Regulation of Insulin Gene Expression by Glucose and Calcium in Transfected Primary Islet Cultures*

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To study the regulation of insulin gene expression by physiological regulators, primary cultures of rat islet cells were transfected with portions of the rat insulin I gene 5′-flanking sequence linked to the reporter gene chloramphenicol acetyltransferase (CAT). Incubation of the cells in increasing glucose concentrations led to a parallel increase in both CAT activity and CAT mRNA levels. Pretreatment of the cells with β-cell-specific toxin streptozotocin reduced CAT activity 97%. β-Cell-specific expression of CAT was also demonstrated by co-staining the transfected cells with antisera to both CAT and insulin. Experiments showing a reduction in the response to glucose in the presence of the calcium channel blocker verapamil suggest that calcium plays a role in the glucose response, possibly via regulation of factors interacting with this limited portion of the insulin gene.

To free themselves from the constraints of continuous food consumption, higher organisms have developed a system for energy storage. In vertebrates, the key regulator of this storage system is insulin. The production and secretion of insulin rise during food consumption and fall during fasting. Increased insulin secretion by the β-cells in the pancreatic islets of Langerhans in response to glucose and other energy sources has been studied extensively, but the mechanism of the glucose-induced transcriptional response is poorly understood. Studies of islets have shown that preproinsulin mRNA levels rise 4–10-fold (varying with experimental design) in response to glucose stimulation (1,2). This rise in mRNA levels apparently results from both an increase in transcription rate and an increase in message half-life (3). These islet experiments are limited to the native, intact insulin genes and therefore cannot examine the role of isolated portions of the insulin gene.

Previous studies have shown that transcription of the insulin gene is dependent on cis-acting sequences in the insulin gene 5′ flank (4–6) and on the proteins which bind these sequences (7,8). We have employed chimeric genes (with the 5′-regulatory region of the insulin gene attached to a reporter gene) to determine whether the glucose effect is mediated through the cis-acting regulatory elements of the enhancer. Unfortunately, the insulinoma tumor cell lines usually used for these studies have little or no transcriptional response to glucose in the physiologic range and therefore cannot be used to study glucose regulated gene expression (9,10). Furthermore, these cells exhibit a very low basal level of insulin gene transcription relative to primary β-cells (9), possibly reflecting some degree of abnormality in the regulation of basal insulin gene expression (independent of the glucose response). Thus we developed a technique for transfecting primary cultured rat islets.

EXPERIMENTAL PROCEDURES

Iset Isolation, Transfection, and Culture—21-day gestation fetal Sprague-Dawley rat islets were isolated and dispersed as described previously (11,12). After growth for 6 h in RPMI 1640 media with 10% fetal calf serum, cells were harvested by pelleting the plates, pelleted, and washed twice in room temperature phosphate-buffered saline, resuspended in 1 ml of phosphate-buffered saline with 20-30 μg of double cesium-purified plasmid DNA and electroporated with a discharge of 175 volts and 2000 microfarads across a 4-mm gap. After 10 min at room temperature, the cells were resuspended in fresh media with the glucose concentrations and additions indicated and incubated for 48 h prior to harvest. The cells were harvested by vigorously rinsing the plates. For the streptozotocin response curve, the cells were harvested by rinsing the plates 3 h prior to transfection, resuspended in 1 ml of fresh media with the streptozotocin concentration shown and incubated for 30 min before washing, resuspending, and incubating in fresh media for another 3 h.

For expression assays, protein was extracted (13) and 5 μg was used for each 2-h CAT assay (13) and 10 μg for each luciferase assay (14). All data points represent the mean of at least three independent transfections ± S.D.

RNA Measurement—After transfection with 25 μg of Ins-CAT plasmid DNA/plate, growth in 1 or 16 mM glucose, and harvesting as in the prior section, total cellular RNA was isolated (15) and treated with ribonuclease-free deoxyribonuclease prior to the final precipitation. Three oligonucleotides complimentary to the rIns-I (364-388 base pairs from the 5' end of the mRNA), CAT (238-255), and rat α-tubulin (553-570) mRNAs were used to make cDNA from 1 pg of RNA, all in the same tube (16). 20% of the cDNA was used as a template for polymerase chain reaction as in Ref. 16, except that the dCDNA was mixed in a 50-μl volume with 50 μCi of [γ-32P]ATP, 1 unit of Thermus aquaticus DNA polymerase, 50 μM dNTP triphosphates, 60 mM KCl, 25 mM Tris-CI, pH 8.3, 4 mM MgCl2, 0.01% bovine serum albumin and 25 pmol each of the six oligonucleotides (one complimentary to each strand of each cDNA: rIns-I, 333-352 and 46-65; CAT, 226-243 and 49-66; rat α-tubulin, 535-552 and 415-432). Annealing was performed at 65°C for 30 s, polymerization at 72°C for 1 min, and denaturing at 94°C for 1 min.

Immunofluorescence Staining—The cells were transfected to multiwell plates containing poly-D-lysine coated coverslips 24 h after transfection with 25 μg of Ins-CAT and stained on these coverslips for another 24 h before immunofluorescence staining as performed in Ref. 17.

RESULTS AND DISCUSSION

We chose to transfect fetal rat islets because of their abundance in the fetal pancreas, the high percentage of β-cells within the islets, and the low prenatal expression of digestive enzymes by the surrounding acinar cells. Transcriptional
Regulation of Insulin Gene Expression by Glucose

Response to glucose was tested by growing the transfected cells in media with the corresponding glucose concentrations for 48 h prior to harvest (Fig. 1). We co-transfected the islet cell cultures with two DNA plasmids: Ins-CAT (4), containing the −410 to +1 enhancer/promoter region from the rat insulin I (rIns-I) gene linked to the coding sequence for chloramphenicol acetyltransferase (CAT); and RSV-Luc (14), containing the nontissue-specific RSV enhancer/promoter linked to the coding sequence for firefly luciferase (Luc). Between 1 and 16 mM glucose, CAT enzyme activity rose 10-fold, with a half-maximal response at 6 mM glucose. There was no significant change in the control Luc enzyme activity over this range.

In order to test whether the glucose-stimulated rise in CAT activity resulted from increased transcription, we quantified the CAT mRNA levels. Because of the extremely low levels of CAT mRNA in the electroporated fetal islet cultures, primer extension and ribonuclease protection assays failed to

![Graph showing CAT enzyme activity vs. mM Glucose](image1)

**Fig. 1.** Effects of glucose concentration on CAT expression. Each plate of cells was transfected with double cesium chloride-purified Ins-CAT plasmid DNA (20 μg) and RSV-Luc (10 μg). CAT enzyme activity is represented by closed circles, luciferase enzyme activity by open circles. All data points represent the mean of at least three independent transfections ± S.D.

![Graph showing CAT enzyme activity vs. mM Streptozotocin](image2)

**Fig. 2.** Analysis by polymerase chain reaction of CAT mRNA in transfected islets. 4-μl aliquots were removed every 5 cycles as shown (lanes 1–10). Lane 11, no cDNA added. Lane 12, 0.2 μg of RNA from cells grown at 16 mM glucose added in place of cDNA. Lane 13, cDNA from cells transfected with plasmid pUC19 in place of Ins-CAT (with no α-tubulin oligonucleotides added). Lane 14, 1 ng each of Ins-CAT and a rIns-I genomic clone added in place of cDNA (only 5% of this product used). This process was repeated twice with independently synthesized cDNA each time, and similar results were obtained.

![Immunofluorescence staining of transfected islet cells](image3)

**Fig. 3.** Effects of streptozotocin treatment on CAT expression. Each plate of cells was transfected with double cesium chloride-purified Ins-CAT plasmid DNA (20 μg) and RSV-Luc (10 μg). CAT enzyme activity is represented by closed circles, luciferase enzyme activity by open circles. All data points represent the mean of at least three independent transfections ± S.D.

![Immunofluorescence micrographs](image4)

**Fig. 4.** Immunofluorescence staining of transfected islet cells. Immunofluorescence micrographs A and B show the same group of cells double-labeled with guinea pig anti-pig insulin (A) and rabbit anti-CAT (B) primary antisera and fluorescein-conjugated goat anti-guinea pig (A) and rhodamine-conjugated goat anti-rabbit (B) secondary antisera. Two of the insulin-positive cells in A are CAT-positive in B. In C the cells on two coverslips are categorized. In D the same procedure was followed with guinea pig anti-pig glucagon antisera (in place of anti-insulin antisera) on two separate coverslips.
detect significant CAT mRNA levels. Therefore, the polymerase chain reaction technique was used to estimate the relative levels of CAT mRNA, rIns-I mRNA, and rat a-tubulin mRNA in islets transfected with Ins-CAT and grown in either 1 or 16 mM glucose for 48 h (Fig. 2). These studies demonstrated a rise in CAT message levels paralleling the rise in CAT enzyme activity.

To minimize the number of contaminating fibroblasts, the free or loosely adherent islet cells were washed from the plates at each step, leaving most of the fibroblasts attached to the discarded plate. Despite this selection, and the rapid loss of acinar cells in culture (11), there was a mixed population of cells in the cultures at the time of harvest. Therefore, to test whether expression of the CAT gene was restricted to the insulin-secreting beta-cells, the beta cell-specific toxin streptozotocin was used to selectively eliminate beta-cells from the population (18) (Fig. 3). With increasing streptozotocin concentration the treated cells lost up to 97% of CAT activity while Luc activity (from the co-transfected RSV-Luc plasmid) dropped below 50% only at 10 mM streptozotocin.

In a second test of cell specificity, the cultured cells were stained with antisera raised to insulin, glucagon, and CAT (Fig. 4). Insulin-positive cells made up 30% of the total population and 63% of the nonfibroblast cells; glucagon-positive cells made up 2.6% of total cells. 11% of insulin-positive cells also stained for the CAT enzyme, while no insulin-negative and no glucagon-positive cells had significant CAT staining. This result suggests that the Ins-CAT construct is expressed largely by streptozotocin-sensitive, insulin-producing beta-cells while the noninsulin-specific RSV-Luc construct is expressed by other streptozotocin-resistant cell types as well.

Transfection with several different plasmids containing limited portions of the rIns-I 5' flank delineated a short segment capable of responding to glucose (Fig. 5). The segment from -85 to +1 (which directs a cell-specific response2) linked to CAT (-85 Ins-CAT) gave only a minimal response to glucose. The sequence from -196 to -247 (which is able to function as a cell-specific enhancer3) was able to impart glucose responsiveness to the unresponsive TK promoter linked to CAT. This sequence does not contain the putative cAMP response element at -184 (19).

The transfected cells were treated with several pharmacologic agents affecting the calcium and cAMP signaling pathways (Table I). Of the agents tested, the calcium channel blocker verapamil had the most significant effect on the response to glucose. Since the effects of adenylyl cyclase inhibition by 2',5'-dideoxyadenosine (20) may actually result from effects on the calcium channel (21), it appears that calcium plays a crucial role in the transcriptional response to glucose.

In these experiments, we have demonstrated a novel form of gene regulation by a metabolic signal. The experimental results suggest that the metabolic or energetic signal from glucose flux is transmitted through the insulin enhancer. The mechanism probably involves trans-acting protein factors which interact with the enhancer element. Activation could result from direct modification of protein factors (phosphorylation, etc.) or perhaps by changes in local, active factor concentrations via a calcium signal. The ability to study the insulin gene 5'-flanking region in glucose responsive primary beta cells will allow the further examination of this process in its natural physiologic setting.

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TABLE I
Effects of physiologic regulators on CAT expression

| Treatment                     | Response to glucose (fold) |
|-------------------------------|---------------------------|
| Control                       | 1.00                      |
| Dibutyryl cAMP (1 mM)         | 2.18 ± 0.83               |
| 2',5'-Dideoxyadenosine (1 mM) | 1.08 ± 0.62               |
| Verapamil (100 μM)            | 0.84 ± 0.21               |
| Verapamil (100 μM) + 2',5'-dideoxyadenosine (1 mM) | 0.086 ± 0.037 |
| Somatostatin (1 μM)           | 0.62 ± 0.12               |

* M. German, L. Moss, and W. J. Rutter, unpublished data.
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Fig. 5. Responses of limited portions of the rIns-I promoter to glucose. Transfections were performed with 25 μg of each of the following plasmids: -85Ins-CAT containing the rIns-I sequences from -85 to +1 linked to CAT (4); TK-CAT containing the herpes thymidine kinase promoter sequence from -109 to +51 linked to CAT; Ins-TK-CAT containing the rIns-I sequence -247 to -196 inserted 5' of TK linked to CAT; 5Ins-TK-CAT containing 5 copies of the -247 to -196 sequence in native orientation inserted 5' of TK linked to CAT. White bars, CAT enzyme activity of transfected cells grown in 2 mM glucose; gray bars, 16 mM glucose. All data points represent the mean of at least three independent transfections ± S.D.

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Regulation of Insulin Gene Expression by Glucose

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