by co-expression of BETA2. When both BETA2/E47 were added to SREBP-1c, the luciferase activity was further enhanced and was close to the authentic maximal activation of the insulin promoter by PDX-1/BETA2/E47 in HepG2 cells, which was reported to be comparable with the intrinsic insulin promoter activity in β cells (38). Fig. 2, *B* and *C*, shows that SREBP-1c and BETA2 dose-dependently contribute to the synergistic activation by either SREBP-1c/BETA2 or SREBP-1c/BETA2/E47 (Fig. 2*C*). However, when SREBP-1c was added to the authentic combination of PDX-1/BETA2/E47, the potent activity was slightly diminished (Fig. 2*B*).

To evaluate the importance of individual SREs and E-boxes in synergistic activation by SREBP-1c and BETA2/E47 more precisely, various mutations in the luciferase constructs were tested in HepG2 cells (Fig. 3). As shown in Fig. 3*B*, mutation of either the E1- or E2-box completely abolished PDX-1/BETA2/E47 activation as previously reported (40), reconfirming that E1- and E2-boxes are essential for PDX-1/BETA2/E47 activation. For activation by SREBP-1c alone, SRE2 considerably contributed to its activation, as already suggested by the deletion study (Fig. 1*D*), but SRE1 was found to be most crucial. Synergistic activation by SREBP-1c/BETA2/E47 was almost eliminated by the disruption of either the E1- or E2-box, whereas mutations in SRE1 or SRE2 only slightly decreased. These data suggested that SREBP activation and SREBP synergism with BETA2/E47 could be mediated through differ-
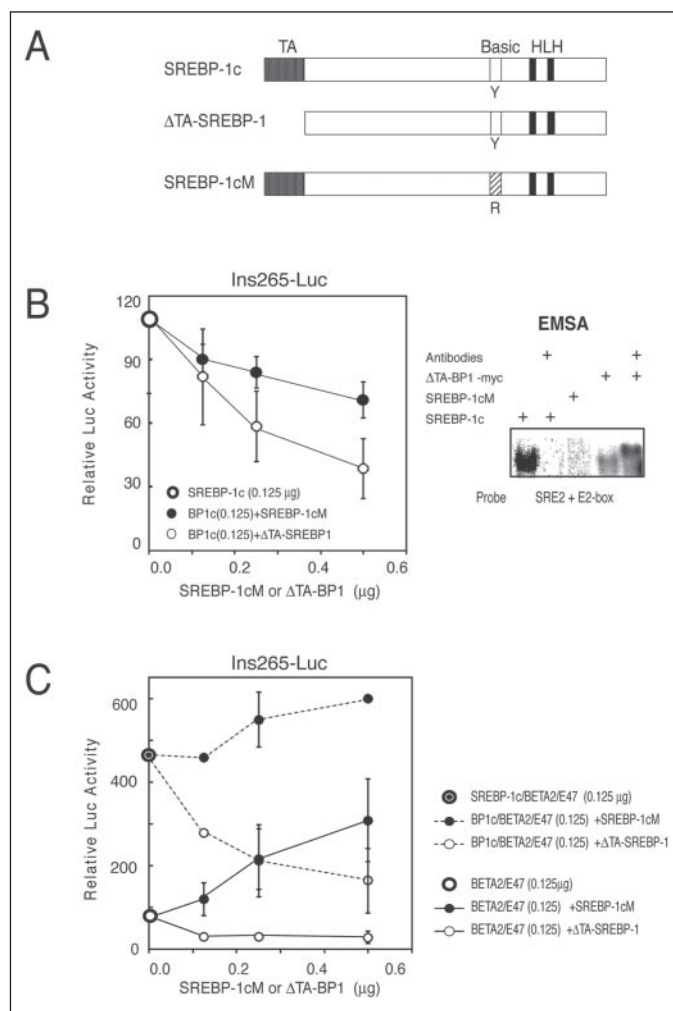

FIGURE 6. **Effects of TA deletion (Δ*TA-SREBP-1*) and Tyr→Arg mutation (*SREBP-1cM*) on the synergistic activation by SREBP-1c/BETA2/E47.** *A*, schematic representation of wild type of SREBP-1c, ΔTA-SREBP-1 in which the amino-terminal transactivation domain of SREBP-1c was deleted, and SREBP-1cM in which arginine (*R*) was substituted for tyrosine (*Y*) residue conserved in the basic region of DNA binding sites of SREBP. *Basic*, DNA binding site; *HLH*, helix-loop-helix structure. *B*, *left*, inhibitory effects of ΔTA-SREBP-1 and SREBP-1cM on the activation of Ins265-Luc by SREBP-1c. Ins265-luc, pSV-β-gal, and pCMV-SREBP-1c (0.125 μg) were transfected with the indicated amounts of ΔTA-SREBP-1 or SREBP-1cM (0–0.5 μg) in HepG2 cells. *Right*, electrophoretic mobility shift analysis (*EMSA*) of DNA-binding activities of SREBP-1c, SREBP-1cM, and ΔTA-SREBP-1. Each of the SREBP plasmids was transfected into HEK293 cells, and nuclear proteins were extracted. The SRE2+E2-box region was used as a probe. Supershift assay was performed using antibodies against SREBP-1 or Myc. *C*, effects of ΔTA-SREBP-1 or SREBP-1cM on the activation of Ins265-Luc by BETA2+E47 or SREBP-1c+BETA2+E47. The indicated amounts of ΔTA-SREBP-1 or SREBP-1cM (0–0.5 μg) were transfected with Ins265-luc and pSV-β-gal in HepG2 cells. The relative Luc activity (mean ± S.E.) was expressed as a fold change to that of the control vector (pCMV7).

ent *cis*-elements. Both the E1- and E2-boxes appear to be indispensable for synergistic activation by SREBP-1c/BETA2/E47 as well as by PDX-1/BETA2/E47 activation.

The Synergistic Activation of SREBP-1 and β Cell-specific Factors Is Mediated through the Inter-region Interaction between E1 and E2 Regions—Effects of sequential deletion of the E1 and E2 regions were estimated in terms of SREBP-1c/BETA2/E47 activation in HepG2 cells. The results highlighted the importance of the E2 region, showing that the E1 region alone does not have any synergistic activation (Fig. 4*A*). Meanwhile, from the evaluation of the construct in which distal E2 region was fused to a SV-40 promoter-driven luciferase reporter gene, it was found that the E2 region alone did not have any enhancer activity (data not shown). All these data indicated that both the E1 and E2
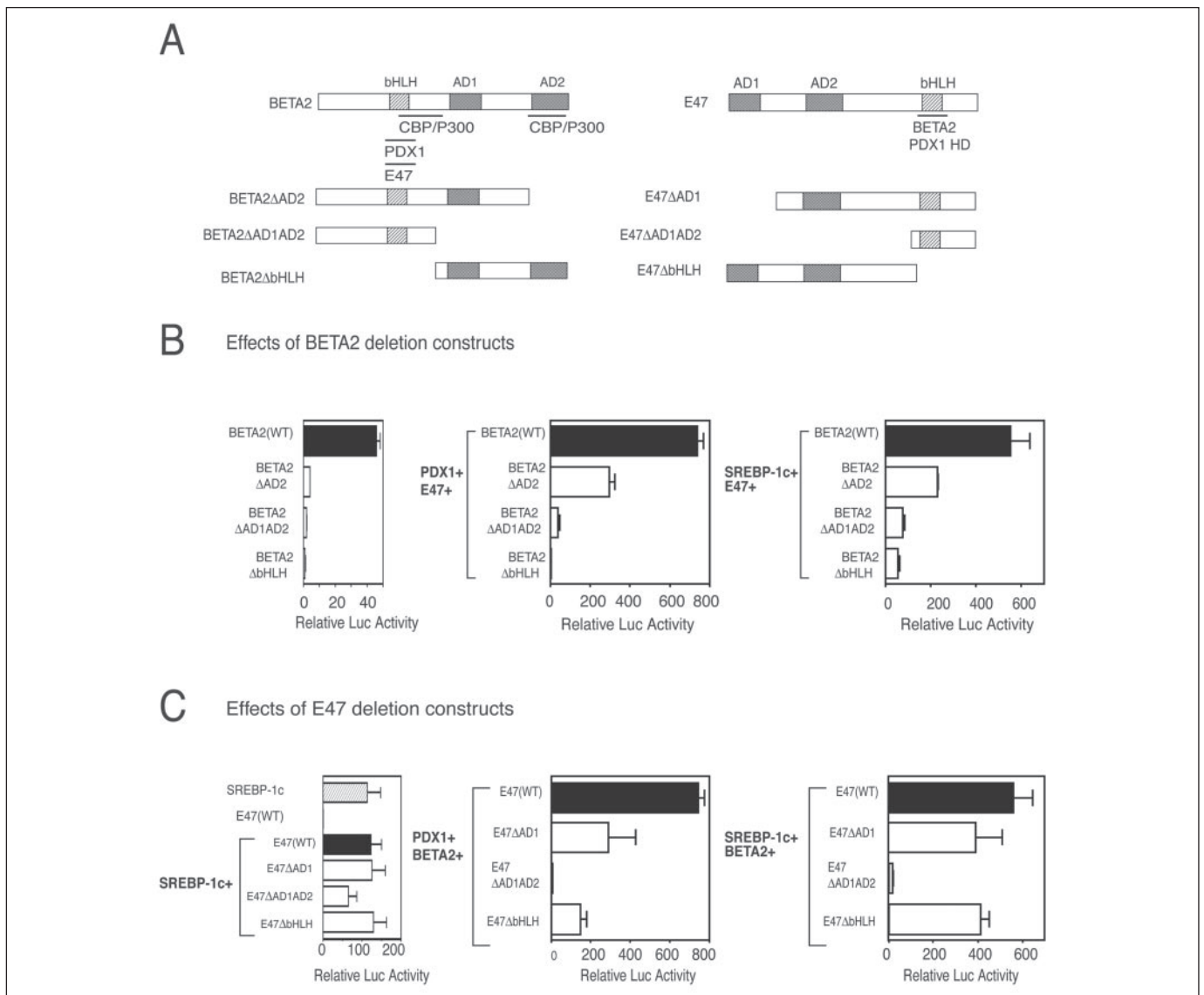
FIGURE 7. **Mapping of essential domains in BETA2 and E47 for the synergistic activation of insulin promoter.** *A*, schematic domain maps of BETA2 (*left*) and E47 (*right*). Both proteins contain two activation domains AD1 and AD2 and a bHLH domain. The *bars* indicate regions for the protein-protein interaction. The constructs are as follows: BETA2-(1–357), BETA2ΔAD2-(1–299), BETA2ΔAD2AD1-(1–170), and BETA2ΔbHLH-(170–357), E47-(1–647), E47ΔAD1-(178–647), E47ΔAD1AD2-(510–647), and E47ΔbHLH-(1–537). *B*, effects of native or truncated BETA2 (0.125 μg) on Ins265-Luc activity with PDX-1+E47 (0.125 μg) or SREBP-1c+E47 (0.125 μg) in HepG2 cells. Basal activities of native and truncated BETA2 were indicated on the *left*. *C*, effects of native or truncated E47 (0.125 μg) on Ins265-Luc activity with SREBP-1c (0.125 μg), PDX-1+BETA2 (0.125 μg), or SREBP-1c+BETA2 (0.125 μg) in HepG2 cells. Basal activities of native and all truncated E47s were 1.0 (data not shown.). The relative Luc activity is expressed as a fold change to that of the control vector (pCMV7).

regions are mandatory for the synergism, implicating that SREBP-1c/ BETA2/E47 as well as PDX-1/BETA2/E47 synergistically activate the insulin promoter through inter-region (E1 and E2 regions) interaction. This hypothesis was supported by experiments showing that the synergistic activation was not observed in the rat insulin II promoter, which does not have an E-box in the E2 region (Fig. 4*B*). To examine the impact of the length between the two regions on inter-region synergism, the effects of deletions in the intervening sequence (hypothetical bending part of the DNA) were evaluated (Fig. 4*C*). In HepG2 cells, synergistic activation by PDX-1/BETA2/E47 or SREBP-1c/BETA2/E47 was essentially abolished in both of the constructs with shorter intervening sequences (-50del, -86del) (Fig. 4*C*, *left*). In HIT cells, the hamster insulinoma cell line, deletion of the intervening sequence affected the insulin promoter activity (Fig. 4*C*, *right*). Thus, the synergism required two separate E1- and E2-boxes with a certain appropriate distance in a bending form presumably to form the complex of DNA and SREBP-1c (or PDX-1)·BETA2·E47.

Direct Interaction of SREBP-1c with BETA2/E47 in Vitro—Based upon the synergistic activation by these bHLH proteins, the physical interaction between SREBP-1c, BETA2, and E47 was estimated using co-immunoprecipitation experiments. As shown in Fig. 5*A*, labeled *in vitro*-translated E47 protein was co-immunoprecipitated with SREBP-1c protein, indicating that E47 and SREBP-1c physically interact. The signal of SREBP-1c/E47 was comparable with that of authentic BETA2/E47 formation (Fig. 5*A*, *a*). Meanwhile BETA2/SREBP-1c binding gave a very weak signal (Fig. 5*A*, *a*). However, labeled BETA2 could be immunoprecipitated by SREBP-1c in the concomitant presence of E47, suggesting the formation of a SREBP-1c·BETA2·E47 complex (Fig. 5*A*, *c*). ΔTA-SREBP-1c also showed similar results, demonstrating that interaction of SREBP-1c with E47 is mediated through the bHLH portion of SREBP-1c and not its transactivation domain (Fig. 5*A*, *b* and *c*). Pull-down assays using GST fusion proteins of the bHLH portion of SREBP-1c and E47 also provided similar results, indicating that the bHLH portions of SREBP-1c and E47 directly interact (Fig. 5*B*). These

FIGURE 8. **Effects of SREBP-1c on rat insulin I gene activity in β cells.** *A, a,* comparison of the activation of wild type (Ins265-Luc) and its various mutation constructs by β cell-specific endogenous factors in HIT cells, the hamster insulinoma cell line. The structure of constructs were as described in the legend to Fig. 3*A*. The delSRE2-Luc SRE2 element but contains SRE1, E1-, and E2-boxes. The A3/4-box (5'-TAATAATCTAATAC-3') was mutated as 5'-TCCTCCTCTGCTAG-3'. The reporter plasmid and pSV-β-gal were transfected in HIT cells. The relative luminescence *versus* Ins265-Luc (set as 1.0) is shown. *b,* activation of Ins265-Luc and mutation constructs by SREBP-1c overexpression in HIT cells. The activity (mean $\pm$ S.E.) was expressed as a fold change to that of the control vector (pCMV7). **, $p < 0.001$; *, $p < 0.01$ when compared with INS265-Luc (*WT*) activity with pCMV7. *B,* Western blot analysis of endogenous SREBP-1c and PDX-1 in HIT cells induced by glucose and T0901317. Cells were incubated with or without T0901317 (10 μM) for 19 h and further incubated in 5.5, 10, and 25 mM glucose in medium for 5 h. Membrane and nuclear proteins were extracted. *C,* Ins265-Luc and pSV-β-gal were transfected in HIT cells in the same condition as Fig. 8*B*. Values are given as relative activity to Ins265-Luc in 10 mM glucose without T0901317 (set as 1.0). *Error bars* represent standard error ($n = 12$). *N.S.,* not significant.
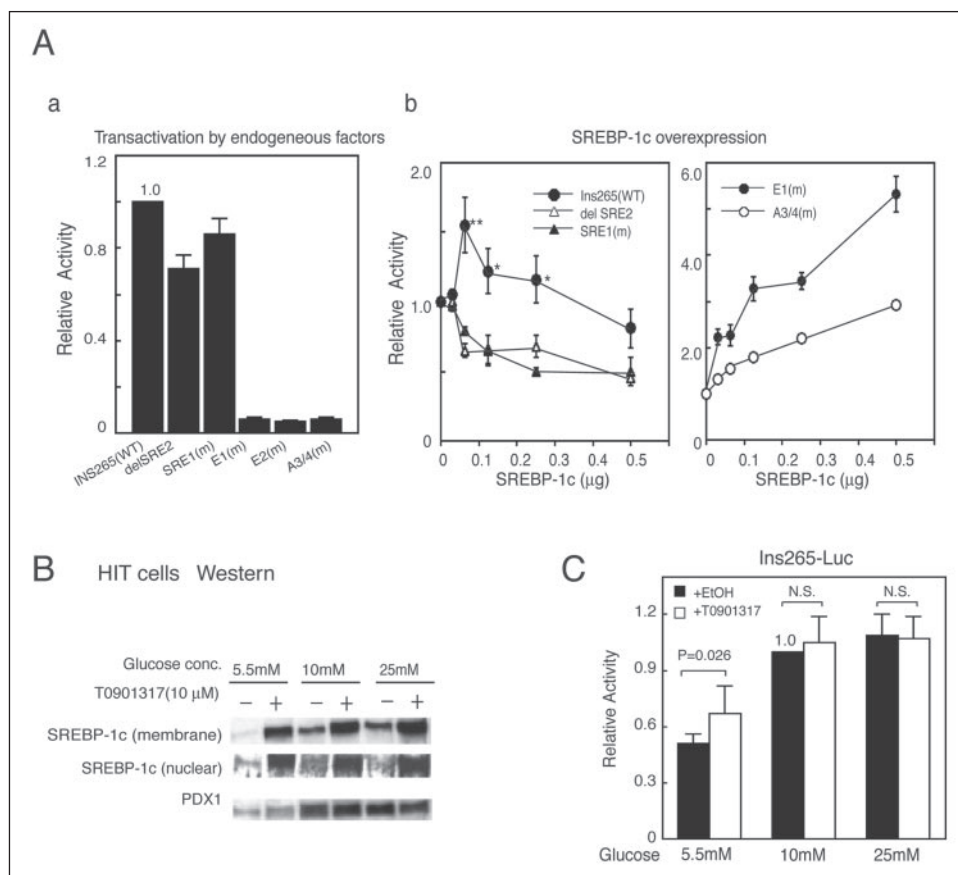
data suggested that SREBP-1c could form the complex with E47 and BETA2 on the insulin promoter.

SREBP-1c Is Involved in Synergistic Activation of Insulin Promoter without DNA Binding—The mutation analysis on E1 and E2 regions indicated that synergistic activation of the insulin promoter by SREBP-1c/BETA2/E47 require both E1- and E2-boxes, rather than SRE1 and SRE2. Thus, SREBP-1c involvement in this synergism could not be mediated through direct DNA binding. We further examined the effect of the SREBP-1c Tyr→Arg mutant (SREBP-1cM) (Fig. 6*A*). By electrophoretic mobility shift analysis, SREBP-1cM does not bind to either SREs or E-boxes of the insulin promoter I (Fig. 6*B*, *right*). Consistent with this, SREBP-1cM, by itself, did not activate the insulin promoter in luciferase assays (Fig. 1*C*). SREBP-1cM only slightly inhibited the activity of SREBP-1c in a dose-dependent manner (Fig. 6*B*, *left*). However, SREBP-1cM robustly activated the insulin promoter synergistically with BETA2/E47, although less efficiently than SREBP-1c (Figs. 2*C* and 6*C*). When SREBP-1cM was added to SREBP-1c/BETA2/E47, the activation by SREBP-1c/BETA2/E47 was further potentiated. Thus, SREBP-1cM did not bind to the insulin promoter but still exhibited the synergistic activation similar to SREBP-1c. These data strongly suggested that the synergistic activation by SREBP-1c and BETA2/E47 on the rat insulin promoter I is mediated through the direct interaction of SREBP-1c with BETA2/E47 on the E-boxes and not through DNA binding of SREBP-1c to SRE elements.

Activation Domains of SREBP-1, BETA2, and E47 Mediate the Synergistic Activation of the Insulin Promoter—Each domain of SREBP-1c, BETA2, and E47 was estimated in terms of their synergistic activation of the insulin promoter. ΔTA-SREBP-1 (ΔTA-BP1) (Fig. 6*A*) has been shown to inhibit the transcriptional activity of SREBP-1 in a competitive way (Fig. 6*B*) (20, 41). By electrophoretic mobility shift analysis, ΔTA-

BP1 binds to SREs of the insulin promoter I (Fig. 6*B*, *right*). ΔTA-BP1, by itself, had no activity for Ins265-Luc (data not shown). ΔTA-BP1 partially but dose dependently inhibited the activation of Ins265-Luc by BETA2/E47 or SREBP-1c/BETA2/E47 (Fig. 6*C*). These data indicate that the transactivation domain of SREBP-1 is crucial for activation of the insulin promoter by SREBP-1 alone and is necessary for full synergistic activation with BETA2/E47.

Fig. 7*A*, *left*, shows the function structure of BETA2. Activation of Ins265-Luc by BETA2 alone was essentially abolished by the deletion of AD2 or bHLH (Fig. 7*B*). Partial deletion of AD2 (BETA2ΔAD2-(1–299)) and lack of both AD1 and AD2 (BETA2ΔAD2AD1-(1–170)) completely attenuated the synergistic activation, suggesting that both AD1 and AD2 are essential for the full synergistic activation of SREBP-1c/BETA2/E47 as well as for PDX-1/BETA2/E47, as previously reported (14). Deletion of bHLH of BETA2 domains (BETA2ΔbHLH-(170–357)), which disrupts heterodimerization with E47, caused a marked reduction in activities in SREBP-1c/BETA2/E47 with some residual activity, reflecting that SREBP-1c alone could bind to the SRE elements and activate the insulin promoter. These data demonstrated that BETA2 contributes to the synergistic activation of the insulin promoter by SREBP-1c/BETA2/E47 through AD1 and AD2.

E47, an alternative splicing form of the *E2A* gene, consists of two activation domains, AD1 and AD2, and a bHLH domain (Fig. 7*A*, *right*) (42). Presumably because of the presence of endogenous E2A products, E47 expression did not enhance the SREBP-1c effect on Ins265-Luc (Fig. 7*C*, *left*). Domain deletion studies indicated that SREBP-1c/BETA2/E47 (and also PDX-1/BETA2/E47) activation of Ins265-Luc was markedly impaired by the deletion of both AD1 and AD2 (E47ΔAD1AD2-(510–647)) in a dominant negative fashion. Taken together, the activation

domains of SREBP-1c, BETA2, and E47 are highly involved in synergistic activation of the insulin promoter by SREBP-1c/BETA2/E47.

Discrepancy in the SREBP Activation of the Insulin Promoter between β Cells and Non-β Cells—To determine whether SREBPs contribute to insulin gene expression in β cells, reporter assays using Ins265-Luc and its mutant constructs were performed in HIT cells. As shown in Fig. 8*A*, *a*, both E-box mutants and the A3/4-box mutant completely abolished the activity, confirming the key roles of PDX-1 or BETA2/E47 in β cells. In contrast, when SRE2 and SRE1 were deleted and mutated, respectively, the activity of the insulin promoter was only marginally reduced (Fig. 8*A*, *a*), suggesting a small, if any, contribution of endogenous SREBP in the insulin promoter activity in β cells. Overexpression of SREBP-1c in HIT cells exhibited a complex, bell-shaped activation of Ins265-Luc with a slight suppression at a higher dose (Fig. 8*A*, *b*, *left*). Conversely, Ins265-Luc with SRE2 deletion or SRE1 disruption, which cancelled the direct binding of SREBP-1c to the promoter, caused dose-dependent inhibition by SREBP-1c, suggesting the inhibitory effect of SREBP-1c on endogenous PDX-1/BETA2/E47 (Fig. 8*A*, *b*, *left*). When the E-boxes or A3/4-box were disrupted to cancel the effects of the β cell-specific factors, exogenous SREBP-1c robustly activated the promoter presumably by binding to intact SRE1 and SRE2 elements, as observed in non-β cells (Fig. 8*A*, *b*, *right*). These data suggested that, although SREBP-1c has a potential to activate the insulin promoter, the presence of β cell-specific factors masked this effect and SREBP-1c overexpression could, rather, inhibit the intrinsic insulin promoter activity.

To further investigate the physiological relevance of SREBP-1c in β cells, the effects of two conditions known to induce endogenous SREBP-1c expression (high glucose and treatment with the T0901317 synthetic LXR agonist) on insulin gene expression were tested. Immunoblot analysis on cell membranes (precursor form of SREBP-1c) and nuclear extracts (mature form) demonstrated that incubation with high glucose and/or treatment with T0901317 additively elevated both membrane and nuclear forms of SREBP-1c protein in HIT cells (Fig. 8*B*). Endogenous PDX-1 protein content was low at 5.5 mM glucose and induced at higher concentrations (10 and 25 mM). T0901317 did not change the PDX-1 protein level. Insulin promoter activity was measured in these conditions (Fig. 8*C*). The activity was increased at high glucose concentrations, presumably reflecting high PDX-1 expression. The LXR ligand slightly but significantly induced the Ins265-Luc at low glucose concentration with low PDX-1 expression but not at higher glucose concentrations with high PDX-1 expression.

Of more physiological relevance, the direct binding of endogenous SREBP-1 to the insulin promoter (containing SRE2 and SRE1 elements) was estimated *in vivo* by ChIP assays of the nuclei from INS1 cells, the rat insulinoma cell line. Both membrane and nuclear SREBP-1 proteins were highly induced by the addition of the LXR ligand, as previously observed in HIT cells (Fig. 9*A*) and liver cells (43). In response to the induction of nuclear SREBP-1 in both 5.5 and 10 mM glucose in medium with T0901317, direct binding of SREBP-1 to SRE2 and/or SRE1 in the insulin promoter was shown by ChIP assay as well as to the fatty acid synthase promoter, an authentic SREBP-1c target (Fig. 9*B*). Finally, insulin gene expression was measured in isolated islets after the SREBP-1c induction. Isolated rat and mouse islets were incubated at 5.5 mM glucose with T0901317 (3 μM). With high induction of SREBP-1c expression, the insulin mRNA level could be increased in both islets (Fig. 9*C*). These data demonstrated that endogenous SREBP-1c could directly bind to the insulin promoter and increase insulin gene expression in β cells in accordance with the findings from non-β cells.
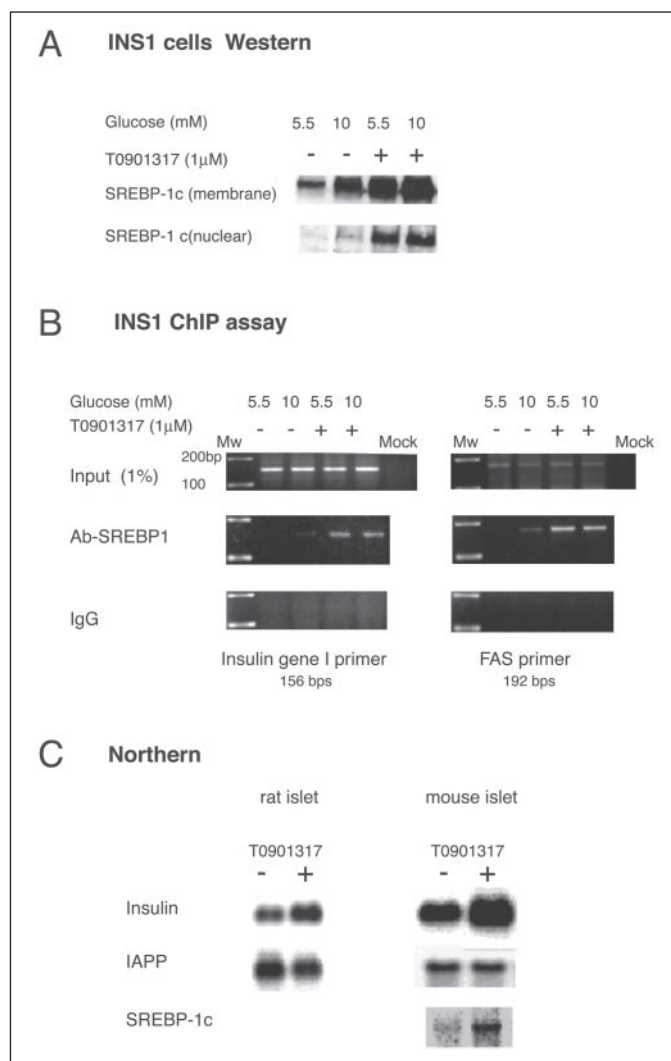

FIGURE 9. **Endogenous SREBP-1c is induced by T0901317, directly binds to insulin promoter, and increases insulin gene expression in β cells.** *A*, Western blot analysis of endogenous SREBP-1c in INS1 cells, the rat insulinoma cell line. Cells were incubated with or without T0901317 (1 μM) for 19 h and further incubated in 5.5 or 10 mM glucose in medium for 5 h. Membrane and nuclear proteins were extracted. *B*, chromatins prepared from INS1 cells (under the same conditions as described for *A*) were subjected to the ChIP assay with anti-mouse SREBP-1 antibody and rabbit IgG as a negative control. Immunoprecipitate samples were subjected to PCR using primers to amplify rat insulin I gene and rat fatty acid synthase (*FAS*) gene promoters. *Mw*, molecular weight. *C*, Northern blot analysis of insulin gene expression in mouse and rat pancreatic islet treated with or without T0901317. Isolated islets were incubated in 5.5 mM glucose with or without T0901317 (3 μM) for 24 h. Amounts of mRNA for insulin, islet amyloid polypeptide (IAPP), or SREBP-1c were measured. Total RNA was isolated and subjected to blot hybridization with the indicated ^{32}P-labeled probe.

DISCUSSION

Identification of Insulin Promoter as an SREBP Target—Our current studies clearly demonstrated that the insulin gene is a novel SREBP target and that SREBP activates the insulin gene promoter, strongly in non-β cells and potentially in β cells. The rat insulin I promoter possesses three novel SRE elements to which SREBP homodimers can bind and which contribute to the SREBP-mediated activation. To date, almost all of the known promoters of the SREBP target genes involved in lipid metabolism have a site(s) for co-activators, NF-Y, and/or Sp1 in the neighboring region to recruit CBP/p300 (44–46). Because β cell factors also use CBP/p300 as a co-activator for insulin gene expression (13, 14), it is plausible that CBP/p300 recruitment is crucial for SREBP-mediated activation. Complete loss of the activity by deletion of the transactiva-

tion domain of SREBP-1, a known CBP binding site, supports this notion.

Synerigistic Activation of SREBP-1 and BETA2/E47—There are a few base pairs overlapping the SRE and E-box in both the E1 and E2 regions. Considering space hindrances in the case of SREBP-1c/BETA2/E47 co-transfection, it is impossible that SREBP-1c binds to SRE and BETA2/E47 binds to E-box in the same region simultaneously. Comparative gel shift assays suggested that, overall, the affinities of BETA2/E47 for E1- and E2-boxes are higher than those of SREBP-1 for SRE1 and SRE2 (data not shown). Thus, when SREBP-1c and BETA2/E47 are both present, BETA2/E47 should dominate the E1 and E2 regions. This could partially explain the finding that the SREBP-1c contribution to insulin promoter activity was small in HIT cells.

As shown by mutation and deletion studies, SREBP-1c/BETA2/E47 synergism requires both the E1 and E2 regions. In particular, E-boxes (and not SREs) in E regions are crucial for the synergistic activation of SREBP-1c/BETA2/E47. Thus, it is BETA2/E47 that directly binds to the insulin promoter (E-boxes). As suggested by immunoprecipitation and SREBP-1cM experiments, SREBP-1c could be committed to this synergism by physically interacting with BETA2/E47 without DNA binding. This illustrates a dual functionality of SREBP-1c; along with being an established transcription factor that binds to SRE, SREBP-1c can also serve as a co-activator for recruitment of CBP/p300. Each component of the SREBP-1c·BETA2·E47 complex has transactivation (TA) domains that can bind to CBP/p300. Thus, the complex with multiple TA domains could efficiently and stably recruit CBP/p300, leading to transcription as high as that seen with PDX-1/BETA2/E47 (13, 14). Transactivation domain deletion studies of SREBP-1c, BETA2, and E47 supported this (Figs. 6 and 7). Furthermore, this synergism could also involve DNA looping so that two sets of BETA2/E47 binding to E1- and E2-boxes could be bridged by SREBP-1c through protein-protein interaction without DNA binding. In many eukaryotic genes, transcription factors bind to promoters at sites distant from one another, yet act synergistically in DNA looping to activate transcription (47, 48). The length between two regions is also important for DNA looping, as evidenced in both PDX-1/BETA2/E47 and SREBP-1c/BETA2/E47 synergism (Fig. 4C).

Potential SREBP Contribution to Insulin Gene Regulation in β Cells—The current data suggested that SREBP-1c could potentially participate in insulin gene regulation. ChIP assays confirmed that endogenous SREBP-1c could directly interact with the insulin gene in β cells. In non-β cells, PDX-1/BETA2/E47 could increase insulin promoter activity to the level of what is seen in β cells (38). SREBP-1c/BETA2/E47 caused a similar effect, suggesting that SREBP-1c could functionally replace PDX-1. However, when both SREBP-1c and PDX-1 were present, they did not synergistically work (Figs. 2B and 8A). Consistent with complex situations, the effect of SREBP-1c expression on the insulin promoter in β cells was biphasic. The molecular mechanism for this apparent antagonism between SREBP-1c and PDX-1 is currently under investigation.

These data question the physiological relevance of SREBP-1c to the regulation of insulin genes under normal conditions. Supportively, as shown in Figs. 8C and 9C, SREBP-1c could contribute to insulin promoter activation and gene expression only when the PDX-1 level is low. Thus, SREBP-1c could have a chance to contribute to the insulin gene regulation in pathophysiological states where PDX-1 function is compromised, as observed in insulin resistance and diabetes. In insulin-resistant animals, SREBP-1c could be increased in the liver and potentially in β cells (21, 49). Hyperglycemia could also increase SREBP-1c expression in liver (50) and a β cell line (22). Potential involvement of

SREBP-1c in β cell lipotoxicity leading to impaired insulin secretion was implicated in the SREBP-1c-inducible INS-1 cell line (22), in adenovirus SREBP-1c-infected MIN6 cells (23) and rat pancreatic islets (24, 25), and β cell-specific SREBP-1c transgenic mice (26). In these SREBP-1c overexpression systems, insulin gene expression was not increased but rather suppressed. However, in our hands, under different conditions, including in β cell lines, β cell-specific SREBP transgenic mice islets, and wild islets incubated with fatty acids, changes in insulin gene expression were not consistent in contrast to decreased insulin secretion. We assumed that this discrepancy was because of differences in the expression levels of PDX-1- and/or β cell-specific factors. It could also be speculated that activation of the insulin promoter by SREBP-1c in our findings could be masked in normal conditions but become overt as a compensatory adaptation for impaired insulin action in insulin resistance or diabetes, especially when associated with decreased PDX-1 expression. Further studies are needed to clarify the physiological and pathophysiological function of SREBP-1 in insulin gene expression and secretion.

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