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Stimulation of pancreatic islet beta-cell replication by oncogenes

(*src*/*myc*/*ras*/electroporation)

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ABSTRACT **Although the growth potential of the pancreatic islet beta cells is limited, glucose, cAMP, and certain polypeptide growth factors have been reported by other workers to exert modest stimulatory effects on beta-cell replication. To further assess means through which beta-cell growth can be stimulated, selected oncogene constructs linked to a rat insulin promoter were introduced by means of electroporation into free islet cells prepared from fetal rats and adult hyperglycemic obese (ob/ob) mice. The uptake and expression of the added oncogenes were sufficiently efficient to exert effects on beta-cell physiology in short-term experiments (≤4 days). Stimulation of islet cell [^{3}H]-thymidine incorporation was observed after transfection with *src* alone or the combination of *myc* and *ras*. The effect observed in the fetal islet cells with *src* was more pronounced than any effect previously reported. Transfection with the *src* oncogene resulted in phosphorylation of lipocortin I and was paralleled by an increased immunofluorescence against src-like immunoreactivity in a majority of the electroporated cells. It is concluded that electroporation can induce sufficiently efficient expression of added oncogene constructs to study their effects on cells that are not readily transformable into continuously growing cell lines. Furthermore, the results suggest that beta-cell replication might be manipulated extrinsically by inserting appropriate growth-promoting genes into these cells.**

Of crucial importance in the development of diabetes, especially type I, is the insufficient extent of islet beta-cell replication occurring after the onset of the disease. Current understanding of the problem suggests that the beta cell has a limited potential for replication, and <15% of fetal beta cells enter a mitotic cycle over a 24-hr period, a figure that rapidly diminishes with the maturation of the beta-cell (for review, see ref. 1). A short-lived, modest stimulation (<150%) of beta-cell mitosis occurs in response to glucose, cAMP, and certain growth factors (1). Furthermore, transgenic mice containing the simian virus 40 T antigen driven by the insulin promoter (2) developed insulin-producing tumors. The viral oncogenes or their normally occurring cellular homologues, the protooncogenes, are recently identified genes that serve as stimulators or regulators of growth in many tissues (3). It thus became relevant to determine whether any of the known oncogenes affect islet cell mitosis in order to understand the processes involved in regulation of beta-cell replication. Three distinct classes of oncogenes have been identified. The products of one group of oncogenes (such as *src*, etc.) are tyrosine kinases (4), a property they share with many growth factor receptors (3), which further emphasizes the relationship of oncogenes to cell growth. On the other hand, the *ras* family of oncogenes encodes GTP-binding proteins (5), which may control the activity of phospholipase C (6), an enzyme intimately involved in many intracellular processes including the proliferation response (7). Finally certain oncogenes, such as *fos* and *myc*, code for nuclear proteins that are acutely expressed when cells enter the mitotic cell cycle (3, 4). Since most of the knowledge on the mode of action of oncogenes or protooncogenes in the regulation of cell growth has been gained by studying the effects of these genes in rapidly proliferating cells, it is not obvious what role these genes may serve in the slowly proliferating beta cell.

We decided to investigate the role of the v-*src*, c-*myc*, and the mutated c-Ha-*ras* oncogenes in islet replication; each gene represents one of the three major groups of oncogenes outlined above. Preliminary studies with the protooncogenes c-*myc* and c-Ha-*ras* showed that the levels of expression of these were very low or undetectable, even in fetal islets stimulated with glucose. Thus, to induce efficient expression, we linked each oncogene to the rat insulin II promoter (RI-2), a beta-cell specific promoter (2, 8, 9). The oncogene constructs were introduced into the islet cells by means of electroporation and subsequently were efficiently expressed. Since we were primarily interested in studying events involved in the initiation of beta-cell proliferation, a transient assay was used for this. The results demonstrate that a slowly or nonproliferating cell in tissue culture can be induced to stimulated growth with this experimental approach.

MATERIALS AND METHODS

Materials. [*methyl*-^{3}H]Thymidine (50 Ci/mmol; 1 Ci = 37 GBq), ^{32}P, and Amplify were from Amersham (Bucks, U.K.). Poly(L-lysine) (M_r 260,000) and RNase A were from Sigma. Restriction endonucleases, T4 DNA ligase, and polymerase were from New England Biolabs and Pharmacia (Uppsala). Soluene was purchased from United Technologies Packard (Downers Grove, IL).

Construction of Oncogene Plasmids. The avian sarcoma virus v-*src* obtained from plasmid pMS484c (10) was linked to the RI-2 sequences [560-base-pair (bp) *Eco*RI–*Mbo* II fragment] with the RI-2 "TATA" box adjacent to the v-*src* translation initiation codon in pSPRIsrc (Fig. 1*A*). The sequences between the RI-2 TATA box and the *src* initiation codon contain the RI-2 transcription start site, an RI-2 splice donor site, and two possible v-*src* splice acceptor sites. Similarly, the human c-*myc* oncogene obtained from a non-Hodgkins lymphoma, pSV2.26 (11), was linked to the RI-2 in pSV2.26RI. Transcription of c-*myc* in pSV2.26RI was anticipated to be driven by the RI-2 (Fig. 1*B*) with the RI-2 first intron sequences ligated directly to the first intron of the c-*myc* gene, thus deleting the first exon of c-*myc*. The first exon of c-*myc* does not contain any protein coding sequences, but it has been implicated in the posttranscriptional regulation of c-*myc* expression (13, 14). Finally, RI-2 was

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Abbreviation: RI-2, rat insulin II promoter.

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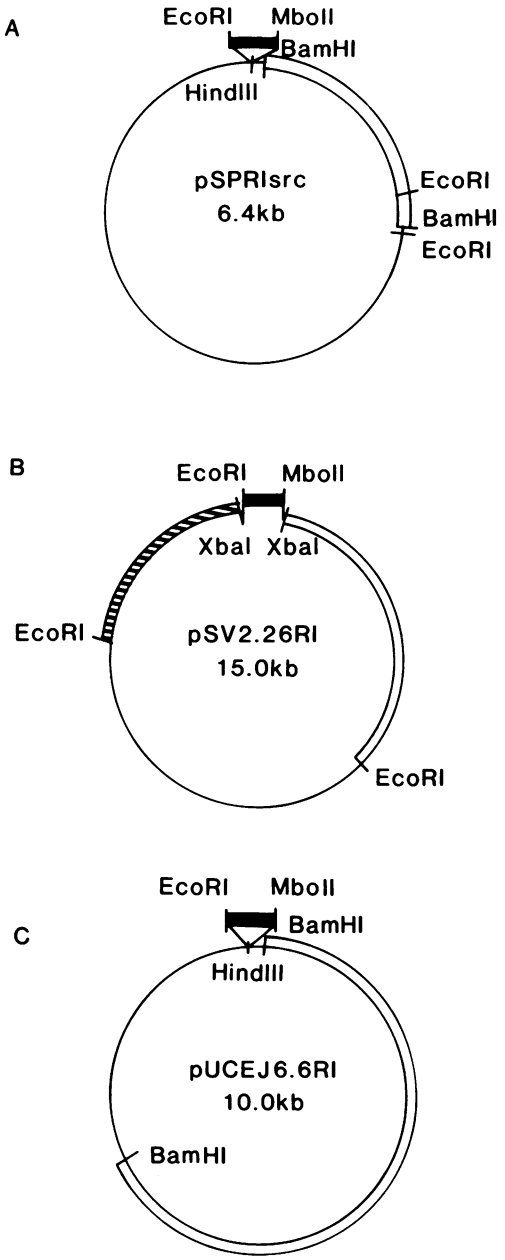

FIG. 1. Construction of plasmids pSPRIsrc (*A*), pSV2.26RI (*B*), and pUCEJ6.6RI (*C*). (*A*) The 2.8-kb *Bam*HI fragment from pMS484c (10) was ligated with T4 DNA ligase into the *Bam*HI site in the polylinker of pSP64, such that the *Eco*RI site at the 3′ end of the v-*src* sequences was 0.6 kb from the *Eco*RI site in pSP64. The 560-bp *Eco*RI–*Mbo* II fragment of RI-2 [containing all promoter sequences necessary for tissue-specific insulin expression (2, 8) and parts of the first intron] was inserted into the *Hin*dIII site of pSP64 after blunt-end formation with T4 DNA polymerase and ligation with T4 DNA ligase. The RI-2 TATA box was adjacent to the *Bam*HI site of pSP64. The open box denotes v-*src* sequences, and the closed box indicates RI-2 promoter sequences. (*B*) The 560-bp RI-2 fragment (see above) was blunt-ended with T4 DNA polymerase together with an *Xba* I digest of pSV2.26 (11) and was ligated with T4 DNA ligase. The *Mbo* II side of RI-2 was ligated to the c-*myc* sequences of the first intron of the c-*myc* gene. The open box shows the 4.6-kb c-*myc* sequences, the hatched box denotes 4.4 kb of the immunoglobulin genome, and the closed box indicates the 560-bp RI-2. (*C*) The 560-bp RI-2 fragment (see closed box in *A*) was ligated into the *Hin*dIII site of pUCEJ6.6 with T4 DNA ligase after formation of blunt ends with T4 DNA polymerase. The open box depicts the 6.6-kb genomic *ras* sequences.

inserted into the plasmid pUCEJ6.6 (12) to form pUCEJ-6.6RI, which makes it possible for the enhancer-like properties of the insulin promoter (8) to drive expression of this mutated c-Ha-*ras* oncogene (Fig. 1*C*). Plasmids were prepared by alkaline lysis (15), with subsequent ammonium acetate precipitation, RNase A treatment (3–12 hr, 50 μg/ml), and phenol extraction followed by extensive dialysis.

Electroporation of Islet Cells. Islet cells were prepared from fetuses of 21-day pregnant rats as described (16); they were collected after 5 days of culture and were dispersed by trypsin/EDTA treatment. Adult obese mouse islets were isolated by collagenase digestion and were dispersed essentially as described (17). The cells (10^6) were placed in a chamber in 300 μl of 120 mM KCl/10 mM NaCl/3 mM glucose/10 mM Hepes, pH 7.0 containing plasmid DNA (50 μg/ml) and were electroporated with a single pulse of 2.5 kV/cm (18). The electroporation was carried out on ice, and the cells remained on ice for 15–20 min, after which they were placed in polylysine-coated multiwell dishes, to which culture medium (RPMI 1640/10% bovine serum) containing either 3 or 17 mM glucose were added. The cells were cultured in 5% CO_2 at 37°C for 3 days, after which the media were changed to media containing [*methyl*-^{3}H]thymidine (0.5 μCi/ml) and the same glucose concentrations. After 24 hr, the media were removed, and the dishes were washed with phosphate-buffered saline (PBS; 154 mM Na^+/4 mM K^+/140 mM Cl^-/10 mM HPO_4^{2-}, pH 7.4). The cells were sonicated for 10 sec in 400 μl of H_2O, and aliquots were taken for DNA determinations (19). The rest of the sonicated sample was precipitated with 10% trichloroacetic acid. The incorporated [^{3}H]thymidine was precipitated with trichloroacetic acid, dissolved in Soluene, and counted by liquid scintillation.

RESULTS

Electroporation has previously been used for efficient transfection and stable transformation of lymphocytes (20, 21) and has also been adopted for islet cell suspensions. After subjecting cells to a brief high-voltage discharge, their plasma membrane is rendered permeable (22). By performing the permeabilization in the presence of extracellular DNA, it is possible for the cells to incorporate and subsequently express that DNA. After a short period (<30 min after the high-voltage discharge), the cells reseal as assessed by trypan blue exclusion (>80% of the cells exclude trypan blue). By comparing the DNA content of aliquots taken directly after electroporation with the recovery of cellular DNA at the end of the experimental period of 4 days, it was found that 17 ± 3% (mean ± SEM) of the cellular DNA remained, suggesting that this is a representative value for the long-term survival in our system. No differences in the DNA contents of the different experimental groups were observed. Finally, >90% of the cells remaining attached at the end of the experimental period were viable as assessed by trypan blue exclusion.

The effects of the presence of pSPRIsrc, pSV2.26RI plus pUCEJ6.6RI, pSV2.26RI, or pUCEJ6.6RI during electroporation on [^{3}H]thymidine incorporation into cells 4 days later [expressed as a percentage of controls (pRI-7, ref. 23)] are shown in Table 1. Optimal effects on [^{3}H]thymidine incorporation were observed during the fourth day after electroporation in preliminary experiments. The experiments were performed in 10% serum, in order to preserve the functional integrity of the islet cells. This high serum concentration has modest stimulatory effects on beta-cell replication in comparison with low serum (1). pSPRIsrc (v-*src*) stimulated [^{3}H]thymidine incorporation during the last 24 hr of the experiment in the presence of 17 mM glucose as compared to the controls in both fetal and adult islet cells. No consistent effects of pSPRIsrc were observed when only 3 mM glucose was present in the medium throughout the experiment. Similarly, the combination of pSV2.26RI plus pUCEJ6.6RI (c-*myc* + c-Ha-*ras*) stimulated [^{3}H]thymidine incorporation into islet cells in a manner similar to that observed with

Table 1. Effects of the presence of pSPRIsrc (v-*src*), pSV2.26RI (c-*myc*) plus pUCEJ6.6RI (c-Ha-*ras*), pSV2.26RI (c-*myc*), or pUCEJ6.6RI (c-Ha-*ras*) during electroporation on the subsequent rates of [^{3}H]thymidine incorporation in fetal rat and adult (ob/ob) islet cells

	[^{3}H]Thymidine incorporation, % of control							
	pSPRIsrc		pSV2.26RI + pUCEJ6.6RI		pSV2.26RI		pUCEJ6.6RI	
Islet cells	3 mM glucose	17 mM glucose	3 mM glucose	17 mM glucose	3 mM glucose	17 mM glucose	3 mM glucose	17 mM glucose
Fetal rat	277 ± 105	414 ± 92*	145 ± 27	341 ± 115	640 ± 322	625 ± 289	87 ± 17	109 ± 36
Adult (ob/ob) mouse	127 ± 27	174 ± 21*	127 ± 33	181 ± 13†	75 ± 13	112 ± 20	137 ± 22	95 ± 16

Islet cells were electroporated in the presence of the given plasmid(s) in medium containing 3 or 17 mM glucose. The [^{3}H]thymidine incorporation was measured 4 days later. The results are expressed as a percentage of the [^{3}H]thymidine incorporation in control islet cells electroporated under identical conditions in parallel experiments with pRI-7 (means ± SEM for 4–6 experiments). The rates of [^{3}H]thymidine incorporation in the controls were 1460 ± 420 and 1620 ± 862 cpm per μg of DNA at 3 and 17 mM glucose, respectively, for the fetal islet cells, and 205 ± 25 and 244 ± 67 cpm per μg of DNA for the ob/ob islet cells in the corresponding groups.

* and † denote $P < 0.05$ and $P < 0.01$, respectively, as determined by a paired Student's *t* test.

pSPRIsrc in the presence of 17 mM glucose. However, due to variability in the results, the effects on the fetal islet cells failed to reach statistical significance (Table 1). Again, no effects were observed in the presence of 3 mM glucose. The c-*myc* oncogene alone (pSV2.26RI) exerted no effects on adult islet cell [^{3}H]thymidine incorporation, and the response in the fetal islets was highly variable with a marked inhibition occurring in three experiments and a strong stimulation in the other three. Also, pUCEJ6.6RI (c-Ha-*ras*) alone did not affect islet [^{3}H]thymidine incorporation.

To assess the extent of the uptake and expression of these oncogene constructs, immunofluorescence of src-like proteins [i.e., proteins detected with monoclonal antibody 273 (33), which is directed against E. coli-produced v-*src*-protein] was measured after electroporation of adult islet cells with pRI-7 (control) or pSPRIsrc (*src*) (Fig. 2). The control cells (Fig. 2 *Upper*) showed weak immunofluorescence, which appeared to arise from fine particulate structures localized mainly in the perinuclear regions. The immunofluorescence was of comparable intensity in the different cells. Fibroblast-like cells also exhibited low src-like immunofluorescence when transfected only with control plasmids (results not shown) or with pSPRIsrc (Fig. 2 *Lower*). Thus islet cells appear to have a basal level of expression of src-like proteins. In agreement with this, normal islets express an RNA of 3.5 kb, which hybridizes to the v-*src* probe when RNA blot-hybridization analysis was performed (results not shown). Islet cells electroporated in the presence of pSPRIsrc (*src*) demonstrated considerably higher levels of immunofluorescence against src-like proteins (Fig. 2 *Lower*). The pattern of immunofluorescence also appeared to be altered; it became much coarser and more uniformly distributed in the cytoplasm, rather than in the perinuclear region (Fig. 2 *Lower*). The majority of the islet cells showed increased src-like immunofluorescence and the levels of fluorescence was uniform throughout the islet.

To directly demonstrate increased tyrosine kinase activity as a result of pSPRIsrc transfection in the islet cells, phosphorylation of lipocortin I, a known substrate for tyrosine kinases (24), was monitored 3 days after electroporation. A M_r 34,000 protein immunoprecipitable with an anti-lipocortin I antibody was phosphorylated only in the *src*-transfected (pSPRIsrc) islet cells (Fig. 3). This direct demonstration of tyrosine kinase activity confirms that electroporation with pSPRIsrc is effective in a high proportion of the islet cells.

DISCUSSION

Our preparations of fetal rat islets (16) and adult obese mouse islets (26) contain a high percentage of beta cells (>90%), and in all instances studied thus far, islet [^{3}H]thymidine incorporation over a 24-hr labeling period in response to various agents has been shown to represent rates of beta-cell mitosis

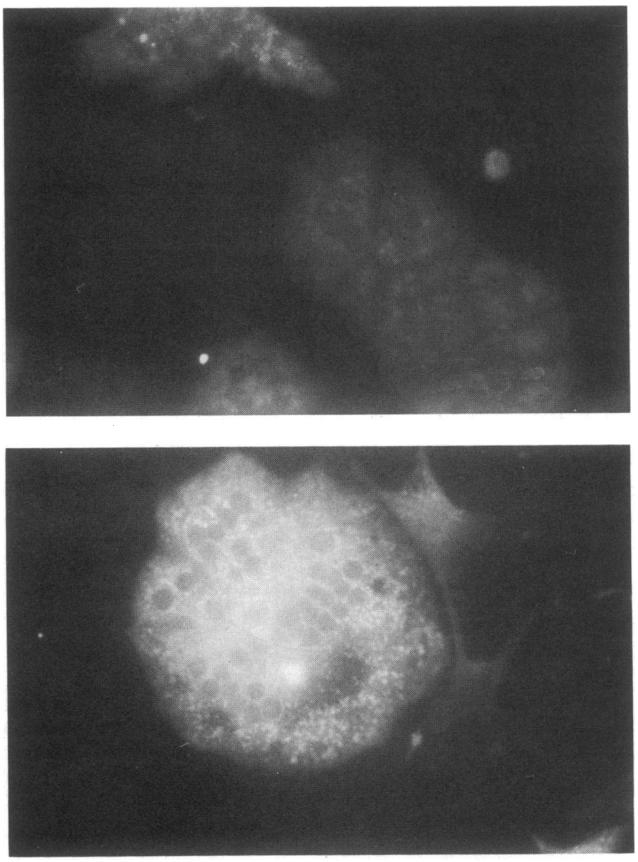

FIG. 2. *src* immunoreactivity in islet cells electroporated in the presence of pRI-7 (*Upper*) or pSPRIsrc (*Lower*). Adult obese mouse islet cells were electroporated in the presence of pRI-7 (control) or pSPRIsrc (v-*src*) as in Table 1. The cells were placed on polylysine coated cover slips and cultured for 3 days in RPMI 1640/10% fetal bovine serum. The cells were then washed in PBS and fixed for 20 min in 3% paraformaldehyde dissolved in PBS. This and all the following steps were performed at 20°C. After two washes in PBS, the cells were permeabilized for 5 min in PBS/0.2% Triton X-100. Following two more washes in PBS, the coverslips were incubated for 30 min with ascites fluid diluted 1:1000 in PBS/0.2% Triton X-100 containing the monoclonal antibody 273 (33) directed against *Escherichia coli* produced v-*src*-protein. The coverslips containing the fixed cells were subsequently washed three times in PBS before a 30-min incubation with fluorescein isothiocyanate-conjugated antibodies against mouse IgG. Finally the coverslips were washed three times in PBS and once in H_2O before being mounted and examined for immunofluorescence.

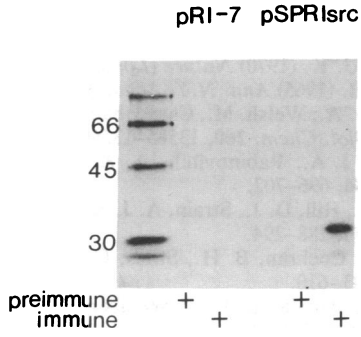

FIG. 3. Effects of pSPRIsrc on the phosphorylation of lipocortin I in islet cells. Adult obese mouse islet cells (5×10^6) were electroporated with either pSPRIsrc (v-*src*) or pRI-7 (control) as described in Table 1. After 3 days, the electroporated cells were labeled with ^{32}P as orthophosphate (1 mCi/ml) in 0.1 mM sodium phosphate in Dulbecco's modified Eagle's medium for 3 hr in the presence of 17 mM glucose at 37°C. After washing the cells in PBS, the cells were lysed and immunoprecipitated using preimmune serum or immune serum against anti-lipocortin I exactly as described (24). The immunoprecipitates were analyzed on 10% $NaDodSO_4$/polyacrylamide gels (acrylamide/bis, 30:0.8) according to Laemmli (25) and were visualized by autoradiography. Molecular size standards ($M_r \times 10^{-3}$) are at left.

as determined by a labeling index (1). A 24-hr labeling period was necessary due to low islet cell [^{3}H]thymidine incorporation rates. These facts, in conjunction with the known tissue specificity of expression of the insulin promoter (2, 8, 9), lead us to conclude that the v-*src* and the c-*myc* plus mutated c-Ha-*ras* oncogenes primarily stimulate beta-cell mitosis. The glucose responsiveness may either reflect glucose-induced oncogene transcription from the insulin promoter (27) or nonspecific anabolic effects of glucose that render the cells more responsive to a mitotic stimulus. We were not able to observe a significant stimulatory effect of glucose on beta-cell replication in the control groups, but this may be due to the use of single-cell cultures rather than intact islets in these experiments.

Our results suggest that a relatively strong mitogenic response of beta cells occurs when islet cells are transfected with an oncogene coding for a protein with tyrosine kinase activity. The mitogenic response of adult beta cells was less pronounced than that of fetal beta cells, which conforms with the view that older beta cells lose their ability to respond to growth stimuli (1). It is also of interest that two growth factors that stimulate beta-cell replication, platelet-derived growth factor (32) and insulin-like growth factor I (28, 29), both activate receptors that function as tyrosine kinases (3). The 4-fold stimulation of [^{3}H]thymidine incorporation into fetal islet cells transfected with *src* is more pronounced than the previously observed effects of the above growth factors on beta-cell replication (1), suggesting that the v-*src* gene may somehow bypass the normal growth factor receptor-mediated pathway of stimulation of beta-cell mitosis. Although c-*myc* appeared to produce strong effects on the growth of fetal beta cells in some experiments, positive results were not consistently obtained (Table 1), in contrast to the consistent positive effects observed in other cell systems (30). Another finding that supports the view that c-*myc* may be an insignificant or inconsistent growth modulator in islets is that the addition of platelet-derived growth factor plus insulin-like growth factor I does not increase the levels of islet c-*myc* expression shortly after addition (M.W., unpublished results). On the other hand, electroporation of *myc* along with *ras* reproducibly stimulated beta-cell mitosis, suggesting that some combinatorial interactions of these two oncogenes may be necessary to consistently induce beta-cell replication. Cooperativity between these two oncogenes has also been noted with respect to tumorigenic transformation of rat embryo fibroblasts (31) and is consistent with the possibility that stimulated mitosis is an early response in the course of transformation.

We have successfully stimulated beta-cell replication in tissue culture by short-term transfection with certain insulin gene–oncogene constructs. To overcome the problem of the limited long-term replication potential of beta-cells, it will be necessary to produce stable transformants. Thus far, despite several attempts, we have not produced continuously growing beta cells by these means. Further studies with other oncogenes or protooncogenes having different or more tissue-specific effects on beta cells, singly or in combination, may aid in overcoming this important limitation. However, besides obtaining a stronger and longer lasting stimulation of beta-cell mitosis, it will be necessary to regulate beta-cell growth in response to the inserted gene(s) in further studies aimed at improving therapeutic efforts in the treatment of diabetes. Protooncogenes linked to inducible promoters (such as the metallothionein promoter) or the use of protooncogenes activated by ligand binding could be valuable in this context. The insulin promoter provides another example of an inducible promoter, since it responds positively to increased glucose (27) and could thus allow the expression of the (proto)oncogene linked to it to be directly related to the glucose concentration of the environment. Finally, our system allows direct studies of the effects of the oncogenes on beta-cell physiology and biochemistry. Since many intracellular processes such as ion fluxes, phospholipid metabolism, protein phosphorylation, and substrate metabolism have been well characterized in islets, electroporation with oncogenes or protooncogenes may reveal mitotic signals that may be specific for insulin-producing cells and also of general interest.

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