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The Insulin and Islet Amyloid Polypeptide Genes Contain Similar Cell-Specific Promoter Elements That Bind Identical β-Cell Nuclear Complexes

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The pancreatic β cell makes several unique gene products, including insulin, islet amyloid polypeptide (IAPP), and β-cell-specific glucokinase (βGK). The functions of isolated portions of the insulin, IAPP, and βGK promoters were studied by using transient expression and DNA binding assays. A short portion (−247 to −197 bp) of the rat insulin I gene, the FF minienhancer, contains three interacting transcriptional regulatory elements. The FF minienhancer binds at least two nuclear complexes with limited tissue distribution. Sequences similar to that of the FF minienhancer are present in the 5′ flanking DNA of the human IAPP and rat βGK genes and also the rat insulin II and mouse insulin I and II genes. Similar minienhancer constructs from the insulin and IAPP genes function as cell-specific transcriptional regulatory elements and compete for binding of the same nuclear factors, while the βGK construct competes for protein binding but functions poorly as a minienhancer. These observations suggest that the patterns of expression of the β-cell-specific genes result in part from sharing the same transcriptional regulators.

In its specialized role of metabolic sensor and regulator, the β cell of the pancreatic islets of Langerhans produces a number of unique gene products, including among others insulin, islet amyloid polypeptide (IAPP), and β-cell-specific glucokinase (βGK). In adult mammals, the insulin gene is transcribed only in β cells (5, 15), while IAPP and βGK are expressed in one or more additional, developmentally related cell types (11, 17, 27). Previous studies of the insulin gene have demonstrated that its unique expression in β cells results in part from the cell-specific activity of its 5′ flanking DNA or promoter, which limits the expression of a linked gene to the β cell (16, 36). Deletion and mutation analyses of the insulin promoter have revealed the presence of multiple *cis*-acting sequence elements (2, 7, 9, 19, 41). These *cis*-acting elements apparently regulate transcription by binding a set of positive and/or negative *trans*-acting protein factors found in the nuclei of insulin-producing and non-insulin-producing cells (2, 24, 29, 40). The unique repertoire of *trans*-acting proteins, therefore, may determine the activity of the insulin promoter in a particular cell type. Because of similarities in expression of the insulin, IAPP, and βGK genes, it seems possible that their respective promoters may be regulated by some of the same protein factors.

The presence of multiple mutationally sensitive *cis*-acting elements, multiple nuclear binding factors, and frequently repeated sequence motifs complicates the study of the intact insulin promoter. We elected therefore to study in detail a portion of the rat insulin (rIns) I promoter between −247 and −198 bp upstream from the transcription start site, the FF minienhancer. Identified functionally in previous work, the FF minienhancer can act as a transcriptional activator when linked to a heterologous promoter (21). The FF minienhancer contains at least two mutationally sensitive elements: the Far element (−241 to −232 bp) and the Far-linked A+T-rich (FLAT) element (−222 to −208) (19). The human IAPP (hIAPP) and rat βGK (rβGK) promoters also contain linked sequence elements similar to the Far and FLAT elements; this allowed us to construct and study human hIAPP and rβGK minienhancers similar to the FF minienhancer.

MATERIALS AND METHODS

Cell lines and transfections. The Syrian hamster simian virus 40-transformed insulinoma line HIT T-15 M.2.2.2 and the Syrian hamster kidney fibroblast line BHK-21 have been previously described (19). Both cell lines were grown in DMEH16 medium with 3 g of glucose per liter and 10% fetal calf serum to a density of 3×10^6 HIT cells or 1×10^5 BHK cells per 100-mm plate at the time of transfection. Transfections were performed by the calcium phosphate technique (19), using 2 μg of double cesium chloride-purified test plasmid and 8 μg of double cesium chloride-purified carrier plasmid DNA (pUC18) for HIT cell transfections and 10 μg of double cesium chloride-purified test plasmid for BHK transfections. Cells were harvested, and protein extracts were prepared 48 h after transfection. Two micrograms of HIT extract or 100 μg of BHK extract was used to test chloramphenicol acetyltransferase (CAT) enzyme activity as previously described (19).

Plasmid construction. The construction of tkCAT (pTE2ΔS/N) and +597tkCAT (pTE2) has been described previously (9). The Ins.ripCAT plasmid contains the rIns I 5′ flanking DNA from −345 to +1 bp linked to the CAT coding sequence; there are engineered *Bam*HI sites at −345 and −85 bp (19). The ripCAT plasmid was constructed from Ins.ripCAT by removing the −345 to −85 *Bam*HI fragment, leaving an intact *Bam*HI site into which the multimerized minienhancers were inserted. This same −345 to −85 *Bam*HI fragment was inserted into the *Bgl*II site in tkCAT to produce Ins-TKCAT.

Minienhancer multimers were constructed from the appropriate oligonucleotides (Table 1) synthesized with restriction

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TABLE 1. Minienhancer sequences[a]

5′ end	Sequence	Name	Gene (reference)
−247	CTTCATCAGGCCATGTGG..CCCCTTGTTAATTAATCTTAATTACCCTAGGTCTA	FF	rIns I (36)
	CTTCATCAGGACCTAGTG..CCCCTTGTTAATTAATCTAATTACCCTAGGTCTA	C	
	CTTCATCAGGCCATCTGG..CCCCTTGTTAATTAATCTAATTACCTGCTTGAGC	D	
	CTTCATCAGGCCATCTGG..CCCCTTGTTAATTAATCGACTGACCCTAGGTCTA	E	
	CTTCATCAGGCCATCTGG..CCCCTTGGTCCGAATCTAATTACCCTAGGTCTA	F	
	CTTCATCAGGCCATCTGG..CAACGTGTTAATTAATCTAATTACCCTAGGTCTA	G	
	CTTCATCAGGCCATCTGG..CCCCTTGTTAATTCCGATAATTACCCTAGGTCTA	H	
	CTTCATCAGGCCATCTGG..CCCCTTGTTAATTAAGAGCATTACCCTAGGTCTA	I	
	CTTCATCAGGCCATCTGG..CCCCTTGTTAATTAATCTACGGCCCCTAGGTCTA	J	
	CTTCATCAGGCCATCTGG..CCCCTTGTTAATTAATCTCAGTACCCTAGGTCTA	K	
	AGGACTCAGGCCATCTGG..CCCCTTGTTAATTAATCTAATTACCCTAGGTCTA	L	
	CTTCATCAGGCCATCTGG..CCCCTTGTTAATTAATCTTAATACCCTAGGTCTA	P	
	TTCATCAGGCCATCTGG..CCCCT	FA	
	TGG.. CCCCTTGTTAATTAATCTAATTACCCTAGGTCTAAGT	FL	
−237	GTTCATCAGGCCACCCAGGAGCCCCTCTTAAGACTCTTAATTACCCTAAGGCTA	R2	rIns II (7)
	GGTCATCAGGCCACCCAGGAGCCCCTCTTAAGACTCGACTGACCCTAAGGCTA	R2.E	
−247	GTTCATCAGGCCATCTGG..TCCCTTATTAAGACTATTAATAACCCTAAGACTA	M1	mIns I (39)
	GTTCATCAGGCCATCTGG..TCCCTTATTAAGACTAGACTGACCCTAAGACTA	M1.E	
−247	GTTCATCAGGCCATCAGGG.CCCCTTGTTAAGACTCTAATTACCCTAGGACTA	M2	mIns II (39)
	GTTCATCAGGCCATCTGGCAGCCCCTCTTAAGACTCTAATTACCCTAAGGCTA	R1R2	
	CTTCATCAGGCCACCCAG..CCCCTTGTTAATTAATCTAATTACCCTAGGTCTA	R2R1	
−176	ACGTTAATATTTACTGATGAGTTAATGTAATAATGACCCATCCGCTTCTGCTGCCTGTGA	AX	hIAPP (28)
	ACGTTAATATTTACTGATGAGTTAATGTAATAATGACCTCGTCGCTTCTGCTGCCTGTGA	AX.N1	
	ACGTTAATATTTACTGATGAGTTAATGTAATAATGACCCATCCGCTGAGTCTGCCTGTGA	AX.N2	
	ACGTTAATATTTACTGATGAGTTAATGTAATAATGACCTCGTCGCTGAGTCTGCCTGTGA	AX.N1N2	
−451	CTTTTTATACACCTTTCCCTTATATCTCCATTTATTCCTGAAGCTTCATGGGATTCAGCC	AD	hIAPP (28)
−147	AGAGAGGCCTTTGGCCATCAGTCCCAGTTTTCTGCATGGTGGCTCTAATGATAGAATGTG	BGK	rβGK (23)

[a] The same oligonucleotides were used in constructs in Fig. 2, 3, and 5 and as probes and competitors in Fig. 4, 6, and 7. All oligonucleotides were designed with restriction endonuclease *Bam*HI and *Bgl*II recognition sites on opposite ends. The gaps in some of the sequences were inserted to allow visual alignment of the sequences. TAAT sequence repeats are boxed, and brackets overlie Nir/Far-related sequences.

enzyme *Bam*HI and *Bgl*II recognition sites on opposite ends. Oligonucleotides were annealed and ligated in the presence of restriction enzymes *Bam*HI and *Bgl*II. Since *Bam*HI and *Bgl*II produce compatible DNA ends, this ligation reaction resulted in exclusively head-to-tail ligation. Multimers were separated by size and inserted as shown in Fig. 1. The first number in each wild-type minienhancer plasmid name in Fig. 2, 3, and 5 represents the number of copies of the minienhancer. The last number represents the orientation of the minienhancer multimer: 1 indicates forward, coding orientation and 2 indicates reverse, noncoding orientation. All mutant constructs contain five tandem copies of the mutant minienhancer in the *Bgl*II site of tkCAT, and therefore these plasmids were simply named for the mutant minienhancer used. Constructs with multiple mutations (such as EF and AX.N1.N2) were made with five tandem minienhancers inserted into the *Bgl*II site in tkCAT; each minienhancer contained all of the mutations shown. InsE.ripCAT was derived from Ins.ripCAT by Kunkel mutagenesis (22).

Electrophoretic mobility shift assays (EMSA). Protein extracts were prepared from HIT T-15 M.2.2.2, BHK-21, and rat islet nuclei by the technique of Dignam et al. (8). Rat islet nuclei were provided by Henk-Jan Aanstoot, University of California at San Francisco; Daudi nuclear extract was provided by Michael Blanar, University of California at San Francisco; canine liver nuclear extract was provided by Scott Weinrich, University of California at San Francisco; and HeLa nuclear extract was provided by Christian Nelson, University of California at Riverside. Five micrograms of each nuclear extract except the islet extract was used per binding assay; 1 μg of islet nuclear extract was used per assay.

The R1Far and AN1 probes (Table 2) were subcloned into pUC19, excised with restriction endonuclease *Bam*HI, labeled with Klenow DNA polymerase and ^{32}P-labeled nucleotides, and purified on a nondenaturing polyacrylamide gel. The FF, AX, and mutant minienhancer probes were end labeled with T4 polynucleotide kinase and [^{32}P]γATP. Unlabeled competitor oligonucleotides were annealed and end filled with Klenow enzyme. The labeled probe and unlabeled oligonucleotide competitors were mixed, prior to addition of a premixture resulting in a final volume of 10 μl, with 100 pg of labeled probe (~10,000 cpm), 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.8), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 3% Ficoll, 100 ng of poly(dI-dC) · poly(dI-dC) per μl, and 1 mg of bovine serum albumin per ml. After incubation at room temperature for 30 min, the mixtures were electrophoresed on 5% nondenaturing polyacrylamide gels (acrylamide/bisacrylamide, 30:1) in 45 mM Tris base–45 mM boric acid–1 mM EDTA (0.5× TBE) (12, 13).

RESULTS

The FF minienhancer functions as a cell-specific enhancer. The oligonucleotides representing the rIns I Far-FLAT region (FF minienhancer; Table 1) were inserted immediately upstream from the non-cell-specific herpesvirus thymidine kinase (TK) promoter linked to the coding sequence for CAT. This allows the indirect measurement of transcriptional activity in transfected cells by measuring CAT enzyme activity. Previous experiments have demonstrated that CAT enzyme activity reflects the rate of transcription of the CAT gene in HIT and BHK cells (9, 19, 24). One, two, or five

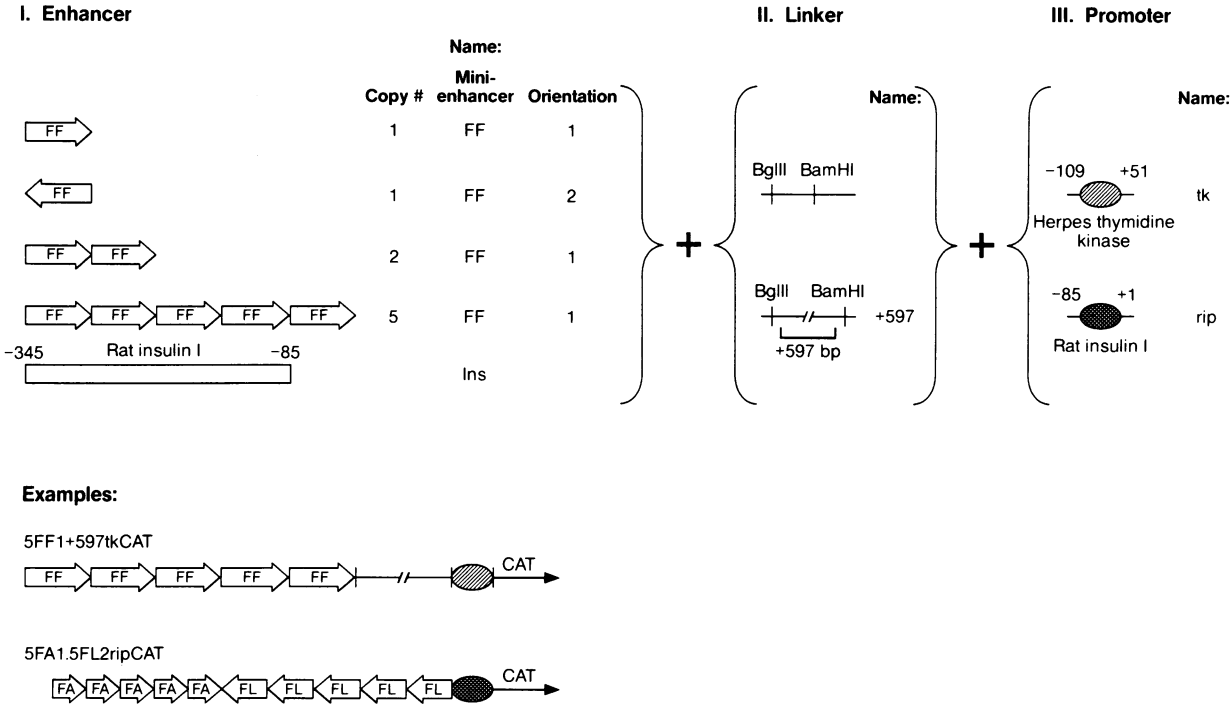

FIG. 1. Design of minienhancer-CAT constructs. As shown, each plasmid was constructed from three elements linked to CAT: an enhancer made from some combination of minienhancers, a linker, and a promoter. For TK promoter (tk) constructs, the enhancers were inserted into the *Bgl*II site; for the rip constructs, the enhancers were inserted into the *Bam*HI site. For plasmids constructed with wild-type minienhancers, the first number in the name represents the number of minienhancer copies; the second number represents the orientation. All plasmids constructed with mutant minienhancers contain five tandem copies of the mutant minienhancer inserted into the *Bgl*II site in tkCAT; therefore, these plasmids are simply named for the mutant minienhancer.

copies of the FF minienhancer were inserted in either the wild-type, coding orientation or reverse, noncoding orientation (Fig. 1). These plasmid constructs were then transfected by the $CaPO_4$ precipitation technique into the Syrian hamster insulinoma cell line HIT T-15 M2.2.2 or the Syrian hamster fibroblast line BHK-21 (Fig. 2A). Constructs with multimers of the FF minienhancer led to increased CAT activity in HIT cells, independent of the minienhancer's orientation. When the FF minienhancer multimer was moved further upstream from the TK promoter by inserting

TABLE 2. Comparison of the Far element and related elements[a]

5′ end	Sequence	Name	Gene (reference)
−241	CAGGCCATCTGGCCC	R1Far	rIns I (19)
	CAGGACCTAGTGCCC	Far-C	
−115	CTCGCCATCTGCCTA	R1Nir	rIns I
−231	CAGGCCACCCAGGAG	R2Far	rIns II (7)
−103	CTGGCCATCTGCTGA	R2Nir	rIns II
−249	TGCTTCATCAGGCCA	CATCAG	rIns I
−151	TGACCCATCCGCTTC	AN1	hIAPP (28)
	TGACCTCGTCGCTTC	AN1.N1	
−145	ATCCGCTTCTGCTGC	AN2[b]	hIAPP
−106	TTGGCCATCAGTCCC	BGK-N	rβGK (23)
−87	TTTCTCATCTGTAAA	Glu-N	Rat glucagon (32)
−87	GGACTCATATGGCAG	Gas-N	Human gastrin (38)
360	TCGGCCATCTTGACT	μE1	Mouse immunogobulin μ heavy chain (10)
392	CCTGCCAGCTGCTGC	μE2	Mouse immunoglobulin μ heavy chain
411	CTTGCCACATGACCT	μE3	Mouse immunogobulin μ heavy chain
540	AACACCACCTGGGTA	μE4	Mouse immunogobulin μ heavy chain
129	TGGGCCACCTGCCTG	κE2	Mouse immunogobulin κ light chain (4)
−65	TAGGCCACGTGACCG	AML-USE[c]	Adenovirus major late promoter (33)

[a] Sequences of the DNA-binding elements used to compete for binding to the Far and AN1 elements are shown. Oligonucleotide competitors were designed with restriction endonuclease *Bam*HI recognition sequences on both ends, resulting in double-stranded DNA fragments 27 bp in length after end filling with Klenow DNA polymerase.

[b] With the *Bam*HI recognition sequence, an AN1 site is recreated on the 5′ end of AN2.

[c] Adenovirus type 2 major late promoter upstream element.

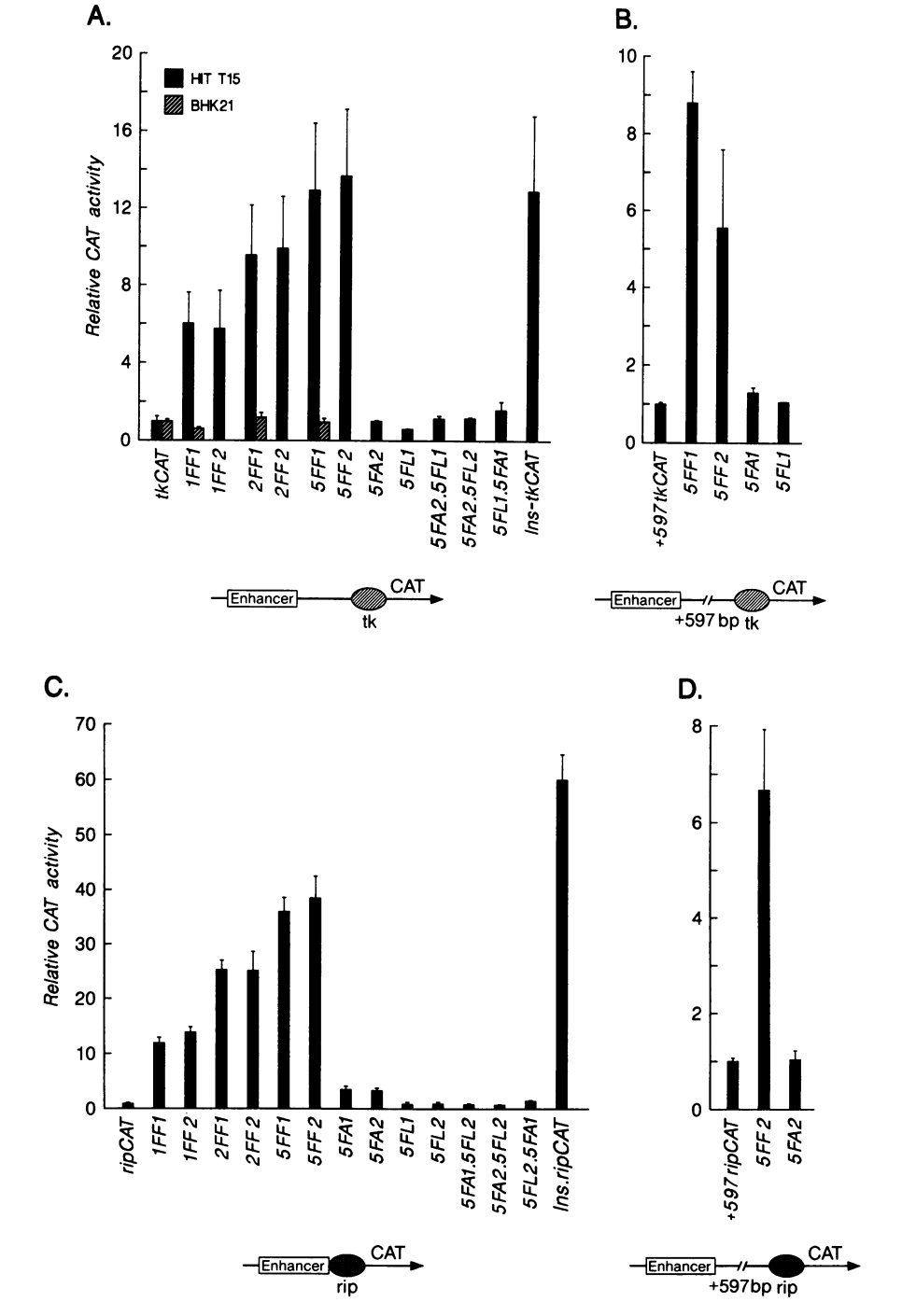
A.
HIT T15
BHK21
Relative CAT activity
tkCAT
1FF1
1FF2
2FF1
2FF2
5FF1
5FF2
5FA2
5FL1
5FA2.5FL1
5FA2.5FL2
5FL1.5FA1
Ins-tkCAT
Enhancer
CAT
tk
B.
+597tkCAT
5FF1
5FF2
5FA1
5FL1
+597 bp tk
C.
Relative CAT activity
ripCAT
1FF1
1FF2
2FF1
2FF2
5FF1
5FF2
5FA1
5FA2
5FL1
5FL2
5FA1.5FL2
5FA2.5FL2
5FL2.5FA1
Ins.ripCAT
rip
D.
+597ripCAT
5FF2
5FA2
+597bp rip
E.
Rat insulin I gene
-247
-196
5' flanking DNA
+1
Preproinsulin coding sequence
FF minienhancer
BamHI
Far element
FLAT element
BglII
GGATCCCTTCATCAGGCCATCTGGCCCCTTGTTAATAATCTAATTACCCTAGGTCTAAGATCT
FA
FLAT-F
FLAT-E
FL

FIG. 2. Activities of wild-type rIns I minienhancers. HIT T15 M.2.2.2 cells and BHK-21 cells were transfected by the calcium phosphate technique with the plasmids shown. Cell extracts were assayed for CAT enzyme activity 48 h after transfection. Each data point represents the average CAT activity of at least three independent transfections ± standard error. Plasmids used in panel A were made by inserting the minienhancer multimers into the *Bgl*II site in tkCAT. Plasmids used in panel B were made by inserting the minienhancer multimers into the *Bgl*II site in +597tkCAT. Plasmids used in panel C were made by inserting the minienhancer multimers into the *Bam*HI site in ripCAT. Plasmids used in panel D were made by inserting the minienhancer multimers into the *Bam* HI site in +597ripCAT. In panel E, the sequences of the FF, FA, and FL minienhancers are shown.

an unrelated 597-bp DNA fragment between the minienhancer multimer and the TK promoter (Fig. 1), the minienhancer could still activate transcription in HIT cells (Fig. 2B). In the non-insulin-producing BHK cells, however, the minienhancer or multimers did not increase CAT activity. Thus, the minienhancer apparently functions in a cell-specific manner.

A parallel set of plasmids was constructed with the rIns I promoter (rip, −85 to +1 bp) in place of the TK promoter. A similar pattern of transcriptional activation by the FF minienhancer independent of position or orientation (Fig. 2C and D) was observed with these plasmids.

The Far and FLAT elements interact synergistically to give full minienhancer activity. To test whether the two known mutationally sensitive regions of the FF minienhancer could function independently as transcriptional enhancers, we designed oligonucleotides containing either the Far or FLAT element (named FA and FL, respectively; Table 1). These were multimerized and inserted into the same CAT plasmid constructs used to test the full FF minienhancer as described above. The FLAT element by itself could not enhance transcription in any of the constructs tested (Fig. 2). Five tandem copies of the isolated Far element did activate weakly when closely linked to the −85 insulin promoter (Fig. 2C), but this activity was lost when the multimerized FA sequence was moved further away (Fig. 2D) or linked to the TK promoter (Fig. 2A and B). We tested shorter oligonucleotides containing the Far element as well as oligonucleotides containing the closely related rIns I Nir element; none of these sequences activated transcription when linked to the TK promoter.

We attempted to reconstitute full minienhancer activity by combining the multimerized FA and FL sequences in various orientations (Fig. 1); none of these combinations resulted in a significant improvement in activity (Fig. 2A and C). These data demonstrate that neither the Far nor the FLAT element has significant enhancer activity alone, but the two function as a unit when properly juxtaposed in the FF minienhancer.

Mutational analysis of the FF minienhancer. To fine map the *cis*-acting elements in the FF minienhancer, we synthesized a series of mutant minienhancers (Table 1). As with the wild-type FF minienhancer, five tandem copies of each mutant minienhancer were inserted immediately upstream of the TK promoter driving the CAT gene. The activities of these constructs were tested in HIT and BHK cells (Fig. 3A). Minienhancers with mutations in the region of the Far element all produced less transcriptional activation than did the wild-type FF minienhancer. The C mutation, which mutates the Far element, resulted in a total loss of minienhancer activity. Mutation of sequences on either side of the Far element (L and G) resulted in only modest decreases in activity.

Mutation of the FLAT region resulted in an interesting set of phenotypes. The E mutation, which mutates the 3′ end of the FLAT element (−209 to −213 bp), produced a marked increase in minienhancer activity. The overlapping J mutation (−208 to −211 bp) had a similar effect. The positive effect of the E mutation was dependent on an intact Far element, as demonstrated by the fact that a minienhancer containing both the C and E mutations (construct CE) resulted in no more activity than did the essentially inactive C mutant minienhancer. The E mutation phenotype was not an artifact of the multimerized minienhancer constructs. This fact was demonstrated by engineering the E mutation in the full rIns I promoter, +1 to −345, driving the CAT gene. The E mutation promoter (InsE.rip) had threefold greater activity than did the wild-type promoter (Ins.rip; Fig. 3B).

The F mutation at the 5′ end of the FLAT element (−218 to −222 bp) also caused increases in minienhancer activity, although this increase was less dramatic than the increase caused by the E mutation. Mutations H and I, which lie between mutations E and F, and mutation K, which overlaps mutation E but involves different bases, caused no significant change in minienhancer activity.

Like the wild-type FF minienhancer, the mutant minienhancers had no activity in the non-insulin-producing BHK-21 cells.

The FLAT element is composed of two negatively interacting elements. Although both the E and F mutations cause increases in minienhancer activity, a minienhancer combining both the E and F mutations (mutant EF) was essentially inactive. These data support the conclusion drawn from the previous set of experiments that the Far and FLAT elements interact to give full minienhancer activity and suggest that the FLAT element is composed of two negatively interacting elements that we have named FLAT-E (−208 to −213 bp) and FLAT-F (−215 to −222 bp). FLAT-E and FLAT-F have marked sequence similarity. Each element contains two copies of the sequence TAAT: FLAT-E contains two overlapping copies on opposite strands of DNA, while FLAT-F contains two copies on the same strand. The E mutation affects bases that are identical between the FLAT-E and FLAT-F elements; the K mutation affects the two bases that are not identical. The P mutation, which changes the FLAT-E element into an identical copy of the FLAT-F element, results in an essentially inactive minienhancer.

Like the combined EF mutant minienhancer, minienhancers containing combined mutations DEFG and CDEFG had no activity in HIT cells.

Protein binding characteristics of the FF minienhancer. Proteins in HIT cell nuclear extracts were tested for the ability to bind to the FF minienhancer by EMSA. HIT cell nuclear extract was mixed with the end-labeled, double-stranded FF DNA fragment, and the resulting protein-DNA complexes were separated on a polyacrylamide gel (Fig. 4; see Materials and Methods for details). This resulted in at least six prominent bands (Fig. 4A, lane 1) representing protein-DNA complexes. The ability of the mutant minienhancers to bind HIT nuclear proteins was tested in a similar fashion. The C probe (Fig. 4A, lane 2) did not produce the third-highest band (band C) seen with the FF probe. Band C

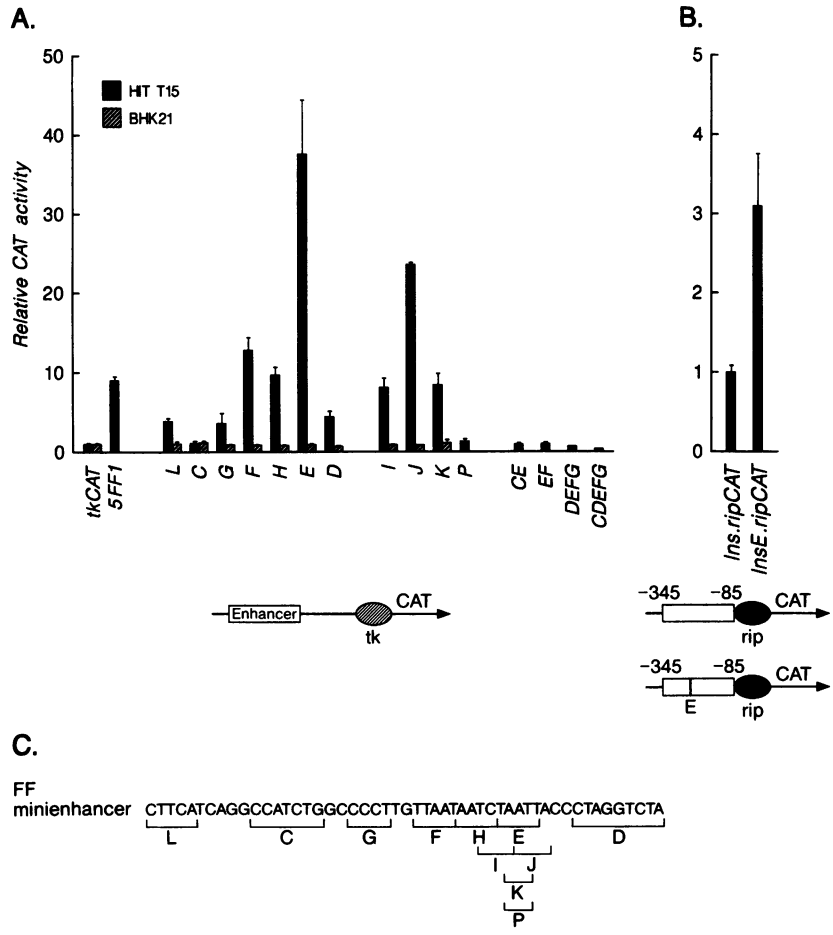

FIG. 3. Activities of mutant rIns I minienhancers. HIT T15 M.2.2.2 cells and BHK-21 cells were transfected by the calcium phosphate technique with the plasmids shown. Cell extracts were assayed for CAT enzyme activity 48 h after transfection. Each data point represents the average CAT activity of at least three independent transfections ± standard error. Plasmids used in panel A were made by inserting five tandem copies of the mutant minienhancer into the *BglII* site in tkCAT. Plasmid Ins.ripCAT in panel B contains the rIns I sequence −345 to +1 linked to CAT; plasmid InsE.ripCAT is the same construct with the E mutation. In panel C, the positions of the FF mutants are shown. For sequences of the mutants, see Table 1.

is actually a doublet resulting from two complexes with very similar mobilities.

The three lowest bands seen with the FF probe (labeled E1, E2, and E3) were markedly decreased or absent when the E probe was used (Fig. 4A, lane 3), while the second-highest band (F2) was more prominent. The two highest bands (F1 and F2) were essentially absent when the F probe was used (Fig. 4A, lane 4), while band E2 increased in intensity. Of the original bands seen with the FF probe, only the C band was present when the EF double-mutation probe was used. These data suggest that the C doublet bands result from protein complexes binding the Far element, while the remainder of the bands result from complexes binding to the FLAT region, with complexes F1 and F2 preferentially binding to the FLAT-F element and with complexes E1, E2, and E3 preferentially binding to the E element. The E1, E2, and E3 bands, however, also appear to bind to the FLAT-F site with lower affinity.

To ensure that the complexes binding to the mutant probe were the same as those which bind the FF probe, we used unlabeled mutant oligonucleotides to compete for binding to the FF minienhancer (Fig. 4B and C). These competition experiments demonstrate that the mutant enhancers do compete for the same complexes with the exceptions noted above. The E mutant oligonucleotide has a markedly higher affinity for the F2 complex than does the FF probe (Fig. 4C and D), and the F mutant oligonucleotide has a slightly higher affinity for the E2 complex than does the FF probe. These increased affinities may explain the increased activation seen when these mutant oligonucleotides are used as minienhancers.

Nuclear extracts from several different cell types were tested by EMSA for binding to the FF minienhancer (Fig. 5E). Only HIT cells produced the F1 complex, and only HIT and rat islet nuclear extracts produced the E3 complex. The F2 complex is detected only in HIT cells, although in the Daudi B-lymphocyte line it may be difficult to detect because of a large band centered immediately below the expected position of the F2 band.

The rIns II minienhancer. The region of the rIns II gene 5′ flanking DNA which corresponds to the Far-FLAT region in the rIns I gene was tested as a minienhancer (R2 minienhancer; Table 1). The FLAT-E element is perfectly conserved in the rIns II gene, but the Far and FLAT-F elements are not conserved. Previous mutational analysis of the rIns II promoter demonstrated that this region is not mutationally

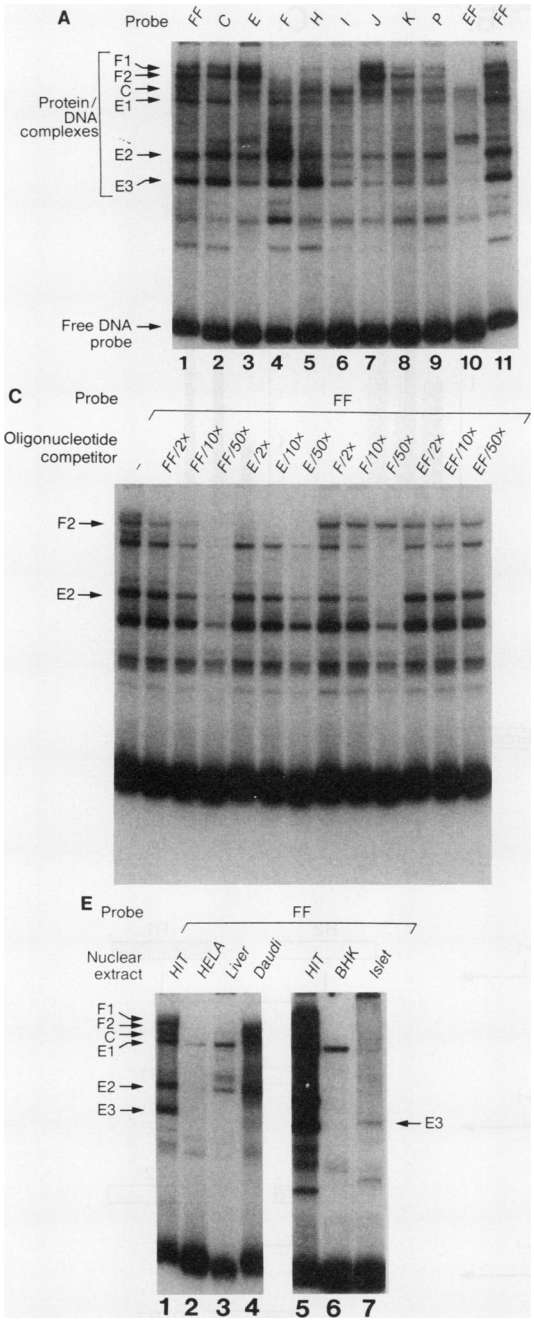

FIG. 4. Binding of nuclear extract to the FF minienhancer. The binding specificities of nuclear proteins were tested by EMSA. See Table 1 for the sequences of probes and competitors. (A) HIT nuclear extract was added to the ^{32}P-labeled probes shown, and the resulting DNA-protein complexes were separated by electrophoresis on a 5% nondenaturing polyacrylamide gel. (B) The binding specificities of the HIT nuclear FF-binding complexes were tested by adding a 20-fold excess of the unlabeled oligonucleotide competitors shown. (C) The relative binding affinities for the E and F sites were tested by adding increasing amounts of the competitor oligonucleotides shown. (D) The binding specificities of the HIT nuclear E probe-binding complexes were tested by adding a 20-fold excess of the unlabeled oligonucleotide competitors shown. (E) The nuclear extracts shown were tested for the presence of FF-binding complexes. Five micrograms of nuclear extract was added to each lane except lane 7, to which only 1 μg of islet nuclear extract was added. Lanes 1 to 4 were exposed for 12 h; lanes 5 to 7 were exposed for 96 h.

sensitive (7, 41). Consistent with this finding, the R2 minienhancer is not active in HIT cells (Fig. 5A). To assess whether this lack of activity resulted from the absence of the Far or FLAT-F element, we tested the activity of two chimeric minienhancers: the Far region for rIns I linked to the FLAT region of rIns II and the converse construct (called R1R2 and R2R1, respectively; Table 1 and Fig. 5E). The substitution of the rIns I Far element made the rIns II minienhancer active (Fig. 5A). These results support the conclusion that an intact Far element must be correctly linked with at least one of the two FLAT elements to produce an active minienhancer.

When used as a competitor for binding to the FF probe (Fig. 6A), the R2 minienhancer was unable to compete for the F2 or C complex. This result is compatible with the lack of conserved Far and FLAT-F elements in the rIns II gene. Presumably because of the lack of a FLAT-F element, mutation of the FLAT-E site in the R2 minienhancer did not result in an increase in minienhancer activity or binding to the F2 complex.

The mIns I and II minienhancers function similarly to their rat homologs. Mouse insulin (mIns) I and II minienhancers (M1 and M2; Table 1) were constructed from the regions of these genes homologous to the rIns I Far-FLAT region. The mIns I gene has a conserved Far element and nearly perfect FLAT-E element, but it lacks a conserved FLAT F element. In the mIns I gene, the FLAT-F element core sequence (TAATAAT) is found on the opposite, noncoding strand of DNA and is 4 bp closer to the Far element than is the rIns I gene. Like the FF minienhancer, the M1 minienhancer functioned as a transcriptional activator, but mutation of the FLAT-E element resulted in a loss of activity (Fig. 5A). The M1 minienhancer was able to compete for binding of the C

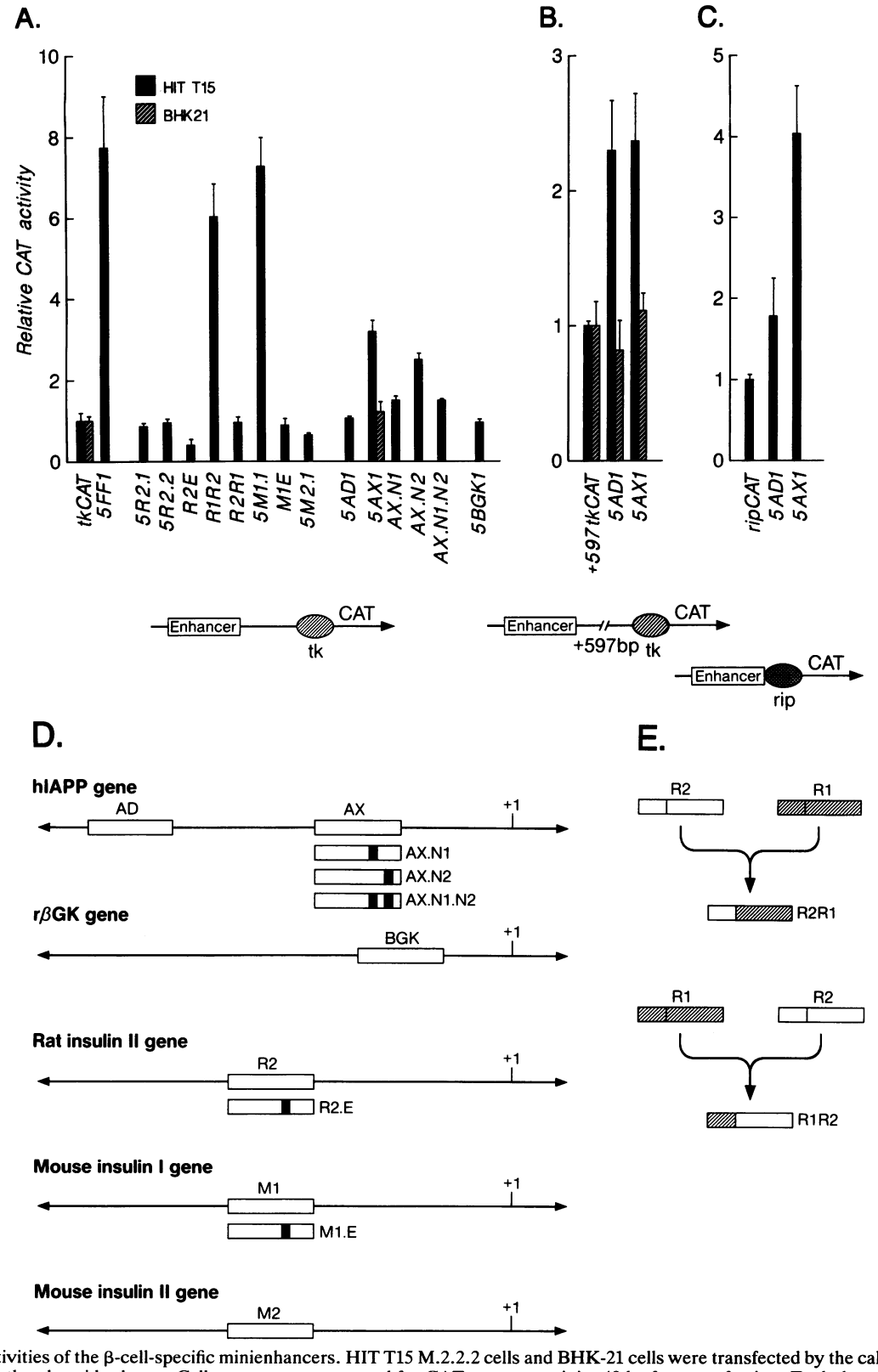

FIG. 5. Activities of the β-cell-specific minienhancers. HIT T15 M.2.2.2 cells and BHK-21 cells were transfected by the calcium phosphate technique with the plasmids shown. Cell extracts were assayed for CAT enzyme activity 48 h after transfection. Each data point represents the average CAT activity of at least three independent transfections ± standard error. Plasmids used in panel A were made by inserting five tandem copies of the minienhancers shown into the *Bgl*II site in tkCAT. Plasmids used in panel B were made by inserting the minienhancer multimers into the *Bgl*II site in +597tkCAT. Plasmids used in panel C were made by inserting the minienhancer multimers into the *Bam*HI site in ripCAT. In panel D, the sources of the minienhancers are shown. In panel E, constructions of the chimeric minienhancers R1R2 and R2R1 are demonstrated.

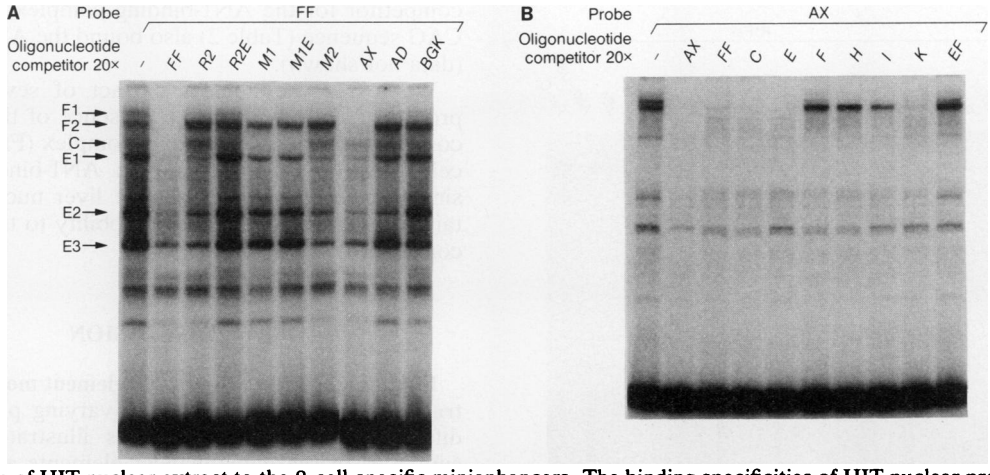

FIG. 6. Binding of HIT nuclear extract to the β-cell-specific minienhancers. The binding specificities of HIT nuclear proteins were tested by EMSA. See Table 1 for the sequences of probes and competitors. (A) HIT nuclear extract was added to the ^{32}P-labeled FF probe and a 20-fold excess of the unlabeled competitor oligonucleotides shown. (B) HIT nuclear extract was added to the ^{32}P-labeled AX probe and a 20-fold excess of the unlabeled competitor oligonucleotides shown.

and E complexes (Fig. 6A) but did not appear to bind the F2 complex. The M1E mutant oligonucleotide competitor had only slightly reduced ability to compete for the E bands, demonstrating that the FLAT-F equivalent site in mIns I binds to the E complexes but is unable to bind the F2 complex.

Like the rIns II gene, the mIns II gene has a conserved FLAT-E element, but it has no Far or FLAT-F element. Like the R2 minienhancer, the M2 minienhancer was inactive when transfected into HIT cells (Fig. 5A) and competed for the E complexes but not the F2 or C complex (Fig. 6A).

The IAPP and βGK minienhancers. Because of their selective expression in β cells, we searched for sequence elements similar to the Far, FLAT-E, and FLAT-F elements in the sequences of the 5′ flanking DNA of the IAPP (28) and βGK (23) genes. In the IAPP sequence, we found a region (AX minienhancer; Table 1) containing three elements similar to FLAT-E and FLAT-F and two elements (AN1 and AN2; Table 1) similar to the Far element. Like the FF minienhancer multimer constructs, five tandem copies of the Ax minienhancer linked to tkCAT displayed activity in HIT but not BHK cells (Fig. 5A), and these AX minienhancer multimers were still active when moved 597 bp further from the TK promoter (Fig. 5B) or when linked to the −85 rIns I promoter linked to CAT (Fig. 5C). Mutations of the AN1 element (AX.N1 minienhancer; Table 1) caused a complete loss of activity, while mutation of the AN2 site (AX.N2 minienhancer; Table 1) resulted in a modest loss of activity (Fig. 5A). The AX oligonucleotide DNA fragment bound all of the complexes that bound the FF minienhancer except the C doublet (Fig. 6A). The FF oligonucleotides in turn were able to compete for all of the major complexes which bound to the AX probe (Fig. 6B).

A more distal region of the IAPP 5′ flanking DNA (AD minienhancer; Table 1) has an A+T-rich region that is juxtaposed to a 12-bp nearly perfect palindrome; a similar palindrome (10 of 12 bp) is found in the rIns II promoter immediately upstream from the conserved rIns II equivalent of the Nir element. In the rIns II promoter, this palindromic sequence (RIPE3b) is markedly sensitive to mutation (7, 18) and interacts with the Nir element in a fashion reminiscent of the Far-FLAT interaction (18, 35). The AD minienhancer was not active when closely linked to the TK promoter but was active in HIT cells but not BHK cells when moved 597 bp further from the TK promoter. The AD oligonucleotides were high-affinity competitors for complex E2 and weak competitors for complex E1.

The rβGK 5′ flanking DNA contains a region (βGK; Table 1) with a sequence similar to that of the Far element (βGK-N; Table 1) linked to an A+T-rich sequence. The βGK minienhancer was not active in HIT cells (Fig. 5A) but did compete for binding of the E1 and E3 complexes (Fig. 6A).

The Far and AN1 sites bind different nuclear factors. End-labeled DNA fragments containing either the rIns I Far element (R1Far; Table 2), the hIAPP AN1 element (AN1; Table 2), or the hIAPP AN2 element (AN2; Table 2) were tested for the ability to bind HIT nuclear proteins. As seen in Fig. 7, the R1Far and AN1 elements each specifically bound a large nuclear complex. The AN2 probe did not significantly bind any nuclear complexes (data not shown). A group of similar binding sites from the 5′ flanking sequences of the islet-specific rIns I, rIns II, hIAPP, βGK, glucagon, and gastrin genes and the mouse B-lymphocyte κ light-chain and μ heavy-chain genes (Table 2) was used to test the binding specificity of the major R1Far-binding complex and the AN1-binding complex. These competition experiments demonstrate that the R1Far-binding complex also binds well to the rIns I and rIns II Nir elements and the μE2 element (Fig. 7A). At higher competitor ratios (50×), the AN2 and μE4 elements also bound the R1Far-binding complex (data not shown).

Like the C complex, the R1Far-binding complex is a doublet (visible when electrophoresis is extended); both complexes are competed for by μE2; the upper band of both doublets is bound by an anti-Pan 2 (E12) antibody, and the lower band is bound by an anti-Pan 1 (E47) antibody (data not shown). We believe that the C and R1Far-binding bands are composed of the same protein complexes.

The rIns II equivalent (R2Far) of the rIns I Far element was unable to bind to the R1Far-binding complex, but it displayed as high an affinity for the AN1-binding complex as did the AN1 site (Fig. 7B). In the hIAPP promoter, the AN2 site overlaps the AN1 site; thus, the AN2 site was a weak

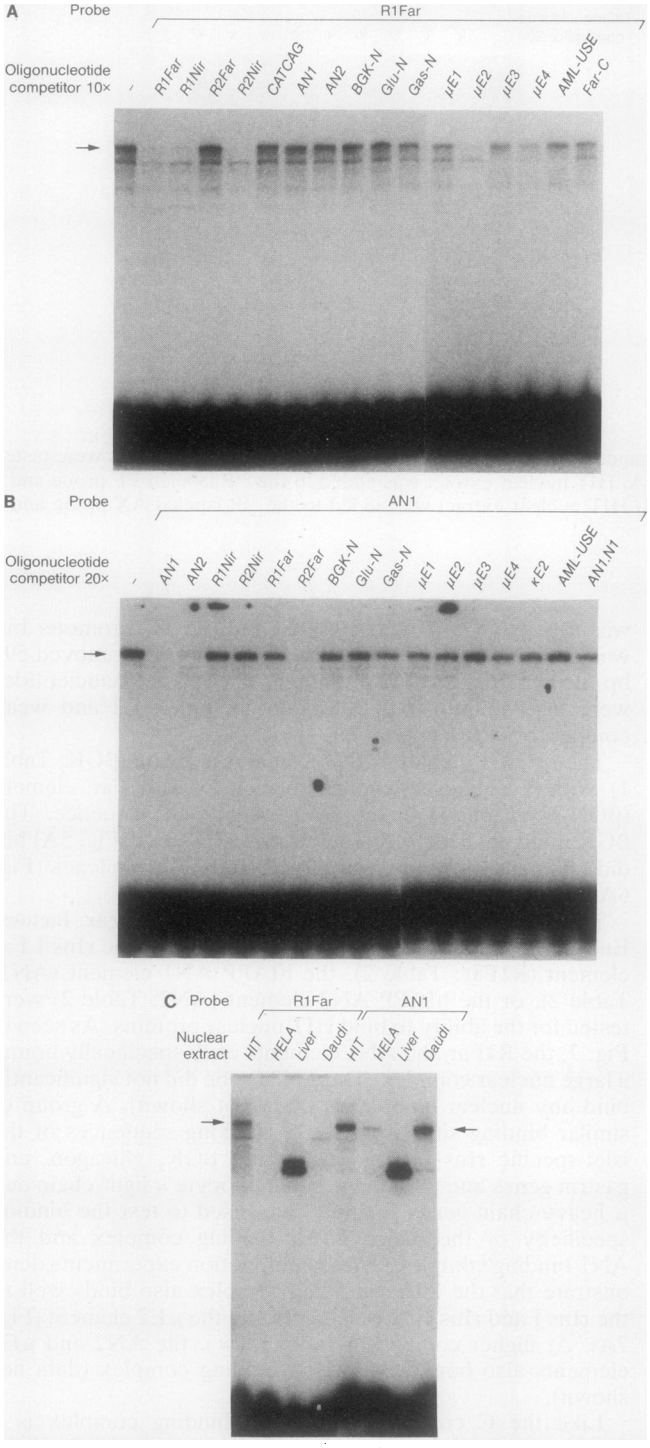

FIG. 7. Binding of HIT nuclear extract to the Far and AN1 elements. The binding specificities of HIT nuclear proteins were tested by EMSA. See Table 2 for the sequences of probes and competitors. (A) HIT nuclear extract was added to the ^{32}P-labeled R1Far probe and a 10-fold excess of the unlabeled competitor oligonucleotides shown. The arrow indicates the major specific binding complex. (B) HIT nuclear extract was added to the ^{32}P-labeled AN1 probe and a 20-fold excess of the unlabeled competitor oligonucleotides shown. (C) The nuclear extracts shown were tested for the presence of R1Far- and AN1-binding complexes. Five micrograms of nuclear extract was added to each lane.

competitor for the AN1-binding complex. The rIns I CAT CAG sequence (Table 2) also bound the AN1-binding factor (data not shown).

We tested the nuclear extract of several non-insulin-producing cell lines for the presence of the R1Far-binding complex and the AN1-binding complex (Fig. 7C). All of the cell types tested contained an AN1-binding complex of similar mobility, while only the liver nuclear extract contained a faint band similar in mobility to the R1Far-binding complex.

DISCUSSION

Interactions within the FLAT element modulate rIns I gene transcription. The dramatically varying phenotypes of the different FLAT region mutants illustrate the regulatory subtleties of the minienhancer elements and the danger of using a single mutation to determine the importance of a promoter element. The FLAT-E/FLAT-F interaction was missed in the original mutation scan of the rIns I promoter because a single block mutation covered both elements and therefore gave a decrease in activity much as did the EF double mutation (19). This may explain why mutations of the FLAT-E-like sites on either side of the rIns I Nir element do not decrease activity; simultaneous mutation of both sites may be required for inhibition. This explanation fits well with evidence from the rIns II gene that the function of the rIns II Nir element requires interaction with the palindromic RIPE3b element which replaces the FLAT-E-like site present immediately upstream of the Nir element in rIns I (18, 35).

The presence of the FLAT-E element decreases the activation through the FLAT-F element, and to a lesser degree the reverse is true. This type of dual-negative interaction allows the β cell to fine tune the contribution of the FF region to the overall transcriptional activity of the rIns I promoter. The binding data suggest that the increased transcriptional activity of the E and J mutants may result from increased binding of the F2 complex, although minienhancers that cannot bind F2 (such as the F mutant) can still function, probably because they bind different activators at the remaining E site. The increased affinity of the E and J mutants for the F2 complex shows that the presence of an intact E site decreases binding at the F site. One simple explanation for this increased affinity may be that occupation of the E site destabilizes F site binding by steric hindrance, by altering the interactions with other proteins, or by changing the DNA conformation.

Alternately, the E site may not need to be occupied in order to destabilize binding at F. The mutants at the E site may cause DNA conformation changes which could increase or decrease binding at F. The similarity of the E and F sequences suggests another possibility: the E site may compete for factors binding at F, but the E-bound complexes might display a high off rate. The P-mutated E site, which more closely resembles the F site, may bind the F-binding proteins, but at a lower affinity and in the wrong position and orientation, resulting in deactivation of the minienhancer. This model is supported by the fact that the P mutant has decreased affinity for the E1, E2, and E3 complexes but still binds the F2 complex. This model is also supported by the fact that the E mutant's affinity for the F2 complex is higher than the wild-type FF mini-enhancer's affinity even when the ratio of binding sites to protein is high (as in Fig. 4C) and the E site is therefore usually unoccupied. The ability of an

unoccupied E site to interfere with binding at F also argues against the simple steric hindrance model.

At our present level of knowlege, we can only speculate on the nature of the E-F interaction at a molecular level. None of the models given above can be ruled out decisively. Mechanistic interpretations are likely to come from additional structural information. When the specific binding proteins become available, the FLAT element will provide an ideal model system for structural studies of protein-DNA interactions. One candidate for such studies could be the neuroendocrine-specific cDNA isI-1, which encodes a homeodomain protein that binds to the FLAT element in vitro (20). Antibody studies, however, suggest that isI-1 is not present in the HIT nuclear complexes which bind the FLAT element in vivo (31).

The IAPP and insulin promoters contain similar elements. The promoters of genes uniquely expressed in the same cell type often contain similar or identical control elements (for example, the immunoglobulin genes [34] and the pancreatic exocrine enzyme genes [3]). Although direct comparison of the known β-cell-specific promoters does not reveal dramatic sequence homology, knowledge of the mutationally sensitive areas of the insulin promoter allows one to recognize parallels with other β-cell promoters. These parallels are especially obvious in the IAPP promoter. Within its 5′ flanking DNA, the hIAPP gene has regions with marked sequence similarity to critical regions in the rIns I and rIns II promoters (25). Similarities between the two regions described in this report (the FF region in rIns I and the AX region in hIAPP) are particularly striking. Not only are the sequences and overall architectures of these regions similar, but our data also show that the functions and interactions of these elements are similar and that they bind the same nuclear proteins.

The HIT nuclear binding complexes. The binding characteristics and cell specificity of the major Far element-binding complex are identical to those of the previously described complexes binding to the rIns I Nir element (24, 30), rIns II Nir element (18, 40), and human insulin Nir and Far elements (2). Antibody binding analysis has shown that this complex contains shPan-1 (14) (the Syrian hamster equivalent of human E47 [26] and mouse A1 [37]) and shPan-2 (the Syrian hamster equivalent of human E12 [26]) basic helix-loop-helix proteins which are widely or ubiquitously expressed. The cell selectivity of the Far-binding complex may result from dimerization of the Pan proteins with a more selectively expressed protein or proteins (1, 6, 14, 35).

The sequence of the AN1 site is similar to the sequences of the Nir and Far elements and of other related elements such as the E boxes in the immunoglobulin genes, but the specific AN1-binding complex has not been previously described. The ability of the rIns II Far element equivalent to bind the AN1-binding complex suggests that the complex may play a role in the rIns II gene expression as well. Complexes binding to the FLAT region (24, 31) and the parallel region of the human insulin gene (2) have also been described, but the binding specificities of these complexes have not been carefully explored. Previous studies of distribution of these complexes indicate that one of the FLAT-binding complexes may be present in β but not α cells (31).

Although some of the nuclear complexes which bind to the FF minienhancer were found only in HIT nuclear extract, clearly non-insulin-producing cell types also contain a variety of different nuclear proteins that can bind to the Far, FLAT-E, and FLAT-F sites. Some of these proteins may be identical to the proteins found in HIT nuclear extract, and many of the other proteins likely function as activators when bound to different promoters. These experiments suggest, however, that the nuclear factors in non-insulin-producing cells do not interact in the exact manner necessary to produce a functioning FF minienhancer. All that is required to shut off the minienhancer is one missing or incorrect member of the group of proteins that interact at the minienhancer or even just an imbalance between alternative binding factors. Given the cell specificity of this minienhancer-activating complex, it is not surprising that other β-cell-specific promoters contain minienhancers of similar architecture. In addition, the cell type expression of the IAPP minienhancer may be broadened by the substitution of the AN1 element for the more restricted Far element.

These data support an increasingly evident rule of cell-specific gene expression: cell-specific transcription results from the complex interplay of multiple *cis*- and *trans*-acting elements. As a result, cell specificity is not dependent on the specificity of any one *trans*-acting protein; rather, it is the complete, interacting complex that is cell specific.

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