# Glucagon Gene Transcription Is Negatively Regulated by Insulin in a Hamster Islet Cell Line

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## Abstract

**Complex interrelationships exist between the four pancreatic islet cell types and their respective secretory products, insulin, glucagon, somatostatin, and pancreatic polypeptide. These hormones are known to interact with the different islet cells and modulate their functions. Insulin inhibits glucagon secretion from the A cell both in vivo and in vitro and, in states of insulin deficiency, high glucagon levels are observed that are normalized by insulin replacement. To determine if insulin also regulates glucagon biosynthesis, we studied its effects on glucagon gene expression. Our studies indicate that insulin, in a dose-dependent fashion decreases steady-state glucagon mRNA levels in a clonal hamster islet cell line, In-R1-G9; this decrease is secondary to an inhibition of glucagon gene transcription as assessed by transcriptional run-on assays and does not involve detectable changes in mRNA stability. Inhibition of glucagon gene transcription is accompanied by corresponding decreases in glucagon immunoreactivity in both cell extracts and medium. We conclude that insulin may not only regulate glucagon secretion but also glucagon gene expression.**

## Introduction

The fact that the four different pancreatic islet cell types A (glucagon), B (insulin), D (somatostatin), and PP (pancreatic polypeptide) are not randomly distributed within an islet, but, on the contrary, are highly organized has led to the hypothesis that such an architecture might serve a functional role and that contiguous cells might influence one another (1–3). In a number of pioneering studies, Samols successively showed that glucagon, the hormonal product of the A cell had stimulatory effects on insulin secretion (4) and conversely that insulin inhibited glucagon release (5). A larger concept of the interrelationships existing between the different islet cells emerged when somatostatin was shown to potently suppress insulin (6) and glucagon secretion (7) and glucagon to enhance somatostatin release (8). Although these interactions between the A, B, and D cells and their secretory products are now well accepted, it is not entirely clear whether they are mediated through paracrine mechanisms, direct sequential vascular perfusion or both. The relative importance of the vascular compartment has recently been illustrated by an increase in glucagon secretion resulting from experimental intravascular depletion of insulin (by anti-insulin antibodies infusion) (9). In addition, Samols, using rat pancreata perfused both anterogradely and retrogradely elegantly underlined the functional roles of the vascular compartment and of the direction of the blood flow in mediating the effects of insulin on glucagon secretion and of glucagon on somatostatin release (10).

A relevant clinical correlate of these experimental observations is the high glucagon levels that characterize untreated diabetes mellitus in humans and animals (11, 12). Hyperglucagonemia is indeed a hallmark of both type I and type II diabetes in man (13). Although fluctuations in blood glucose levels have been proposed to be the main determinant in the regulation of glucagon secretion (14), more recent data have shown that these effects are mostly secondary to the increased release of insulin, which appears to exert its effects independently of ambient glucose concentration (15). The tonic inhibition of glucagon secretion by insulin and the chronically elevated glucagon levels in states of insulin deficiency has led to the hypothesis that insulin might not only affect the release of glucagon but might also be involved in the control of glucagon biosynthesis (3). Insulin is known to affect the levels of certain cellular mRNAs through a direct interaction with its cell surface receptors (16, 17). To better understand insulin action on the A cell, we studied the effects of insulin on glucagon gene expression. Using a clonal hamster islet cell line, In-R1-G9, producing high levels of glucagon and no detectable insulin (18) we show here that insulin decreases steady-state glucagon mRNA levels in a dose-dependent fashion and that this decrease reflects a direct inhibition of glucagon gene transcription.

## Methods

*Cell culture.* In-R1-G9 cells were previously cloned from hamster insulinoma cells (In-111-R1) (18). These cells grow in monolayers in RPMI 1640 at a glucose concentration of 1,800 mg/liter, supplemented with 10% newborn calf serum (NCS), 100 U/ml penicillin and 100 μg/ml streptomycin. $3 \times 10^6$ cells were plated on 100 mm dishes for RNA extraction and experiments were run 3–4 d later. Cells were incubated with porcine insulin in RPMI 1640 supplemented with 0.5% BSA.

For all experimental conditions, cells were switched from RPMI 1640 medium containing 10% NCS to the RPMI 1640 with 0.5% BSA at time −1 h.

*Northern blot analysis.* Cells were lysed in guanidine thiocyanate and RNA was extracted through a cesium chloride gradient by centrifugation (19). 20 μg of total RNA, representing each experimental condition, was denatured in glyoxal, size-fractionated on a 1.4% agarose gel and electroblotted on a nylon membrane (Nytran; Schleicher and Schuell, Keene, NH). Blots were baked for 2 h at 80°C, prehybridized in 1 M NaCl, 1% SDS, 10% Dextran sulfate at 50°C for 2 h, hybridized with oligonucleotide cDNA probes ($5 \times 10^5$ cpm/ml) in the same solution for 24 h at 50°C and washed at 55°C in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate), 1% SDS; they were exposed to Kodak X AR films at −70°C with an intensifying screen.

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Oligonucleotides from 25 to 43 bases, complementary to glucagon and to β-actin RNAs (three oligonucleotides for each sequence) were synthesized by an automated oligonucleotide synthesizer (Applied Biosystems, Foster City, CA) (19); they were 5′ end-labeled with [$^{32}$P]γ-ATP by $T_4$ polynucleotide kinase to a specific activity of 2–4 × $10^8$ cpm/μg (19).

*Nuclear run-on experiments.* Gene transcription rates were determined by quantification of nascent glucagon and β-actin RNA transcripts in nuclei (the equivalent of 300–350 μg of DNA) from control and insulin-treated cells, as previously described (20). Briefly, cells were washed with ice-cold PBS and scraped into 2 ml of 0.25 M sucrose, 20 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1% N P-40, 1 mM dithiothreitol. The nuclei were isolated by disrupting the cells in a Dounce homogenizer and pelleted through a step gradient of 1 ml 0.3 M sucrose, 20 mM Tris-HCl (pH 7.9) layered over 1.5 ml of 0.5 M sucrose, 20 mM Tris-HCl (pH 7.9). Nuclear transcripts were labeled with [$^{32}$P]α-UTP and extracted as described (20). Labeled RNA (> 90% of the counts were precipitable by trichloroacetic acid) was hybridized to an excess of cloned specific DNA (5 μg) (rat glucagon cDNA [21] and as control, chicken β-actin cDNA [20]) bound to nitrocellulose using a slot-blot apparatus. Filters were prehybridized and hybridized for 48 h at 60°C (20).

*Glucagon mRNA stability.* Glucagon mRNA half-life was determined from an actinomycin D decay curve. Actinomycin D (5 μg/ml) used to prevent RNA synthesis was added to the medium 30 min before addition of insulin. Both control and insulin-treated cells were lysed after 4, 12, and 24 h, total RNA was extracted and analyzed by Northern blot as described above.

*Hormone biosynthesis and secretion.* Glucagon immunoreactivity was measured in both cell extracts and culture medium as previously described (22) using a rabbit antiserum to glucagon (Amersham Corp., Arlington Heights, IL). Insulin immunoreactivity was assessed in the culture medium using an insulin radioimmunoassay kit (Amersham Corp.).

## Results

*Phenotypic expression of In-R1-G9.* In-R1-G9, a glucagon-secreting clonal hamster islet cell line has recently been isolated (18) and characterized (18, 23). This cell line was chosen for the stable and high expression level of the glucagon gene and for the absence of insulin and somatostatin production, as assessed by both mRNA analysis and radioimmunoassay (23). However, when cells were passaged multiple times, measurable amounts of insulin immunoreactivity were occasionally detected in the culture medium (up to 0.5 ng/2 × $10^5$ cells/24 h); these cells were not considered for further experiments.

*Glucagon biosynthesis is inhibited by insulin.* To first determine if insulin could be affecting glucagon biosynthesis, we monitored glucagon-like immunoreactivity from control and insulin-treated In-R1-G9 cells in both medium and cell extracts. 5 × $10^5$ cells were incubated with $10^{-8}$ M insulin for 6, 12, and 24 h. There was a time-dependent decrease in glucagon immunoreactivity in both medium and cell extracts (Fig. 1); however, more pronounced decreases were noted at 6 h in the medium compared to cell extracts; insulin inhibition of glucagon secretion from In-R1-G9 cells, in addition to decreases in biosynthesis, is probably the explanation for this observation. At 24 h, the decrease in glucagon immunoreactivity reached 64% of control values in cell extracts and 69% in medium.

A progressive increase in glucagon-like immunoreactivity was observed between 6 h and 24 h in both control cell extracts and culture medium. Such an increase was expected in the medium since it probably did result from accumulated peptide

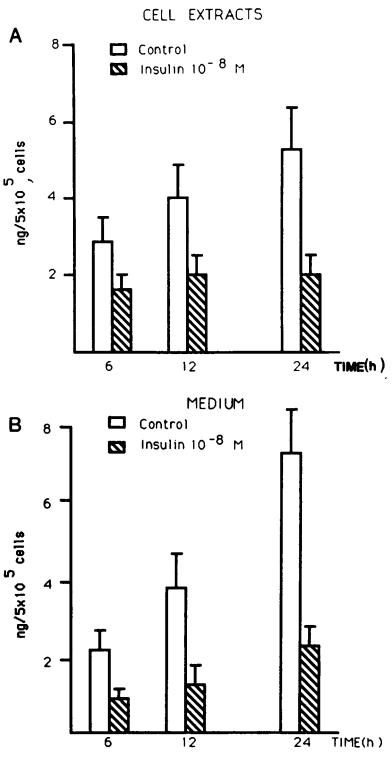


*Figure 1.* Effect of insulin on glucagon immunoreactivity. Culture medium was switched from RPMI 1640 with 10% NCS to RPMI 1640 with 0.5% BSA at time −1 h. Insulin ($10^{-8}$ M) was added to 5 × $10^5$ In-R1-G9 cells for 6, 12, and 24 h. Glucagon was measured by radioimmunoassay in both cell extracts (*A*) and medium (*B*). Assays were run in triplicate. Results represent the means±SEM of three separate experiments.

secreted over time. By contrast, the twofold increase measured in cell extracts could have been due to the removal of low concentrations of insulin present in NCS with a consequent disinhibition of glucagon biosynthesis.

*Insulin decreases steady-state glucagon mRNA levels.* To further investigate the mechanisms by which insulin affects glucagon biosynthesis, insulin action on glucagon gene expression was examined at the mRNA level. Insulin was added to In-R1-G9 cells at different concentrations in RPMI 1640 medium containing 0.5% BSA; these cells can be maintained in serum-free medium for at least 48 h without significant cell loss (data not shown). Total RNA was extracted from the cells after a 24-h incubation period with insulin, separated by electrophoresis on agarose gel and analyzed by Northern transfer. Insulin decreased glucagon mRNA levels in a dose-dependent manner (Fig. 2). At the lowest concentration, $10^{-11}$ M, glucagon mRNA levels were at 70% of the control values and further reductions down to 22% were seen as the concentrations of insulin were increased to $10^{-8}$ M. Between $10^{-8}$ and $10^{-6}$ M insulin no change in the abundance of glucagon mRNA levels could be detected. Others have also noted that some effects of insulin do not change or even decrease at markedly high hormone concentrations (24, 25).

The relative quantity of actin mRNA was also assessed to control for the amount of total RNA electrophoresed in each lane and for the specificity of the insulin effect; the level of actin mRNA was found to be comparable (within 20% for each experimental condition) (Fig. 1). The values of the intensity of the glucagon mRNA signal given by laser densitometry was thus corrected by the relative amount of actin mRNA in its respective lane.

To investigate the time-dependency of the insulin-mediated decrease in glucagon mRNA, we incubated In-R1-G9 cells in the presence of insulin ($10^{-8}$ M) for 6, 14, 24, and 48 h.

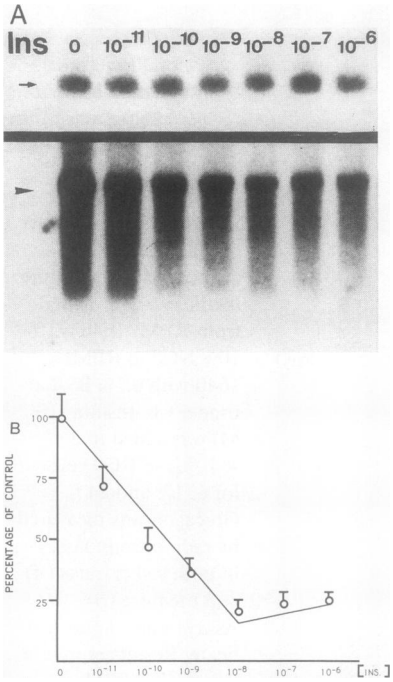


*Figure 2.* Effect of insulin on glucagon mRNA. ~ $10^7$ In-R1-G9 cells were incubated with 0, $10^{-11}$, $10^{-10}$, $10^{-9}$, $10^{-8}$, ~ $10^{-7}$, and $10^{-6}$ M insulin for 24 h in RPMI 1640 with 0.5% BSA (changed from RPMI with 10% NCS at time − 1 h). Total RNA was extracted and analyzed by Northern blot (*A*). Each lane contains 20 μg of total cellular RNA. Blots were hybridized with oligonucleotide cDNA probes complementary to glucagon mRNA and, after elimination of the glucagon-specific probes by boiling the filter membrane with cDNA probes complementary to β-actin mRNA. → points to β-actin mRNA and ➤ to glucagon mRNA. (*B*) Quantifications of each mRNA were done by densitometric scanning of the autoradiographs. Means±SEM of three experiments are shown. Values are derived from three different experiments and represent the percentage of the control value.

There was a time-dependent reduction in glucagon mRNA abundance; the maximal effect was observed at 24 h with a 76% decrease (Fig. 3). Of note the mRNA levels found at 48 h was slightly higher (up to 52%) than at 24 h, which probably indicates a significant degradation of insulin in the medium. The quantity of the control actin mRNA was not grossly altered throughout the time course.

*Insulin negatively regulates glucagon gene transcription.* To determine whether the observed changes in glucagon mRNA levels induced by insulin were mediated at the transcriptional level or, alternatively, through a decrease in glucagon mRNA stability, we performed nuclear run-on assays. We examined the rate of nascent glucagon and β-actin RNA transcript formation in isolated nuclei from control In-R1-G9 cells and cells treated with insulin ($10^{-8}$ M); this system assesses the ongoing rate of transcription (26). 5 μg of rat glucagon and chicken β-actin cDNAs and pGem 3 (the cloning vector used for glucagon cDNA) were immobilized on nitrocellulose filters and hybridized with labeled nuclear RNA transcripts. Insulin induced a fourfold decrease in glucagon gene transcription; this effect was already seen at 1 h and persisted up to 24 h (Fig. 4). In comparison, the β-actin signal remained unchanged and pGem 3 elicited no signal. The addition of α-amanitin at 2 μg/ml to the nuclei (to inhibit RNA polymerase II [26]) abolished transcription, indicating that the signals observed resulted from RNA polymerase II-mediated gene transcription.

Glucagon mRNA half-life was then compared in both control and insulin-treated cells. Actinomycin D (5 μg/ml), which completely blocks RNA synthesis in islet cells (27) was first added to In-R1-G9 cells, which were then incubated in the

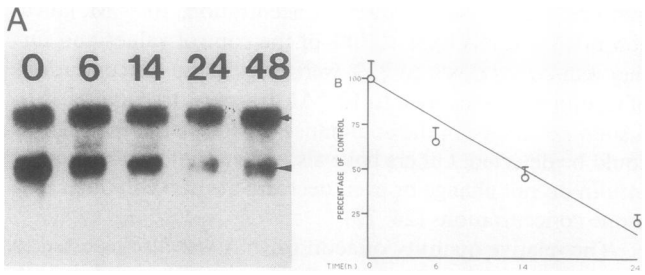


*Figure 3.* Time course of insulin effects on glucagon mRNA ~ $10^7$ In-R1-G9 cells were incubated with $10^{-8}$ M insulin in RPMI 1640 with 0.5% (as detailed in Fig. 2) for 6, 14, 24, and 48 h. Total mRNA was analyzed as detailed in Fig. 2. (*A*) Blots were hybridized directly with both oligonucleotide cDNA probes complementary to glucagon and β-actin mRNA. → points to β-actin mRNA and ➤ to glucagon mRNA. (*B*) Quantifications of mRNAs were done by densitometric scanning of the autoradiographs. Means±SEM of three experiments are shown. Values represent the percentage of the results obtained at time 0.

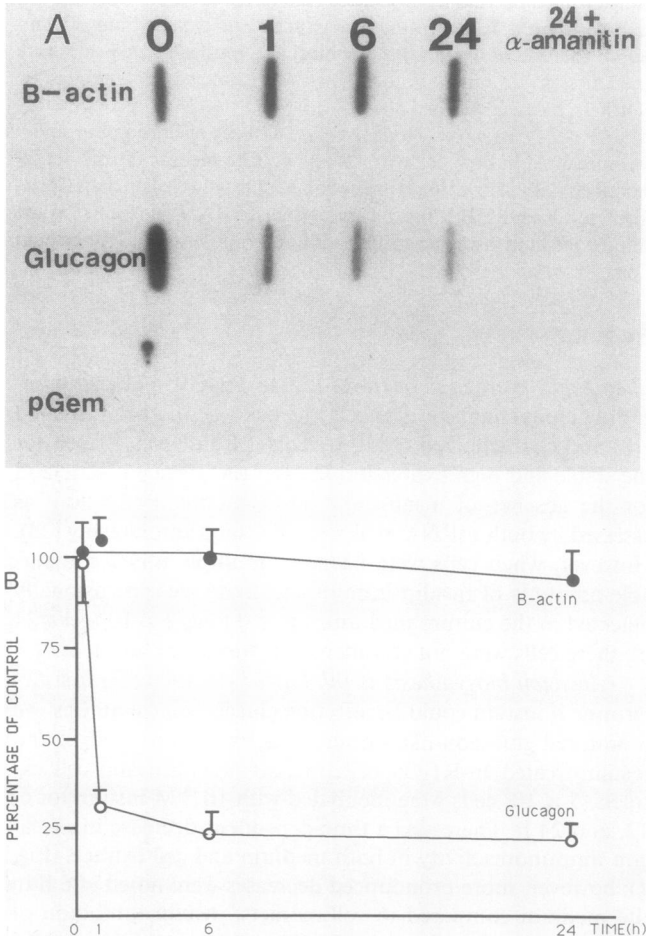


*Figure 4.* Effect of insulin on glucagon gene transcription In-R1-G9 cells were incubated with $10^{-8}$ M insulin in RPMI 1640 with 0.5% BSA (as detailed in Fig. 2) for 0, 1, 4, and 24 h. Nuclear run-on transcription assays were performed as described in Methods. (*A*) Labeled nuclear RNA transcript were hybridized to rat glucagon and chicken β-actin cDNAs, immobilized on nitrocellulose filters using a minifold dot blot. (*B*) Quantifications of the autoradiographic signals were done by densitometric scanning. Values represent the means±SEM of three experiments.

presence (for 4, 12, and 24 h) or absence of insulin; total RNA was extracted and analyzed by Northern blot (Fig. 5). Glucagon-specific mRNA disappearance rates were similar in both control and insulin-treated cells. The half-life of glucagon mRNA was estimated to be ~ 12–14 h, similar to what was previously found in a rat islet cell line (20). These results indicate that insulin effects on glucagon gene expression are mediated predominantly or entirely at the level of transcription of the glucagon gene and not by modulating the stability of the mRNA.

## Discussion

Our results show that insulin induces a rapid and substantial decrease in glucagon gene expression and that this effect occurs at the level of transcription of the gene. Previous studies indicate that insulin decreases glucagon secretion both in vivo and in vitro independently of glucose concentration (9, 10, 15). In the described experimental system, in the presence of 180 mg/dl of glucose, both glucagon secretion (18) and glucagon gene expression are modulated by insulin; we, indeed, observe, in the presence of insulin a dose-dependent decrease in steady-state glucagon mRNA levels; this effect is already detected at an insulin concentration of $10^{-11}$ M (similar to the concentrations previously shown to affect phosphoenolpyruvate carboxykinase (PEPCK) gene expression [16]) and maximal effects are seen at $10^{-8}$ M. Of note, Takaki reported a decrease in glucagon secretion from In-R1-G9 cells at even lower insulin concentrations ($10^{-14}$ M) (18); whether the process of glucagon secretion from In-R1-G9 cells is more sensitive to insulin than glucagon gene transcription is unclear; this differential response might also be explained by concentrations of contaminating insulin (produced by the cells) not detected by radioimmunoassay. The low concentrations of insulin capable of inhibiting both glucagon secretion and glucagon gene transcription in In-R1-G9 cells are in contrast to the very high concentrations usually necessary in vitro for suppressing the A cell (28–30). However, the concentrations of insulin released into the core capillaries of the pancreatic islets and thus perfusing the A cells in the mantle are estimated to be in the range of $10^{-9}$ to $10^{-7}$ M (3), roughly corresponding to the concentrations inducing maximal suppression of glucagon gene expression in In-R1-G9 cells. It is thus possible that the glucagon gene in the normal A cell is under tonic inhibition by these high insulin levels. Furthermore, conditions which reduce endogenous insulin secretion such as diabetes mellitus facilitate A cell inhibition by exogenous insulin (3); in addition, the canine gastric A cells, chronically exposed to much lower insulin concentrations than the islet A cells show a higher sensitivity to exogenous insulin (31). The high insulin sensitivity of In-R1-G9 cell could thus be explained by a change in insulin receptor number or affinity compared to normal A cells, due to an absence of previous exposure to insulin, thereby permitting maximal derepression of the glucagon gene. Conflicting reports have appeared on the presence of insulin receptors on A cells (32, 33) and it is unknown if the number or the functionality of these receptors is altered in chronic insulin deficiency such as in diabetes mellitus. Our data indicate that insulin induces a decrease in glucagon biosynthesis. The time-dependent decrease in glucagon immunoreactivity differs slightly, however, in cell extracts compared to culture medium. Glucagon in the medium is indeed more rapidly depressed than in the cell extracts; this probably indicates that glucagon values in the medium are the results of two different processes: inhibition of cell secretion and of biosynthesis. It is unclear if intracellular glucagon, once secretion is blocked by insulin, is eventually slowly released in the medium or if, alternatively, it undergoes intracellular degradation, as it has been proposed for insulin (34). It is of interest to note a substantial increase in glucagon immunoreactivity in cell extracts between 6 and 24 h. A possible explanation for this increment may be the disinhibition of glucagon biosynthesis induced by the removal of very low concentrations of insulin contained in NCS. Since such low concentrations of insulin do not influence glucagon mRNA levels, it may be hypothesized that insulin could not only affect glucagon gene transcription but also glucagon biosynthesis at the translation of its mRNA.

In contrast to the combined inhibition of glucagon secretion and biosynthesis induced by insulin, changes in hormone release are not always accompanied by changes in hormone biosynthesis. For instance, thyroid and glucocorticoid hormones regulate growth hormone (GH) levels without any effect on secretion (35). In addition, effects on secretion, such as those induced by growth hormone releasing factor (GHRF) on GH secretion appear to be independent of GHRF action on GH gene transcription (36). Hormone secretion and biosynthesis can thus be regulated independently and differentially, implicating that different mechanisms operate in modulating both processes. The insulin-induced decrease in glucagon biosynthesis occurs through a specific inhibition of the transcription of the glucagon gene, as assessed by run-on assays; glucagon mRNA half-life doesn't appear to be affected by insulin and the level of inhibition of glucagon gene transcription can account for the decrease in steady-state glucagon mRNA levels. The decrease in transcription is remarkably rapid, since after 1 h of treatment, glucagon RNA transcripts are down by 70%; such a rapid effect of insulin on transcription has been

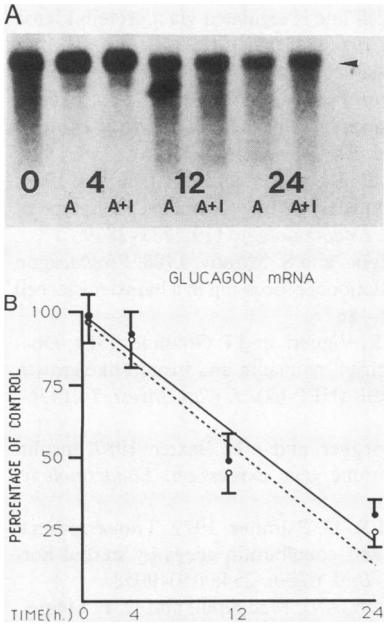


*Figure 5.* Effect of insulin ($10^{-8}$ M) on glucagon mRNA stability. In-R1-G9 cells were incubated in RPMI 1640 with 0.5% BSA as detailed in Fig. 2. Half-life of glucagon mRNA was determined in the absence (- - - ) or presence (——) of $10^{-8}$ M insulin. (*A*) Actinomycin D (5 μg/ml). (*I*) insulin $10^{-8}$ M. Actinomycin D (5 μg/ml) was added to In-R1-G9 cells 30 min before time 0. Total RNA was extracted from control and insulin-treated cells ($10^{-8}$ M) at times 0, 4, 12, and 24 h. (*A*) Northern blot analysis of total RNA; ➤ points to glucagon mRNA. (*B*) Glucagon-specific mRNA was quantitated by densitometric scanning of the autoradiograms of Northern blots. Values are the means±SEM of three experiments.

noted for the glucokinase, PEPCK and GAPDH genes (16, 17, 37), among others, and does not require protein synthesis (17). Although the signalling pathways used by insulin to control gene expression are not known, insulin appears to act through specific sequences within gene promoters (37, 38); it is conceivable that preexisting nuclear factors that bind to these sequences, are modified by a cascade of phosphorylation-dephosphorylation reactions induced by insulin; modulating the phosphorylation state of *trans*-acting factors has recently been shown to be important in regulating transcription of specific genes (39, 40). It is interesting to note that, although the *cis*-acting DNA sequences responsible for the tissue-specific expression of the glucagon gene appear to be different in the A cell of the pancreas compared to the L cell of the intestine (41, 42), the same DNA element could mediate insulin effects on glucagon gene regulation in these two tissues. Indeed, insulin effects on glucagon secretion do not appear to be limited to the A cell of the pancreatic islets, but are probably also observed on the canine gastric A cells (31) and the intestinal L cells (43). This model thus offers the possibilities to identify discrete DNA sequences through which insulin exerts negative regulatory effects and the *trans*-acting factors interacting with them. It must be pointed out, however, that In-R1-G9 cells are clonal hamster islet cells, originally immortalized by the BK virus. Although In-R1-G9 cells can be considered as a useful model to study the molecular mechanisms of glucagon gene expression our results should be interpreted with reservations in regard to the transformed state of these cells.

Finally, our data may provide a potential explanation for the hyperglucagonemia observed in insulin deficiency characteristic of diabetes mellitus; the high glucagon levels seen in these conditions can be indeed rapidly and completely corrected by adequate insulin therapy. We hypothesize that the rapid restoration of normal glucagon levels in diabetic states results not only from an inhibition of glucagon hormone secretion but also from a direct inhibition of glucagon gene transcription.

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