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The Insulin Gene Contains Multiple Transcriptional Elements That Respond to Glucose

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The β cells in the pancreatic islets of Langerhans increase insulin gene transcription in response to increased glucose concentration. We have mapped sequences within the rat insulin I gene 5′-flanking DNA (rInsI promoter) that direct this transcriptional response to glucose. When linked to chloramphenicol acetyltransferase and expressed in cultured β cells, no single mutation of the rInsI promoter removes its ability to respond to glucose, although several mutations cause marked reductions in basal chloramphenicol acetyltransferase expression. A 50-bp sequence isolated from the rInsI promoter, the Far-FLAT minienhancer, can confer glucose responsiveness to nonresponsive promoters. Fine mapping of this minienhancer further localizes a glucose response to the sequence GGCCATCTGGCC, or the Far element. Nuclear extracts from islets grown in various glucose concentrations demonstrate a glucose-stimulated increase in a protein complex that binds the Far element and contains the transcription factors Pan-1 and Pan-2. Overexpression of intact or partially deleted Pan-1 ablates the Far-directed transcriptional response to glucose. We conclude that the full glucose response of the insulin promoter involves the interaction of multiple sequence elements. Part of this response, however, results from activation of a complex binding at the Far element.

The β cells in the pancreatic islets of Langerhans are uniquely designed to sense the metabolic state of the organism, largely by sensing the circulatory glucose concentration. The β cells respond to elevated glucose levels by increasing insulin synthesis and secretion. Increased insulin synthesis results from the combined effects of increases in insulin gene transcription (34), preproinsulin mRNA half-life (44), and preproinsulin mRNA translation (43). The 5′-flanking DNA, or promoter, of the insulin gene can confer glucose responsiveness to a linked gene when expressed in β cells, demonstrating that at least part of the transcriptional response to glucose is encoded in this relatively small piece of DNA (16).

The insulin promoter also functions in a distinctly cell-specific manner: it exclusively limits transcription of any linked gene to the β cell (20, 41). The *cis*-acting elements within the promoter that are required for cell-specific expression in insulin-producing tumor cell lines have been studied extensively for the rat insulin I (9, 24), rat insulin II (5, 46), and human insulin (2) genes. The rat insulin I gene 5′-flanking DNA (rInsI promoter), extending approximately 400 bp upstream from the transcription start site, contains two identical 8-bp sequences, the Nir element at −104 to −111 bp and the Far element at −231 to −238 bp, respectively. In insulin-producing tumor cells, mutation of either of these sequence elements results in a 10-fold loss of transcriptional activity, while mutation of both sites results in a nonfunctioning promoter (24).

The Far element has very little activity when isolated from the remainder of the promoter, but it functions in a markedly synergistic fashion when included with its downstream flanking sequence, the AT-rich FLAT element (17). The 50 bp of DNA containing the Far and FLAT elements—the Far-FLAT (FF) minienhancer—form a potent, cell-specific transcriptional enhancer (17, 25). The Nir element (also called IEB-1 [26], ICE-1 [45], or RIPE3a [22]) also functions synergistically with a linked site (RIPE3b) in the context of the rInsII promoter (22, 39).

The Nir and Far elements contain the core sequence CANNTG, or E box, originally found in the immunoglobulin genes (3, 11) but since recognized in numerous genes. Like many other E boxes, the Nir and Far elements bind the ubiquitous transcription factors Pan-1 and Pan-2 (33) (Pan-1 is also termed E47 [32] or A1 [42], and Pan-2 is also termed E12 [32]). These proteins contain both a helix-loop-helix (HLH) domain that is required for dimerization and DNA binding and a basic domain that is required for DNA binding. Previous studies suggest that in β cells the Pan proteins dimerize with a protein of limited distribution, resulting in a unique complex that binds the Nir and Far elements (1, 4, 15, 39).

Several cDNAs also have been described that encode homeodomain-containing proteins that bind the FLAT element (10, 18, 25). One of these proteins, Lmx-1, dramatically activates the FF minienhancer in combination with the Pan proteins (18).

The insulin-producing tumor cell lines used in most studies of the insulin promoter have little or no secretory or transcriptional response to glucose. We have developed a technique for transfecting primary cultured islet cells and have used this technique to show that both the full rInsI promoter and the isolated FF minienhancer (one, two, or five copies in tandem) can respond to glucose (16). In the present study with primary cultured islet cells, we have used a series of mutations in the rInsI promoter and the FF minienhancer to map glucose-responsive sequences more carefully and determine the role(s) of their cognate binding proteins.

MATERIALS AND METHODS

Plasmid construction. Construction of the rInsI promoter mutants (24), the cytomegalovirus (CMV) promoter-driven expression plasmids containing Pan-1 (15) and *lmx-1* (18)

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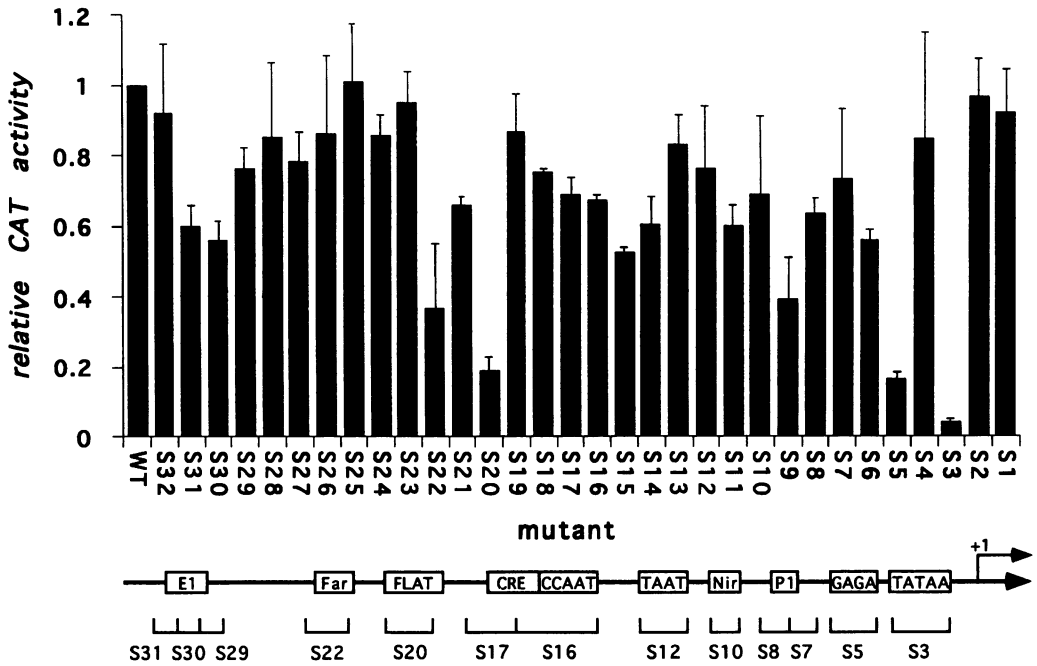

FIG. 1. Activities of mutated rInsI promoters in primary cultured β cells. Cultured fetal rat islets were transfected with the mutated insulin promoter-CAT plasmids shown and grown in medium with 11 mM glucose. CAT enzyme activity was assayed with equal amounts of protein 36 h after transfection. Each bar represents the mean + standard error of the mean of three independent transfections. The CAT activity of cultures transfected with the wild-type promoter plasmid was arbitrarily set at 1.0.

cDNAs, and the minienhancer plasmids (17) has been described previously. The pFOX-CAT plasmid was constructed in multiple steps. A *Sau*3AI restriction endonuclease fragment containing the entire chloramphenicol acetyltransferase (CAT) coding region (19) was inserted into the polylinker of the pBluescript SK+ plasmid (Stratagene Cloning Systems, La Jolla, Calif.). The simian virus 40 polyadenylation signal was amplified by PCR, one copy was inserted downstream from the CAT sequence at the T3 end of the polylinker, and two copies were inserted between the upstream *Pvu*I site and the 5′ end of the CAT sequence (removing the M13 forward sequencing primer site and the polylinker). An oligonucleotide containing the M13 forward universal primer site and multiple restriction endonuclease sites was then inserted between the double polyadenylation sites and the 5′ end of the CAT gene. This construct does not contain an intron. We found, as have others (21), that the simian virus 40 small-T intron used in many CAT plasmids reduces the expression of functional CAT enzyme.

Islet transfection. Fetal (21-day-gestation) Sprague-Dawley rat islets were isolated and transfected as previously described (16). Each 10-cm-diameter plate of cultured islet cells was transfected with 25 μg of double cesium-chloride-purified CAT plasmid DNA and 10 μg of the cotransfected expression plasmids shown. The transfected cells were grown in RPMI 1640 medium with the additives shown for approximately 36 h prior to harvesting and protein extraction. CAT enzyme assays were performed with 10 μg of protein for 2 h (9).

Electrophoretic mobility shift assays (EMSAs). Fetal islets used for making nuclear extracts were isolated and cultured by a protocol identical to that used for transfections, with the single exception that the cells were not subjected to electroporation. The handpicked adult rat islets were generously provided by M. Ma, J. Wang, and G. Grodsky, University of California at San Francisco. After overnight culture in medium containing 5.5 mM glucose, the islets were transferred to media with the glucose concentrations shown and cultured for 24 h prior to harvest. Nuclear extracts were prepared by the method of Dignam et al. (8).

The Far and FLAT probes (for sequences, see Fig. 3) were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding was performed in a 10-μl volume with 2 μg of nuclear extract, 100 pg of labeled probe (approximately 10,000 cpm), 10 mM HEPES (pH 7.8), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 nM dithiothreitol, 3% Ficoll, 200 mg of poly(dI-dC)·poly(dI-dC) per ml, and 1% polyvinyl alcohol. After incubation at room temperature for 30 min, the mixtures were electrophoresed on 5% nondenaturing polyacrylamide gels (acrylamide-bisacrylamide, 30:1) in 0.5× TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA) (12, 13).

RESULTS

Mutation analysis of the intact rInsI promoter. A series of plasmids incorporating sequential block replacement mutations in the rInsI promoter linked to the CAT coding sequence was previously used to map *cis*-acting regulatory elements in the promoter by transfection and transient expression in the hamster insulinoma cell line HIT-T15 M2.2.2 (24). We transfected this same series of mutant plasmids into cultured islets and measured CAT activity after 36 h of culture in RPMI 1640 medium with 11 mM glucose (Fig. 1).

The majority of these replacement mutations cause a modest reduction in the transcriptional activity of the promoter, but four mutations cause large drops in activity. The first of these mutations, S3, includes the TATAA element and causes the loss of 96% of transcriptional activity. S5, the second most deleterious mutation (removes 84% of wild-type activity), replaces an interesting purine-rich, or GAGA, sequence that

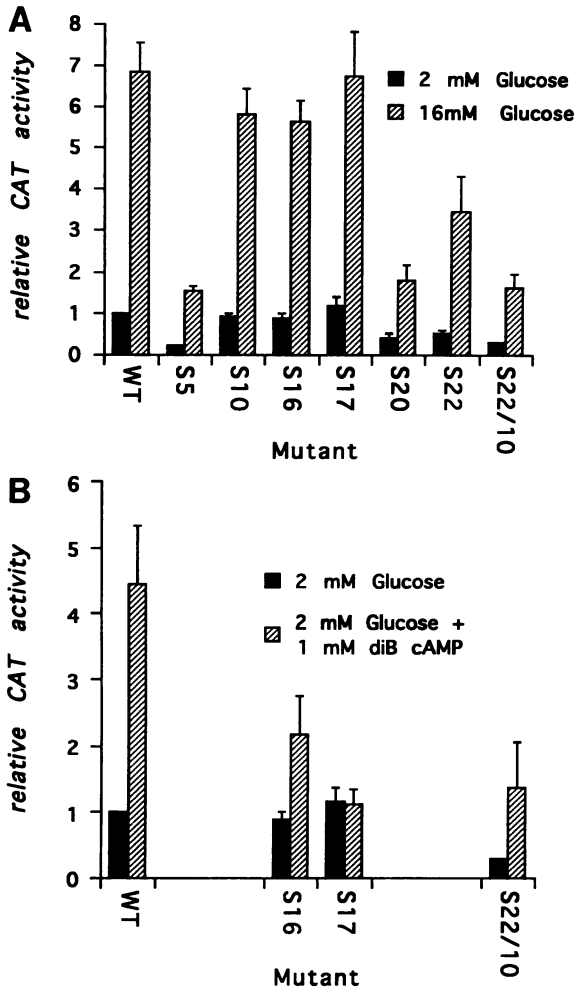

FIG. 2. Glucose and cAMP regulation of selected mutant promoters. (A) Cultured fetal rat islets were transfected with the mutated insulin promoter-CAT plasmids indicated and grown in 2 or 16 mM glucose. CAT enzyme activity was assayed with equal amounts of protein 36 h after transfection. Each bar represents the mean + standard error of the mean of three independent transfections. The CAT activity of cultures transfected with the wild-type promoter plasmid and grown at 2 mM glucose was arbitrarily set at 1.0. (B) Cultured fetal rat islets were transfected with the mutated insulin promoter-CAT plasmids shown and grown in 2 mM glucose alone or with 1 mM dibutyryl (diB) cAMP. WT, wild type.

has been studied in detail (27). Mutations of the Far element (S22 [removes 65% of wild-type activity]) and the FLAT element (S20 [removes 81% of wild-type activity]) also disable the promoter. In contrast, the S10 promoter with a mutation in the Nir element, a sequence element that is critical for the function of the rInsI, rInsII, and human insulin promoters in insulinoma cell lines, retains 69% of wild-type activity in primary cultured islets.

We tested selected block replacement mutants for the ability to respond to glucose by growing the transfected islet cells in either 2 or 16 mM glucose for 36 h prior to harvesting and performing CAT enzyme assays (Fig. 2). None of the mutations eliminates the glucose response, as represented by the ratio of CAT activities at 16 and 2 mM glucose. Only the FLAT element mutation (S20) causes any significant reduction in the glucose response (4.1-fold versus 6.9-fold for the wild-type promoter). The mutation that causes the greatest absolute reduction in transcriptional activity, S5, actually causes a small increase in glucose response.

TABLE 1. Glucose responses of cells transfected with plasmids with mutant promoters[a]

Promoter	Relative CAT activity[b] of cells grown with the following concn of glucose:		Stimulation (fold)[c]
	2 mM	16 mM	
None	1	1.2	1.2 ± 0.05
S (wild type)	237	1,183	5.2 ± 0.7
S12	238	995	5.2 ± 1.7
S20	90	606	6.6 ± 2.0
S22/10	32	149	4.6 ± 0.6
S22/16/10	1.7	9.3	4.1 ± 1.1
S22/17/10	4.8	20	4.5 ± 0.6
S22/20/10	2.4	6.4	2.7 ± 0.2
S20/12	144	564	3.7 ± 0.6

[a] The wild-type or mutant rInsI promoters were inserted upstream of the CAT gene in the pFOX-CAT plasmid. Cultured fetal rat islets were transfected with these plasmids and grown for 36 h in 2 or 16 mM glucose for 36 h prior to harvesting.

[b] Relative CAT activities are shown, with each value representing the mean of at least three independent transfections. The CAT activity in cells transfected with the promoterless control plasmid (pFOX-CAT) and grown in 2 mM glucose was arbitrarily set at 1.0. CAT enzyme activity was assayed with equal amounts of protein.

[c] Each stimulation (fold) value represents the mean ± standard error of the mean of the ratios of at least three pairs of independent transfections grown at 2 and 16 mM glucose.

The inability of any single replacement mutation to eliminate the rInsI promoter's transcriptional response to glucose suggests that there are multiple glucose-regulated elements in the promoter. We therefore constructed and tested promoters with two or more block replacement mutations. Combined mutation of the identical Nir and Far elements (double-mutant plasmid with S22 and S10 mutations [S22/10]) does not completely disable the promoter (Fig. 2A) as it does in HIT cells (24), and it causes only a small reduction in the glucose response.

Because the combination of multiple mutations produces promoters with low intrinsic transcriptional activity, we constructed several of these promoters in a plasmid, pFOX-CAT, with two simian virus 40 polyadenylation signals upstream of the promoter in order to reduce background activity. The glucose responses of some of these constructs are shown in Table 1. The addition of mutation S17, which includes part of the cyclic AMP (cAMP) response element (CRE) (38), or S16, which includes both part of the CRE and the CCAAT element, to the S22/10 double-mutant promoter causes a dramatic drop in absolute transcriptional activity despite the fact that neither S16 nor S17 causes large decreases in activity by itself. Both of these triple mutants retain the response to glucose despite their low intrinsic levels of activity. These data suggest that the Nir and Far elements and the CRE and CCAAT elements function in an independent or additive fashion, rather than a synergistic fashion.

None of the other combinations tested removed the glucose response. The S22/20/10 combination caused a significant decrease (2.7-fold versus 5.2-fold from wild type), but the activity of this promoter is very low, approaching that of the promoterless parent vector, pFOX-CAT.

The CRE is not required for glucose response. Both mutants S16 and S17 cover part of the CRE found in the rInsI

promoter. These two mutants as well as the wild-type and S22/10 promoter plasmids were tested for response to the cAMP agonist, dibutyryl cAMP. The S16 mutant has a reduced response to dibutyryl cAMP, and the S17 mutant does not respond at all (Fig. 2B). Since neither CRE mutation causes a reduction in the response to glucose (Fig. 2A), we conclude that the CRE must not be essential for the transcriptional response to glucose.

Fine mapping of the FF minienhancer. To circumvent the complexity of the intact insulin promoter, we studied the 50-bp FF minienhancer (Fig. 3C) (17, 26) in detail. Five copies of the minienhancer were linked in tandem to the herpes simplex virus thymidine kinase (TK) promoter driving CAT gene expression. By itself, the TK promoter does not respond to glucose, but the addition of five copies of the FF minienhancer (5FF1) results in a sixfold response to glucose (Fig. 3A).

We have previously described a series of mutant minienhancers with small replacement mutations that span the length of the minienhancers (Fig. 4) (17). As with the wild-type minienhancer, five copies of each mutant minienhancer were linked to TK-CAT. We tested the responses of these mutant minienhancers to glucose in transfected primary islet cell cultures (Fig. 3A). Only the C mutation, which mutates the Far element, removes all response to glucose. The EF mutation, which mutates both of the binding sites in the FLAT element (17), retains some response to glucose (3.0-fold), despite a lower level of activity than that of the TK promoter alone.

The TK promoter functions in most cell types and therefore might be expected to function in the non-β cells present in these islet cultures (16). For this reason, we also tested the multimerized mutant minienhancers linked to a minimal rInsI promoter. This promoter (labeled RIP in Fig. 3B) is truncated at −85 bp. The −85 RIP, which includes the sequences from S1 to S8 (Fig. 1), has low intrinsic activity and exhibits almost no response to glucose (Fig. 3B) but retains β-cell specificity (15a). As with the TK-linked constructs, five copies of the wild-type minienhancer stimulate a marked response to glucose. Again, mutant C, the Far element mutant, completely lacks any glucose response, while mutant EF, the FLAT element mutant, retains some response to glucose (Fig. 3B).

Shorter minienhancers including only the Far element (FA) or only the FLAT element (FL) were also tested. Despite low intrinsic activity, the FA minienhancer responds to glucose (3.7-fold) while the FL minienhancer does not (1.5-fold) (Fig. 3B).

Three additional control plasmids were tested: (i) five copies of the AX minienhancer (17), a β-cell-specific minienhancer from the human islet amyloid polypeptide promoter (35) linked to −85 RIP-CAT; (ii) a portion of the Rous sarcoma virus long terminal repeat (19) linked to −85 RIP-CAT; and (iii) the murine mammary sarcoma virus (MSV) long terminal repeat (7) linked to −85 RIP-CAT. Despite high levels of activity, neither the AX nor the Rous sarcoma virus construct responded to glucose. Surprisingly, the MSV enhancer construct demonstrated a significant (5.2-fold) response to glucose. The insulin promoter and MSV enhancer share no obvious sequence similarities, and the MSV enhancer includes no Far element or E-box sequence.

Overexpression of the Pan cDNAs ablates the response to glucose. We next tested the effects of two DNA-binding proteins, Pan-1 and Lmx-1, on glucose regulation of the minienhancer. Pan-1 binds the Far site (15), and Lmx-1 binds the FLAT E site with high affinity and the FLAT F site with lower affinity (18). The cDNAs encoding these two proteins were inserted in separate plasmids downstream of a powerful eukaryotic promoter, the human CMV immediate-early gene

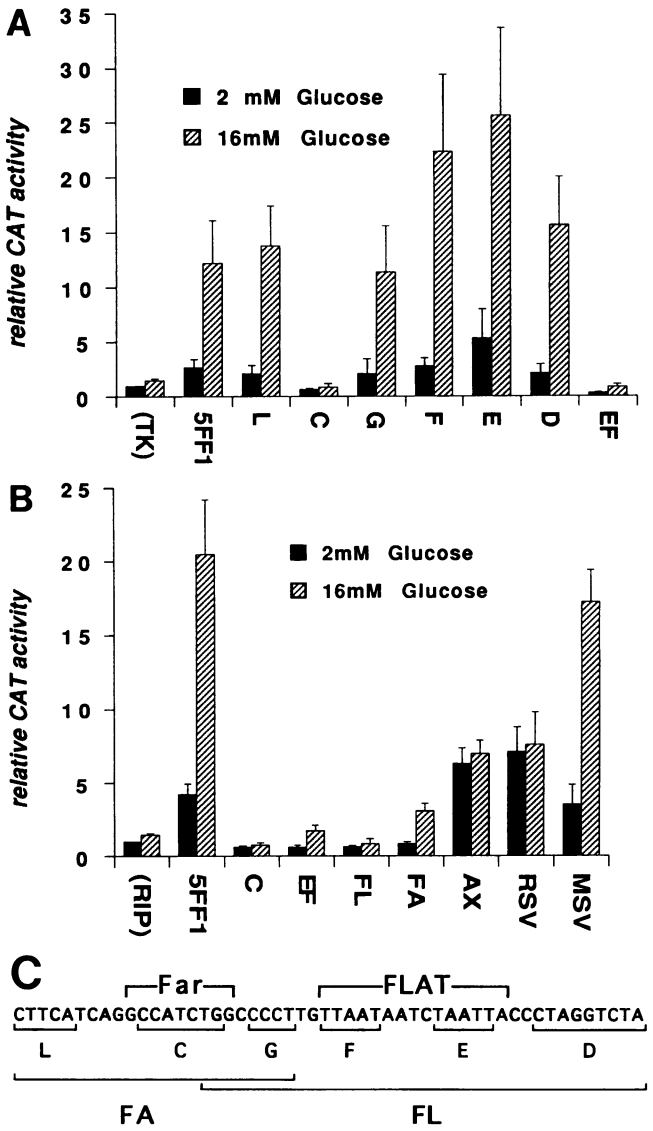

FIG. 3. Glucose regulation of the FF minienhancer. (A) Cultured fetal rat islets were transfected with plasmids containing five tandem copies of the wild-type (5FF1) or mutated (L through EF) minienhancers linked to the minimal TK promoter driving the CAT gene and grown in 2 or 16 mM glucose. CAT enzyme activity was assayed with equal amounts of protein 36 h after transfection. Each bar represents the mean + standard error of the mean of at least three independent transfections. The CAT activity of cultures transfected with the enhancerless TK-CAT plasmid (TK) and grown at 2 mM glucose was arbitrarily set at 1.0. (B) Cultured fetal rat islets were transfected with plasmids containing five tandem copies of the wild-type (5FF1), mutated (C and EF), or partially deleted (FL and FA) insulin minienhancers, five copies of the human islet amyloid polypeptide minienhancer (AX) (17), or a single copy of the Rous sarcoma virus or MSV enhancer linked to the minimal rInsI promoter truncated at −85 bp (RIP) driving the CAT gene and grown in 2 or 16 mM glucose. CAT enzyme activity was assayed with equal amounts of protein 36 h after transfection. Each bar represents the mean + standard error of the mean of at least three independent transfections. The CAT activity of cultures transfected with the enhancerless RIP-CAT plasmid (RIP) and grown at 2 mM glucose was arbitrarily set at 1.0. (C) The sequence of the FF minienhancer is shown with the positions of the Far and FLAT elements, the replacement mutations, and the truncated FA and FL minienhancers.

5'end	Sequence	Name
-247	CTTCATCAGGCCATCTGGCCCCTTGTTAATAATCTAATTACCCTAGGTCTA	FF
	AGGACTCAGGCCATCTGGCCCCTTGTTAATAATCTAATTACCCTAGGTCTA	L
	CTTCATCAGGACCTAGTGCCCCTTGTTAATAATCTAATTACCCTAGGTCTA	C
	CTTCATCAGGCCATCTGGCAACGTGTTAATAATCTAATTACCCTAGGTCTA	G
	CTTCATCAGGCCATCTGGCCCCTTGGTCCGAATCTAATTACCCTAGGTCTA	F
	CTTCATCAGGCCATCTGGCCCCTTGTTAATAATCGACTGACCCTAGGTCTA	E
	CTTCATCAGGCCATCTGGCCCCTTGTTAATAATCTAATTACCTGCTTGAGC	D
	CTTCATCAGGCCATCTGGCCCCTTGGTCCGAATCGACTGACCCTAGGTCTA	EF
	CTTCATCAGGCCATCTGGCCCCT	FA
	TGGCCCCTTGTTAATAATCTAATTACCCTAGGTCTAAGT	FL

FIG. 4. Sequences of the wild-type and mutant insulin minienhancers. Changes in the mutant minienhancers are underlined.

promoter. These plasmids direct high-level expression of the full-length proteins when transfected into eukaryotic cells.

In islet cultures transfected with CMV-Pan-1, the 5FF1 minienhancer construct shows a marked reduction in glucose response (Fig. 5). This reduction results from increased activity at 2 mM glucose and decreased activity at 16 mM glucose. FA, the Far element minienhancer, has no response to glucose in cells overexpressing Pan-1. Pan-1 overexpression has no significant effect on general regulation of the MSV enhancer, which does not include a canonical Pan-binding site, and Lmx-1 overexpression has no effect on glucose regulation of the FF minienhancer (Fig. 5).

We also tested the effect of expressing partially deleted Pan proteins. Three proteins with deletions were tested. (i) Pan-SS contains a 3′ deletion that results in expression of a carboxy-terminally truncated protein without an intact HLH domain. In vitro, Pan-SS cannot dimerize or bind DNA (1a, 15). (ii) The amino-terminally truncated Pan-SH dimerizes and binds DNA with high affinity (1a). (iii) The Pan-AA cDNA lacks the same coding sequences as Pan-SH does; in addition, within the basic domain two codons for the basic amino acid arginine have been replaced with codons for alanine. The resulting protein can form dimers but cannot bind DNA. In vitro, Pan-AA can prevent full-length Pan proteins from binding DNA by forming non-DNA-binding heterodimers (1a). We expect that expression of Pan-AA in vivo should prevent the endogenous Pan proteins (and possibly other HLH proteins as well) from binding DNA.

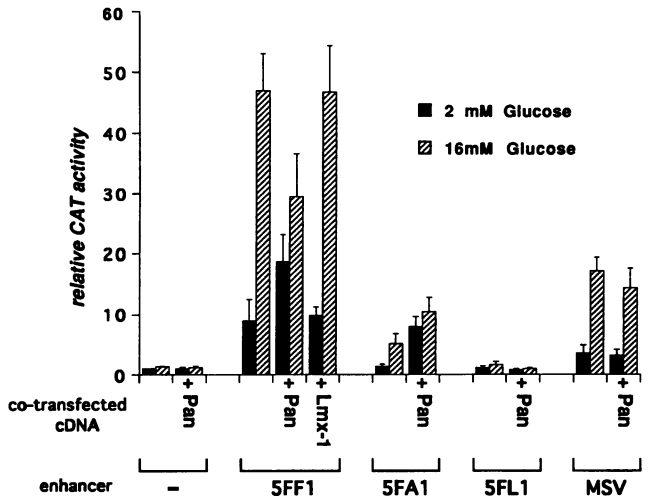

FIG. 5. Effects of transcription factor expression on the transcriptional response to glucose. Cultured fetal rat islets were cotransfected with two plasmids: (i) one plasmid containing either no enhancer (−), five tandem copies of the FF (5FF1), FA (5FA1), or FL (5FL1) minienhancer, or a single copy of the MSV enhancer linked to the minimal rInsI I promoter truncated at −85 bp (RIP) driving CAT gene expression; and (ii) a second plasmid with the CMV promoter driving the expression of either no cDNA, the Pan-1 cDNA, or the *lmx-1* cDNA. After transfection, these cells were grown in 2 or 16 mM glucose for 36 h prior to assaying CAT enzyme activity with equal amounts of protein. Each bar represents the mean + standard error of the mean of at least three independent transfections. The CAT activity of cultures transfected with the enhancerless RIP-CAT plasmid and the control CMV plasmid and grown at 2 mM glucose was arbitrarily set at 1.0.

As expected, expression of Pan-SS has no effect on the function of the FA minienhancer. Both DNA-binding Pan-SH and non-DNA-binding Pan-AA cDNAs dramatically reduce the FA minienhancer's response to glucose (Fig. 6).

The Pan-containing DNA-binding complex increases with increasing glucose concentration. In order to test for glucose effects on nuclear DNA-binding proteins, we made nuclear extracts from both fetal and adult rat islets after growth in various glucose concentrations. The fetal islets were grown and harvested in the same manner as the transfected islet cultures, including trypsin disruption of islets prior to growing these cells in 2 or 16 mM glucose for 36 h. Purified and handpicked adult islets were grown overnight in 5.5 mM glucose prior to 24 h of culture in 2 or 16 mM glucose. Nuclear extracts were tested for the ability to bind labeled double-stranded oligonucleotides containing either the Far or FLAT binding sites by an EMSA. Figure 7 shows a representative EMSA using adult rat islets. Complex C1, the slowest migrating complex that binds the Far probe, increases in intensity with increasing glucose concentration.

Complex C1 has the same mobility and binding specificity (data not shown) as those of the Pan-containing complex described previously in nuclei from β-cell tumor lines (4, 15, 17, 31, 36, 37, 39, 45). When antisera to Pan-1 and Pan-2 are mixed with nuclear extract from islets grown at 16 mM glucose, the formation of complex C1 is blocked (Fig. 7), indicating that complex C1 contains one or both of the Pan protein epitopes. A similar result is seen with nuclear extract from cells grown at 2 mM glucose (data not shown).

None of the major FLAT-binding complexes showed a significant response to glucose. This result was confirmed by quantification of the complexes with a PhosphorImager (Table 2). We repeated these experiments three times with fetal islets

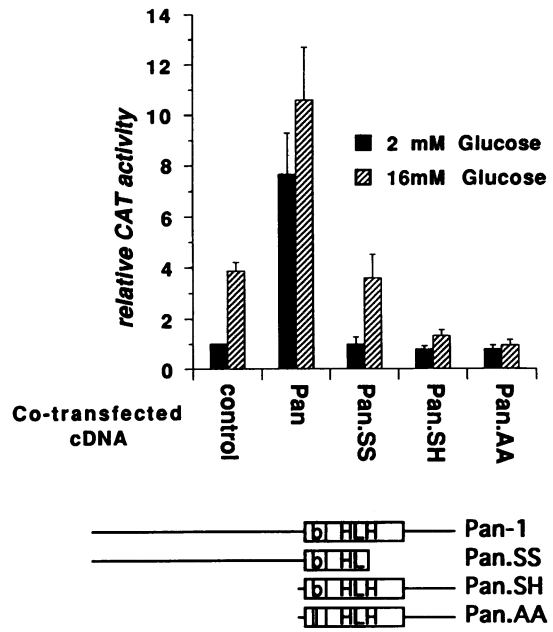

FIG. 6. Effects of expression of partially truncated Pan proteins on the transcriptional response to glucose. Cultured fetal rat islets were cotransfected with two plasmids: one plasmid containing five tandem copies of the FA minienhancers linked to the minimal rInsI promoter truncated at −85 bp (RIP) driving CAT gene expression; and a second plasmid with the CMV promoter driving the expression of either no cDNA (control), the Pan-1 cDNA, or the partially truncated Pan-1 cDNAs shown. After transfection, these cells were grown in 2 or 16 mM glucose for 36 h prior to assaying CAT enzyme activity with equal amounts of protein. Each bar represents the mean + standard error of the mean of at least three independent transfections. The CAT activity of cultures transfected with the control CMV plasmid and grown at 2 mM glucose was arbitrarily set at 1.0. b, basic.

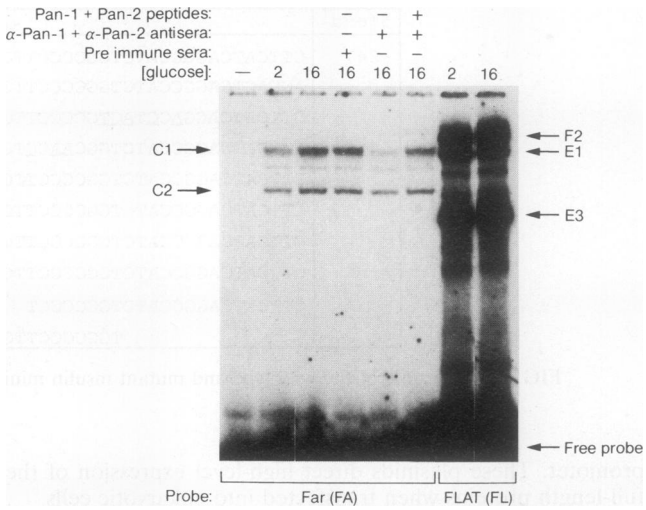

FIG. 7. DNA binding of nuclear extracts from adult rat islets grown in various glucose concentrations. Islets were isolated from adult rats and grown overnight in 5.5 mM glucose, prior to changing to the glucose concentrations (in millimolar) shown above the lanes. After 24 h in culture with the glucose concentrations shown, nuclear extracts were obtained. The nuclear extracts were mixed with the radiolabeled FA or FL probe, and the resulting protein-DNA complexes were separated by polyacrylamide gel electrophoresis. The leftmost lane contains no nuclear extract. Pan-1 and Pan-2 peptides, antisera against Pan-1 (α-Pan-1) and Pan-2 (α-Pan-2), and preimmune sera were included (+) or not included (−).

and obtained the same results: only complex C1 increases with increasing glucose concentration (data not shown).

DISCUSSION

Metabolic signals in β cells. Glucose plays a central role among the molecules that signal the metabolic state of the organism. Because of the unique metabolism of the β cell, it is the preeminent metabolic signal molecule at physiologic concentrations. As in other cells, glucose passively enters the β cell through a glucose transporter and is catabolized via glycolysis to pyruvate, which undergoes oxidative phosphorylation in the mitochondria (Fig. 8).

The β cells sense glucose indirectly, using the levels of the products of glucose catabolism to gauge glucose levels. By altering β-cell glucose metabolism, we have previously shown that the β-cell must catabolize glucose before it can stimulate insulin gene transcription (14). To a lesser degree, other energy sources such as leucine also can stimulate insulin gene transcription, but certain intermediates of glycolysis, which in the β cell are almost exclusively the products of glucose catabolism, are necessary for a maximum response (14). Because other nutrients can mimic the glucose effects, we prefer the term metabolic response element to describe the *cis*-acting promoter elements that respond to glucose.

β-cell glucose metabolism activates a variety of intracellular signaling pathways, and more than one of these pathways probably regulate insulin gene transcription. Because cAMP can activate the insulin promoter and raise insulin mRNA levels, it has been proposed that the cAMP pathway may contribute directly to glucose stimulation of insulin gene transcription (23, 38). Our data, however, do not support this model. Since the S17 promoter, which do not respond to cAMP, responds normally to glucose, cAMP must not be necessary for the glucose response. These data fit well with our previous finding that cAMP agonists and antagonists do not alter the relative activation of the insulin promoter by glucose (16). If cAMP plays any role in the metabolic regulation of the insulin promoter, it may modulate the signal supplied by other intracellular messengers. On the other hand, we have previously shown that a signal provided by calcium influx contributes to the activation of insulin gene transcription by glucose (16). Figure 8 outlines one possible pathway for glucose stimulation of insulin gene transcription.

TABLE 2. Glucose responses of promoter-binding complexes[a]

Complex	Increase (fold)[b]	*P* value[c]
C1	2.48 ± 0.23	<0.03
C2	1.16 ± 0.19	NS
F2	0.87 ± 0.09	NS
E1	0.87 ± 0.18	NS
E3	0.85 ± 0.27	NS

[a] Islets were grown overnight in 5.5 mM glucose and then changed to 2 or 16 mM glucose for 24 h prior to harvesting, extracting nuclear proteins, and analyzing by EMSA as in Fig. 7. Radioactivity in each Far- and FLAT-binding complex was measured with a PhosphorImager.

[b] The increase (fold) is the ratio of the activities of each band at 2 and 16 mM glucose. Each value is the mean ± standard error of the mean of three independently purified sets of nuclear extract.

[c] The paired *t* test was used to calculate *P* values. NS, not significant.

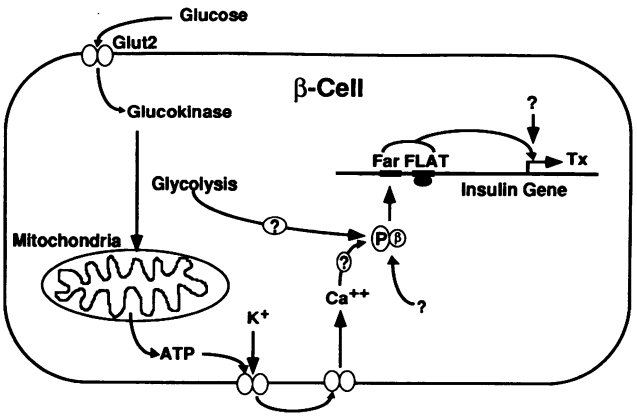

FIG. 8. Glucose activation of insulin gene transcription. A schematic representation of one possible pathway for glucose regulation of insulin gene transcription is shown. P represents the Pan-1 or Pan-2 protein, and β represents the selectively expressed heterodimer partner of Pan found in β cells. Question marks are shown to indicate unknown steps in the pathway or the probable intersection with other unknown pathways. Glut2, glucose transporter 2; Tx, transcription.

Mapping a metabolic response element. We used a combination of mutation, minienhancer, and deletion analyses to map a metabolic response element. This element, the Far element, is also critical for cell-specific function of the insulin promoter (24). Since the rInsI promoter contains two copies of the Far element sequence, the Nir and Far elements, it is not surprising that mutation of the Far element alone does not reduce the intact promoter's response to glucose. In fact, minienhancers that were designed from the rInsI and human insulin promoters and contain the Nir element and its upstream flanking sequence, the RIPE3b element, can also respond to glucose (17a). However, combined mutation of the Nir and Far elements, even in combination with other mutations, does not ablate the promoter's response to glucose. These data demonstrate that there are additional metabolic response elements within the rInsI promoter.

Even within the FF minienhancer, the Far element may not be the only metabolic response element. Because the FLAT element has no transcriptional activity on its own, we could not test it directly for glucose regulation. Other evidence, however, suggests that the FLAT element may be regulated by glucose: the S20 FLAT element mutation and the combined S22/20/10 mutations reduce the response to glucose.

Interestingly, other investigators have found that the FLAT element responds to glucose (30). Melloul et al. (30) used adult rather than fetal islets, which may explain why they were able to detect activity of the isolated FLAT element. The role of the Far element was not directly tested. These investigators also pointed out that fetal islets normally have a blunted response to glucose. However, 21-day-gestation fetal rat islets rapidly develop adult phenotype in culture. We have found that after 48 h in culture, the transfected fetal β cells have an adult type secretory response to glucose (17a).

Role of HLH proteins. Glucose presumably regulates the metabolic response elements by regulating the protein factors that bind to them. We have shown that overexpression of the HLH protein, Pan-1, that binds to the Far element ablates the Far element's response to glucose. There are four simple models that explain the effect of Pan-1 overexpression.

First, Pan-1 may supplant glucose-responsive proteins that normally bind to the Far element in vivo. If this is true, these glucose-responsive Far-binding proteins may also be HLH proteins, since the Pan-AA protein, which heterodimerizes with HLH proteins but does not bind DNA, interferes with the function of these proteins. Second, Pan overexpression may simply overwhelm the normal mechanism for modification by glucose of the Far element-activating machinery. Third, the normal complex of Pan-1 or Pan-2 and a β-specific HLH protein may be replaced by Pan-1 homodimers because of the excess Pan-1. If only the β-specific subunit were regulated by glucose, this new complex would not respond to glucose. Finally, the levels of functional Pan-containing complexes may vary with the glucose concentration secondary to alteration in levels of or modifications to the Pan or β-specific subunits.

The results of the EMSA with islet nuclear extract support the last two models, but this binding data must be interpreted cautiously. Changes in the binding complex abundance in vitro do not necessarily translate into changes in the transcriptional activity of the binding site in vivo. Conversely, the transcriptional activity of complexes may be altered by glucose without any change in abundance or binding affinity. For example, any of the FLAT element-binding complexes that appear unaltered by glucose in an EMSA may be modified by glucose to increase their transcription activation capability without changing their abundance or DNA binding affinity. In addition, glucose activation could involve nuclear factors that do not bind directly to DNA or that appear insignificant by EMSA.

Interestingly, Melloul et al. (30) obtained a different result in a similar experiment: they found that a FLAT-binding complex responded to glucose and they were unable to detect any Far-binding complexes. The nuclear extract used for these experiments was obtained from adult rat islets cultured after isolation for 1 to 2 h in high- or low-glucose medium. The timing of changes in the nuclear complexes in response to glucose is an important issue, since rapid changes imply the modification of proteins that are already present, rather than the production of new proteins. However, since islets can be generally insensitive to glucose for the first hour after the stress of isolation, Melloul et al. may be observing a different phenomenon. For this reason, we placed all islet cultures in equal glucose concentrations for at least 8 h prior to testing glucose responsiveness. Because CAT activities were measured after 36 to 48 h at the same glucose concentration in both studies, we believe that the changes in binding complexes that we observed at 24 h are more likely to explain the observed levels of CAT expression. As discussed above, however, both the Far element and the FLAT element and their cognate binding complexes may contribute to the net response to glucose.

The β cell is not the only cell that must sense and respond to glucose. As part of the liver's role in regulating glucose fluxes, some hepatocyte genes are also regulated by glucose. In some cases, this regulation is indirect via insulin, as in the case of the phosphoenolpyruvate carboxykinase promoter (29). In contrast, transcription from the liver pyruvate kinase (LPK) promoter responds to glucose in the absence of changes in insulin concentration (6, 28). This effect has been mapped to a short piece of DNA with remarkable functional similarity to the Far-FLAT minienhancer. A similar sequence encodes a glucose response in the liver S14 gene as well (40). Like the FF minienhancer, the LPK minienhancer consists of two synergistic elements. One of these elements, termed the MLTF-like element, encodes the glucose response and may be regulated by an HLH protein (28). The MLTF-like element also contains two sequences with 5 of 10 nucleotides identical to those in the Far element. Since the LPK promoter is also active in β cells,

it will be interesting to learn if a Pan-containing complex binds and regulates this site in both cell types.

In summary, the insulin promoter contains several *cis*-acting sequence elements that respond to changes in glucose concentration. One of these metabolic response elements is a cell-specific HLH protein-binding site, the Far element. The combination of cotransfection data and binding data suggests that the Pan-containing complex that binds the Far element is regulated by glucose. From investigation of glucose regulation of the Pan-containing complex, we may derive a general model for glucose regulation of gene expression.

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