Cell-specific and Ubiquitous Factors Are Responsible for the Enhancer Activity of the Rat Insulin II Gene*

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Pancreatic β-cell-specific expression of the insulin gene is mediated, at least in part, by an enhancer element termed the rat insulin promoter element 3 (RIPE3) found within the rat insulin II gene between positions -126 and -86. Here we identify three distinct factors interacting with RIPE3, namely 3a1, 3a2, and 3b1, which bind to the sequences between -100 to -90, -108 to -99, and -115 to -107, respectively. Factors 3a1 and 3b1 are β-cell specific whereas 3a2 is ubiquitously distributed. The 3a1 site contains the consensw binding sequence (CANNTG) for a group of DNA-binding proteins called basic-helix-loop-helix proteins. We showed in this study that the 3a1 binding activity contains E12/E47, a member of the basic-helix-loop-helix protein family, or an E12/E47-like protein. Sequence comparison of the 3a2 and 3b1 binding sites suggests that they are unique and may bind to novel transcription factors. Mutation analysis of each individual binding site in transient expression experiments indicates that all of the three binding sites contribute to the enhancer activity of the RIPE3 in β-cells. Mutation in any one of the three binding sites not only disrupts binding of the corresponding factor but decreases RIPE3 enhancer activity by 4–7-fold. The results suggest that interactions among the 3a1, 3a2, and 3b1 factors are required for maximum enhancer activity of the RIPE3 in insulin-producing cells.

The insulin gene is expressed only in pancreatic β-cells. Its expression is not only tissue and cell type specific but is also developmentally regulated (Alpert et al.). Previous studies, including transient expression in cultured cells and gene transfer into mouse embryos, have demonstrated that β-cell-specific expression is regulated at the level of transcription and is directed by the 5′-flanking sequence of the insulin gene (Edlund et al., 1985; Hanahan, 1985; Walker et al., 1983). The mammalian insulin genes are highly homologous within the 5′-flanking region (for review, see Steiner et al., 1985). It is therefore likely that they are controlled by similar regulatory mechanisms. In fact, cis-acting elements, including cell-specific enhancers and promoters, have been defined, in most cases, within 300 base pairs upstream from the transcription initiation sites of several insulin genes (Boam et al., 1990; Crowe and Tsai, 1989; Edlund et al., 1985; Karlsson et al., 1987; Whelan et al., 1989). Accumulating evidence shows that by interacting with trans-acting factors, these cis-elements can confer either positive regulation in insulin-producing cells or negative regulation in nonproducing cells (Hwung et al., 1988; Karlsson et al., 1989; Ohlsson et al., 1988; Whelan et al., 1989, 1990).

There are two nonallelic insulin genes in rats: the rat insulin I gene and the rat insulin II gene. Both genes are regulated coordinately in vivo (Giddings and Carnaghetti, 1988). It has been proposed that the rat insulin I gene was derived from the insulin II gene by a retrotransposition event (Soares et al., 1985). To characterize the cis-acting elements important for β-cell-specific expression of the insulin II gene, deletion and linker-scanning (LS) mutation analysis of the 5′-flanking region were carried out in our laboratory (Crowe and Tsai, 1989). From these studies several important regions were defined (termed the rat insulin promoter elements (RIPEs)). Mutations in these regions resulted in decreased gene activity in insulin-producing cells. Two of the linker-scanning mutants, LS-102/91 and LS-129/110, had the most drastic effects. These two regions were combined and termed RIPE3 (-126 to -86). Further studies on the RIPE3 element demonstrated that RIPE3 alone can confer cell type-specific expression in either orientation in insulin-producing cells. However, two subregions of RIPE3, RIPE3a (-110 to -86) and RIPE3b (-126 to -101), displayed only marginal activities (Hwung et al., 1990). It is therefore likely that the RIPE3 element contains multiple protein binding domains that interact with multiple trans-acting factors and together contribute to the cell type-specific expression of the insulin gene.

In this study we have used nuclear extracts from insulin-producing and nonproducing cells to show that several factors interact with RIPE3. By gel mobility shift assays and methylation interference experiments we demonstrate that there are at least three protein binding sites on the RIPE3 element. Two of them bind factors that are uniquely present in β-cells. The other is recognized by a ubiquitous factor. By point mutation in each individual site we also show that each of them has positive regulatory function in β-cells. These results indicate the involvement of multiple factors, including β-cell-specific and ubiquitous, in regulating β-cell-specific expression of the insulin gene.

MATERIALS AND METHODS

Cell Culture and Preparation of Nuclear Extracts—Hamster insulinoma cells, HIT-T15 M2.2.2 (Santerre et al., 1981) and kidney fibroblast cells, BHK-21, were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units of penicillin, and 100 μg/ml streptomycin. The abbreviations used are: LS, linker scanning; RIPE, rat insulin promoter element; HEPES, 4-(2-hydroxyethyl)-1-piperazinediethanesulfonic acid; CAT, chloramphenicol acetyltransferase; HIT, hamster insulin tumor; BHK, baby hamster kidney; B-HLH, basic-helix-loop-helix; USF, upstream stimulatory factor.
penicillin/ml, and 100 μg of streptomycin/ml. Nuclear extracts were prepared essentially as described by Ohlsson and Edlund (1986) except that cells were grown in tissue culture flasks prior to the preparation of extracts. HeLa cells were grown in spinner cultures in S-MEM JOKLIK minimum essential medium (Irvine Scientific) supplemented with 5% horse serum and antibiotics as described above. HeLa nuclear extracts were prepared basically as described by Dignam et al. (1983).

**Gel Mobility Shift Assays**—The gel mobility shift assays were performed as described (Ohlsson et al., 1988) with a few modifications. Two double-stranded oligonucleotides, RIPE3 (−126 to −86) and RIPE3b (−126 to −101), were end labeled with α−32P−deoxyribonucleotide and Klenow enzyme (Promega) and then were used as probes in the assays. Binding reactions contained 25 mM HEPES (pH 7.8), 60 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 10,000 cpm of end-labeled, double-stranded oligonucleotide, 5 μg of nuclear extract, and either 1 μg (for the RIPE3b probe) or 2 μg (for the RIPE3 probe) of poly(dI-dC)−poly(dI-dC) (Pharmacia LKB Biotechnology Inc.). After binding, the samples were loaded onto a 5% nondenaturing polyacrylamide gel (acrylamide:bisacrylamide ratio, 19:1) and run in 0.5 X TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8.5). After electrophoresis, the gel was dried and subjected to autoradiography.

**Methylation Interference Analysis**—For these experiments, oligonucleotides were 5′ end labeled with [γ−32P]ATP and polynucleotide kinase (Promega) and then annealed with a 2-fold excess of the complementary oligonucleotide. The labeled double-stranded oligonucleotides were then purified from the gel, partially methylated with dimethyl sulfate as described (Boam and Docherty, 1988; Maxam and Gilbert, 1980), and used as probes in the binding reaction. The binding reaction was performed in the same way as described for gel mobility shift assays, except that it was scaled up 10-fold and 200,000 cpm of probe was used. The specific complexes were eluted from the gel in 0.3 M sodium acetate (pH 7.2) overnight. The bound oligonucleotides were purified by passing through the Elutip-D-column (Schleicher & Schuell), ethanol precipitated, and cleaved with 1 M piperidine at 90 °C for 90 min. Samples were freeze-dried and run on 15% polyacrylamide sequencing gels.

**Plasmid Constructions**—The vector PVOCAT-50 used in the transfection experiments contains the chicken ovalbumin gene minimal promoter (−50 to +40) fused to the reporter chloramphenicol acetyltransferase (CAT) gene and simian virus 40 (SV40) splice-polyadenylation sequences (Bradshaw et al., 1988). The oligonucleotides, RIPE3, RIPE3a, RIPE3b, and point mutants of RIPE3 were inserted in various copy numbers and orientations to the upstream of the ovalbumin minimal promoter as described by Hwung et al. (1990).

**Transfection and CAT Assays**—Transient transfection experiments were carried out by the calcium phosphate coprecipitation method as described (Edlund et al., 1985; Hwung et al., 1988). Cells were harvested 44–48 h post-transfection and assayed for CAT activity as described previously (Gorman et al., 1982).

**RESULTS**

**Both Cell-specific and Ubiquitous Factors Bind to the RIPE3 Enhancer**—It has been demonstrated that RIPE3 is important for the rat insulin II gene expression in HIT cells (Crowe et al., 1989) and that this element is sufficient to stimulate β-cell-specific expression from a heterologous minimal promoter (Hwung et al., 1990). To characterize the nuclear factors that bind to RIPE3 and are thus involved in directing β-cell-specific expression, nuclear extracts were prepared from insulin-producing HIT cells and analyzed by gel mobility shift assays. The binding reaction was conducted in the presence of an extract and a α−32P-labeled RIPE3 oligonucleotide corresponding to the rat insulin II promoter sequences from −126 to −86. Two RIPE3-specific retarded complexes, 3a1 and 3a2, were observed (Fig. 1A, lane 2). Their formation was abolished by the presence of excess unlabeled RIPE3 oligonucleotide but not by excess unrelated RIPE7 oligonucleotide (−305 to −281) (compare Fig. 1A, lanes 3 and 7). Both 3a1 and 3a2 complexes were reduced by competition with RIPE3a that contains only a part of RIPE3, namely sequences from −110 to −86 (lane 4). No competition was observed with a mutated RIPE3a oligonucleotide in which part of the sequence (from −99 to −92) was replaced by a SacI linker (lane 5). This suggests that the sequences from −110 to −86 contained the binding sites responsible for the formation of both complexes. However, excess RIPE3 oligonucleotide containing a 10-base pair insertion at the position −107 (mRIPE3(I)) inhibited the formation of complex 3a1 but not complex 3a2 (Fig. 1A, lane 6), suggesting that the proteins responsible for the 3a1 and 3a2 complexes are distinct factors. In addition to 3a1 and 3a2, there were also several other retarded complexes that were either nonreproducible or not competed by RIPE3 (i.e. nonspecific). The identities of those complexes therefore were not pursued any further.

To dissect more closely the exact protein contact sites in the 3a1 and 3a2 complexes, methylation interference experiments were carried out. Fig. 1B shows that G −93, G −100 on
the top strand and G –92, G –95 on the bottom strand are important for the binding to 3a1 protein (Fig 1B, lanes 2 and 4). Consistent with the results of the mobility shift assays, 3a2 binds to a different site on RIPE3, with strong interference at G –99 and weak interference at G –105, –106, and –107 on the bottom strand (Fig. 1B, lane 5). We could not find any G residue contact on the top strand for the 3a2 complex (data not shown).

To determine the tissue distribution of 3a1- and 3a2-binding proteins nuclear extracts were prepared from non-insulin-producing BHK and HeLa cells. Gel mobility shift assays clearly demonstrated that the 3a1 complex was formed with HIT cell nuclear extracts but not with HeLa or BHK extracts (Fig. 2). In contrast, 3a2-binding protein was present in HIT, BHK, and HeLa cells (Fig. 2, lanes 1, 5, and 9). The 3a2 complexes formed with BHK and HeLa extracts were specific because they can be competed by increasing amounts of unlabeled RIPE3 (Fig. 2, lanes 5–8 and 9–12). Methylation interference analysis of 3a2 complexes obtained with BHK and HeLa extracts showed that they bind exactly the same nucleotides as the 3a2 complex obtained with the HIT cell nuclear extract (Fig. 1B, lanes 5, 6, and 7). However, we did observe a slight difference in the mobility of 3a2 complexes from insulin-producing and nonproducing cells (compare Fig. 2, lanes 1, 5, and 9). The cause of such a difference remains unclear. From these results we conclude that at least two factors interact with RIPE3, one of which (3a1) is β-cell-specific, and the other of which (3a2) is ubiquitous and of unknown identity.

E12/E47 or an E12/E47-like Protein Is Involved in β-Cell-specific 3a1 Complex Formation—Since the β-cell-specific binding site 3a1 contains the core sequence CANNTG or “E box” that was originally identified in immunoglobulin gene enhancers (Ephrussi et al., 1985; Lenardo et al., 1987), we were interested in determining whether the 3a1-binding protein belongs to the E box-binding protein family. It has been shown by others that the E box is not only present in the insulin gene promoter but also in the immunoglobulin and muscle creatine kinase gene enhancer elements (Bushkin and Hanschka, 1988; Ephrussi et al., 1985). A common amino acid sequence motif is present in the proteins that bind to the E box sequence. This motif consists of a basic region followed by a helix-loop-helix domain (B-HLH) which is important for DNA binding and protein-protein dimerization (Murre et al., 1989a, 1989b). A typical member of this family is E12/E47, which binds to the immunoglobulin κE2 enhancer. It has been shown that E12/E47 is ubiquitous and can form heterodimers with other members of the B-HLH protein family (Murre et al., 1989b). To determine whether the pancreatic-specific complex 3a1 contains E12/E47, anti-E12/E47 antiserum was used in the gel mobility shift assay.

As shown in Fig. 3, the formation of a DNA complex by the cell-specific factor 3a1 can be inhibited by preincubation of the HIT cell nuclear extract with anti-E12/E47 antiserum; in contrast, the ubiquitous 3a2 complex was not affected. Antibodies against other members of the B-HLH family, human USF (adenovirus major late transcription factor) (Gregor et al., 1990) and sea urchin USF (suUSF), did not affect either 3a1 or 3a2 complex formation. We conclude that the 3a1 complex contains either E12/E47 or a protein that is antigenically related to E12/E47.

An Additional β-Cell-specific Factor Binds to RIPE3b—The results of previous linker-scanning mutagenesis on the 5′-flanking region of the rat insulin I (Karlsson et al., 1987) and the rat insulin II genes (Crowe and Tsai, 1989) suggest that multiple cis-elements are involved in determining the β-cell-specific expression of the insulin gene. Indeed, deletion of the sequences from –100 to –90 (corresponding to the 3a1 binding site) did not completely abolish β-cell-specific expression (Whelan et al., 1989). In addition, mutation in the region from –126 to –110 (RIPE3b) also reduced insulin gene expression. Furthermore, RIPE3b alone directed β-cell-specific expression although to a lesser degree than the intact RIPE3 (–126 to –86) (Hwang et al., 1990). It is possible that some cellular factor(s) interacts with RIPE3b and acts together with the RIPE3a-binding protein to establish the full cell-specific enhancer activity of RIPE3.

To characterize the factors that bind to RIPE3b, gel mobility shift assays were performed using the labeled RIPE3b oligonucleotide as probe. As shown in Fig. 4A, two specific retarded complexes, 3b1 and 3b2, were observed. Formation of the 3b1 complex can be inhibited by excess unlabeled RIPE3b oligonucleotide (Fig. 4A, lane 2) but only weakly competed by excess unrelated RIPE7 oligonucleotide (lane 3) or mutant RIPE3b in which RIPE3b sequences from –118 to –111 were replaced by a ScaI linker (lane 4). Since formation of the 3b2 complex is only slightly competed by excess RIPE3 (see Fig. 6C, lane 3) the identity of the 3b2 binding factor was not pursued further. Methylation interference analysis conducted on the RIPE3b1 complex revealed strong interference at G –107, –108, –111 and weak interference at G –114 on the bottom strand (Fig. 4B). No interference at glycine residues was detected on the top strand.

To determine the tissue distribution of the RIPE3b1 binding factor, nuclear extracts from BHK and HeLa cells were

**Fig. 2.** Tissue distribution of the factors that bind to the RIPE3. Nuclear extracts prepared from HIT cells (lanes 1–4), BHK cells (lanes 5–8), and HeLa cells (lanes 9–12) were used in gel mobility shift assays. Increasing amounts of unlabeled RIPE3 oligonucleotide (from 25-fold to 100-fold molar excess) were included in the binding reactions as competitor.

**Fig. 3.** Anti-E12/E47 antibody inhibits formation of the β-cell-specific 3a1 complex. Gel mobility shift assays were conducted with 32P-labeled RIPE3 oligonucleotide and HIT cell nuclear extracts. The anti-E12/E47 antiserum (Murre et al., 1989b) and anti-USF antiserum were preincubated with nuclear extracts for 2 min at room temperature before the binding reaction was initiated.
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FIG. 4. Analysis of protein binding to RIPE3b. A, gel mobility shift and competition analyses of complexes formed by factors in the HIT cell nuclear extract with the labeled RIPE3b oligonucleotide, 50-fold molar excesses of unlabeled oligonucleotides, as indicated on top of each lane, were used as competitors in the binding reactions. B, methylation interference analysis of the specific 3b1 complex formed by the factor in HIT cells. Residues that interfere with protein binding are indicated with closed circles; open circle denotes partial interference.

FIG. 5. Tissue distribution of the RIPE3b binding factors. Similar gel mobility shift experiments were conducted as described in the legend of Fig. 2 except that the labeled RIPE3b oligonucleotide was used as probe.

used in the mobility shift assay. The results clearly show that the RIPE3b1 binding factor is unique to insulin-producing HIT cells. It is not present in non-insulin-producing BHK or HeLa cell extracts (Fig. 5). In addition to 3b1 and 3b2, we also observed other retarded complexes that seemed to be competed well by excess RIPE3b (Figs. 4A and 5). Since those complexes were not very reproducible, they were not studied in any detail.

The Binding Sites on RIPE3 Have Positive Regulatory Function in Pancreatic β-Cells—Fig. 6A summarizes the three binding sites on RIPE3. The 3a2 site is bound by a ubiquitous factor, and it is flanked at both ends by cell-specific binding sites, 3a1 and 3b1. Also shown in the figure are the positions of the previously described linker-scanning and insertion mutants. These mutants substantially reduced the insulin promoter activity (Crowe and Tsai, 1989), suggesting that some, if not all, binding proteins identified so far are important for the RIPE3 enhancer activity. To examine further the functional significance of each binding site, three mutant oligo-

FIG. 6. A, summary of the protein binding sites on the RIPE3. Also shown in the figure are the position of the linker-scanning mutants LS-127/110, LS-100/91 (Crowe and Tsai, 1989), and the insertion mutant mRIPE3(1). The point mutations are depicted as well. B, gel mobility shift and competition analyses of the 3a1 and 3a2 complexes using wild-type or mutated RIPE3 oligonucleotides as competitors. C, gel mobility shift and competition analyses of the 3b1 and 3b2 complexes using wild-type or mutated RIPE3 oligonucleotides as competitors.
nucleotides based on the RIPE3 sequence were designed. The RIPE3a1m contains a point mutation in the 3a1 binding site, changing the G at position −93 to a T. The RIPE3a2m contains three nucleotide mutations with -102TGG-100 changed to GAT, which are located within the 3a2 site. Finally, the RIPE3b1m contains two nucleotide changes within the 3b1 binding site with -112TC-111 changed to CG.

To ensure that each mutant only affects the binding of the individual protein, the gel mobility shift assays were performed using either wild type or mutant oligonucleotides as competitors (Fig. 6, B and C). The results clearly demonstrated that excess RIPE3a1m competed with the binding of 3a2 and 3b1 but had no effect on the binding of 3a1 (Fig. 6B, lane 3; Fig. 6C, lane 4). The same was true for the other mutated oligonucleotides; that is, excess RIPE3a2m as competitor did not affect 3a2 binding, and excess RIPE3b1m did not effect 3b1 binding (Fig. 6, B and C). Thus these three RIPE3 mutants each specifically affects a single protein binding site.

The wild-type and mutated oligonucleotides were then inserted into the BglII site of the pOVCAT-50 vector in various copy numbers and orientations to drive the expression of CAT gene from an ovalbumin minimal promoter. The resulting plasmids were transfected into insulin-producing HIT cells or nonproducing BHK cells. The transient expression of the CAT gene was then measured. CAT enzyme activity from the test plasmid was normalized in both HIT and BHK cells to the activity obtained from a SV40 enhancer-CAT expression plasmid (pSV2CAT) (Gorman et al., 1982) transfected in parallel.

The results (Fig. 7 and Table I) indicated that all mutants show reduced activities in HIT cells. Three copies of the wild-type RIPE3 can restore over 75% of the activity of the intact promoter (INSCAT), which contains 448 base pairs of the rat insulin II gene 5′-flanking sequence. However, three copies of the mutant RIPE3 showed 4–7-fold decreased activity compared with that of the wild type. Moreover, the RIPE3a1m and the RIPE3b1m showed about the same activity as that of the RIPE3b and RIPE3a3, respectively, which contain only two of the three protein binding sites on RIPE3 (Fig. 7A, lane 11; Fig. 7C, lane 12). In BHK cells none of the RIPE3 mutants expressed significant amount of CAT activity over the vector pOVCAT-50 (data not shown). Neither did the wild-type RIPE3 drive any CAT activity in BHK cells (Hwang et al., 1990). Moreover, the mutants no longer functioned as well in the reverse orientation as they were in the native orientation, even in three copies (Fig. 7 and Table I). These results indicate that the three binding sites revealed in the previous binding assays all have positive regulatory function in HIT cells. The mutation that disrupted the binding of factors also affected the enhancer activity in transient expression experiments. Interestingly, with only one site mutated, the enhancer still maintained its cell-specific activity, suggesting that more than one binding site controls the cell-specific expression. It is very likely that the interactions between the factors that bind to 3a1, 3a2, and 3b1 give rise to the maximum enhancer activity of RIPE3 in insulin-producing cells.

**DISCUSSION**

The RIPE3 is an enhancer element important for β-cell-specific expression of the rat insulin II gene (Hwang et al., 1990). In this study we have shown that at least three factors, 3a1, 3a2, and 3b1, bind to the RIPE3; two of them are β-cell-specific (3a1 and 3b1) whereas the other one is generally expressed (3a2). Mutation in any of the binding sites not only affects the binding of the factor but also disrupts the activity of the element in β-cells. The discovery of more than one binding site in one regulatory element is not uncommon. The best known example is the SV40 enhancer, which is composed of multiple protein binding motifs (for a review, see Maniatis et al., 1987). By cooperating with each other, these motifs together give rise to the full enhancer activity (Fromental et al., 1988). Since disruption of any of the three binding sites affects the activity of expression in β-cells, we conclude that multiple protein-protein interactions are necessary for the full RIPE3 activity.

The location of 3a1 binding site is consistent with the result of previous linker-scanning mutagenesis which greatly reduced rat insulin II gene expression (Crowe and Tsai, 1989). It also overlapped with the insulin control element defined by Whelan et al. (1988, 1990) in the rat insulin II gene and with the “Nir box” in the rat insulin I gene (Moss et al., 1988). All these elements contain the consensus sequence CANNTG, or E box, which was originally identified in immunoglobulin gene enhancers and shown to be important for enhancer activity (Ephrussi et al., 1985; Lenardo et al., 1987). Much effort has been concentrated on the identification of the factor that binds to the insulin E box CANNTG (sequences from −109 to −104 in the rat insulin I gene and −108 to −103 in the rat insulin II gene), or the 3a1 binding site characterized in this report. In fact, three laboratories including ours have
cloned cDNAs from mouse (Walker et al., 1990), rat (Shibasaki et al., 1990), and hamster† which encode the proteins that bind to this conserved sequence. All of these proteins turn out to be members of the B-HLH protein family. Other members of this family include factors important in muscle differentiation (MyoD, myogenin, Myf-5) and proteins of Drosophila which regulate the development of the peripheral nervous system (achaete-scute complex, daughterless, hairy, and extradentatae) (for recent reviews, see Jones, 1990; Olson, 1990). Moreover, all three proteins were shown to be ubiquitous. It is intriguing to explain how a ubiquitous factor can bind to a supposedly cell-specific binding site and direct the tissue-specific expression. It has been suggested that the B-HLH proteins be divided into three classes: class A (E12/E47, daughterless), class B (MyoD, achaete-scute complex), and class C (myc) (Murre et al., 1989a). Class A proteins are ubiquitous; they can form homodimers or heterodimers with members of the tissue-specific class B proteins and bind to tissue-specific enhancers (Murre et al., 1989a). Evidence presented in this report shows that the β-cell-specific complex 3α1 contains E12/E47 or an E12/E47-like protein (Fig. 3). Our results are consistent with a model in which a β-cell-specific factor forms a heterodimer with E12/E47 or an E12/E47-like protein and then binds to the RIPE3α1 site. Similar results described by Cordle et al. (1991) also support this model.

In addition to the 3α1 complex, we also found another β-cell-specific complex, which we call 3b1. The binding sequence for 3b1, GCTTCAGCCC, is distinct from that for 3α1. Since the oligonucleotide carrying the 3α1 site cannot compete for binding of 3b1 in the mobility shift assay (data not shown), the factor that binds to the 3b1 site must be different from the one that binds to the 3α1 site. Furthermore, mutation in the RIPE3b1 site decreased the RIPE3 enhancer activity by 5–7-fold (Fig. 7C and Table I) in HIT cells. These results suggest that the RIPE3b1 binding protein is a cell-specific, positive regulator for rat insulin II gene expression. The reason we did not observe the 3b1 complex when the full-length RIPE3 was used as probe is still not clear. However, during the process of investigation we repeatedly found that the 3b1 complex signal was weaker than the 3α1 complex in the mobility shift assays. It is possible either that the binding affinity of the 3b1 complex is weak or that its concentration is low in the nuclear extract so that its shifted band is obscured by some other band. In any event, the results of competition (shown in Fig. 6C, lane 3) clearly demonstrate that RIPE3 can compete with RIPE3b for the 3b1 binding. Since an intensive GenBank search for the 3b1 binding sequence did not show any consensus match, we feel that the 3b1 binding factor is a novel DNA-binding protein. Further characterization of the 3b1-binding protein requires cloning of the factor, which is now under way in our laboratory.

The role of the ubiquitous binding site 3α2 in a tissue-specific regulatory element is intriguing. Two hypotheses can be proposed. One is that the presence of the 3α2 functions as a bridge between the two cell-specific factors 3α1 and 3b1, thus stabilizing the whole binding complex and increasing the gene expression. Alternatively, 3α2 might function as a negative regulator in non-insulin-producing cells and further tighten the control of tissue-specific expression of the insulin gene. The results of our studies support the first hypothesis. However, we cannot exclude the possibility that since the basal level expression of the test plasmids in the BHK cell was so low, we might have overlooked any minor increase of expression when the mutant constructs were applied.

We conclude that three protein binding sites, 3α1, 3α2, and 3b1, are present on the RIPE3. The 3α1- and 3b1-binding proteins are β-cell specific whereas the 3α2-binding protein is ubiquitous. All three proteins are important, positive regulators of RIPE3 enhancer activity, which act cooperatively to confer maximum tissue-specific expression.

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REFERENCES


Table I

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*All the oligonucleotides were inserted into the vector pOVCAT-50 in various copy numbers and + or – orientation. The resulting plasmids were transfected transiently into HIT cells, and CAT activity was measured.

†Values depicting CAT activity were obtained by quantitating the percent conversion of [14C]chloramphenicol to its acetylated derivatives from data presented in Fig. 7. The activities expressed are relative to that of the vector pOVCAT-50. Values are averages from two or more experiments.

**M. Peyton and M.-J. Tsai, unpublished result.**
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