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Insulin regulation of the glucagon gene is mediated by an insulin-responsive DNA element

(gene regulation/gene transcription)

JACQUES PHILIPPE

Departments of Microbiology and Medicine, Centre Médical Universitaire 9, avenue de Champel, 1211 Geneva 4, Switzerland

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ABSTRACT **Diabetes mellitus is characterized by insulin deficiency and high plasma glucagon levels, which can be normalized by insulin replacement. It has previously been reported that glucagon gene expression is negatively regulated by insulin at the transcriptional level. By transfection studies, I have now localized a DNA control element that mediates insulin effects on glucagon gene transcription. This element also confers insulin responsiveness to a heterologous promoter. DNA-binding proteins that specifically interact with this insulin-responsive element are found in both glucagon- and non-glucagon-producing cells; and the pattern of binding, as assessed by the gel retardation assay, is not modified by prior insulin treatment.**

Insulin regulates cell growth and promotes energy storage by interacting with its cell surface receptors (1). Some insulin effects occur through changes in gene expression; multiple examples of gene regulation by insulin at both transcriptional and posttranscriptional levels have been described in the last few years (2). However, the molecular mechanisms by which insulin mediates its cytoplasmic and nuclear effects are poorly understood. Characterization of cis-acting DNA sequences mediating insulin-induced modifications in gene transcription is a fundamental step leading to a better understanding of these mechanisms. Insulin-responsive elements (IREs) have recently been identified for the c-*fos*, phospho-*enol*pyruvate carboxykinase, amylase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes (3–6). In contrast to most sequences that control common regulatory events, these IREs do not share any homology.

Glucagon is a 29-amino acid peptide synthesized by the alpha cell of the pancreas and is involved in glucose homeostasis by directly activating gluconeogenesis and glycogenolysis. In diabetes mellitus, insulin deficiency is associated with high plasma glucagon levels, which rapidly return to normal with insulin treatment. *In vitro*, glucagon secretion and biosynthesis are inhibited by insulin (7–9). It has recently been shown that insulin effects on glucagon biosynthesis are mediated through a decrease in glucagon gene expression, which occurs at the transcriptional level (8, 9). I now report on the identification of an IRE within the glucagon gene promoter that displays sequence homology with a previously characterized IRE of the GAPDH gene.

METHODS

Plasmids. Deletion mutant plasmids containing different lengths of the 5′ flanking region of the rat glucagon gene linked to the coding sequence of the bacterial chloramphenicol acetyltransferase (CAT) gene have been described (10). DNA sequence from base pairs (bp) −274 to −234 with *Bam*HI compatible ends, which contains the wild-type or the mutated DNA element G_3, was inserted into a *Bam*HI site 5′ of the glucagon promoter (bp −136 to +51) and the herpes simplex virus (HSV) thymidine kinase (TK) promoter; the latter promoter consists of the first 196 bp cloned into poCAT (11). Mutant G_3 oligonucleotides were generously provided by W. Knepel and G. Waeber (Harvard University, Cambridge, MA).

Sequences of the Oligonucleotides.

Wild-type G_3

GATCCTGAAGTAGTTTTTCACGCCTGACTGAGATT-
GAAGGGTGTAGC

G_3 mutant (G_3M_3)

GATCCTGAAGTAGTTTTTCACGCCTGACTGAACGC-
GAAGGGTGTAGC

G_3 mutant (G_3M_6)

GATCCTGAAGTAGTTTTTCAATTATGACTGAGATT-
GAAGGGTGTAGC

Cell Culture and Transfection Studies. In-R1-G9, HeLa, and JEG-3 (a choriocarcinoma cell line) cells were cultured in RPMI 1640 containing 11 mM glucose, 5% fetal calf serum, and 5% newborn calf serum. In-R1-G9 cells were transfected in suspension by the DEAE-dextran method (10) with 3 μg of indicator plasmid and 1 μg of each control plasmid, pxGH5 and pSV_2Apap, to monitor transfection efficiency (12, 13). pSV_2Apap is a plasmid containing the human placental alkaline phosphatase gene driven by the simian virus 40 long terminal repeat, and pxGH5 contains the human growth hormone (GH) sequence under the control of a metallothionein promoter. pRSVCAT and poCAT (11) were used as positive and negative controls, respectively. Cell extracts were prepared 48 hr after transfection and analyzed for CAT and alkaline phosphatase activities; immunoreactive GH was also determined in the culture medium for some of the experiments by using a radioimmunoassay kit (Nichols Institute, San Juan Capistrano, CA). Alkaline phosphatase assays were done as reported (13) except that cell extracts rather than cell suspensions were used. In brief, cells were scraped into TBS buffer (154 mM NaCl/50 mM Tris·HCl, pH 7.5/1 mM $MgCl_2$), pelleted, and resuspended in the same buffer. They were then subjected to three freeze–thaw cycles, and cell debris was pelleted. Aliquots of the supernatant were heated for 60 min at 65°C and incubated at 30°C with the substrate *p*-nitrophenyl phosphate for 4 hr. Absorbance was measured at 405 nm. No background activity of untransfected cells was detectable at this incubation time. CAT activity was corrected for the protein concentration of the lysate and per

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Abbreviations: IRE, insulin-responsive element; CAT, chloramphenicol acetyltransferase; GH, growth hormone; HSV, herpes simplex virus; TK, thymidine kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

unit of alkaline phosphatase or 100 ng of immunoreactive GH. Insulin did not significantly influence expression of the plasmids pxGH5 and psV$_2$Apap. The transcriptional start site was mapped as described (10).

Cell Extracts and Gel Retardation Assays. Nuclear extracts were prepared from In-R1-G9, JEG-3 choriocarcinoma, and HeLa cells by the method of Dignam *et al.* (14) and Schreiber *et al.* (15), and whole-cell extracts were prepared by the method of Manley *et al.* (16). Gel retardation assays were performed as described (17), with poly(dI-dC) and single-stranded *Escherichia coli* DNA as nonspecific competitor.

RESULTS AND DISCUSSION

To examine how insulin exerts its effects on glucagon gene expression, deletion mutant plasmids containing various lengths of the 5′ flanking region of the rat glucagon gene fused to the CAT gene (10) were transiently transfected into In-R1-G9 cells. This islet cell line produces high levels of glucagon and is known to be responsive to insulin (9, 18, 19). Insulin effects on CAT activity were then examined; insulin treatment of In-R1-G9 cells transfected with a plasmid containing 2400 bp (bp −2400 to +51) of the 5′ flanking region of the rat glucagon gene resulted in a 67% decrease in CAT activity; under the same conditions CAT activity measured from pRSVCAT-transfected cells was slightly increased (Fig. 1*a*). No significant change in insulin effect was observed despite progressive deletions from −2400 bp to −269 bp; most of these effects were, however, lost at −256 bp (Fig. 1*a*). Only minor changes (14%) were indeed seen for −256 CAT and for shorter deletion mutants. Because the latter effect was of unknown importance, we focused our attention on the sequences between −269 and −256 bp; this region corresponds to the 5′ distal part of a DNA control element, G$_3$, that was previously identified by functional and binding assays (10). G$_3$ was localized by DNase I footprinting assays between −268 bp and −238 bp, just 42 bp upstream of the most proximal enhancer, G$_2$ (Fig. 1*a*). Its removal by 5′ deletion did not result, however, in a significant drop in basal transcriptional activity. By contrast, when G$_3$ was removed from its tandem arrangement with G$_2$ and placed directly upstream of its own promoter or of the HSV TK promoter, it was able, in an orientation-dependent manner, to increase CAT activity nearly as much as G$_2$ (10).

To verify the property of G$_3$ to function as an IRE, we linked sequences −274 bp to −234 bp to the first 136 bp of the glucagon promoter (containing the upstream promoter element, G$_1$) or to the heterologous HSV TK gene promoter (pUTCAT) (11). In both cases, insulin decreased CAT activity by 61% and 70%, respectively, without significantly affecting activity from pUTCAT alone; the decrease conferred by G$_3$ was strongly orientation dependent (Fig. 2). In addition, two G$_3$ elements placed in tandem were not able to amplify the insulin effect observed from a single G$_3$, in analogy to the absence of additional activity conferred by two G$_3$ elements compared to a single G$_3$ (20). Neither the Rous sarcoma virus enhancer nor G$_2$, linked to the HSV TK gene promoter, could confer insulin responsiveness (Fig. 2).

To more precisely map the critical insulin-responsive region of G$_3$, the transcriptional activity obtained from both

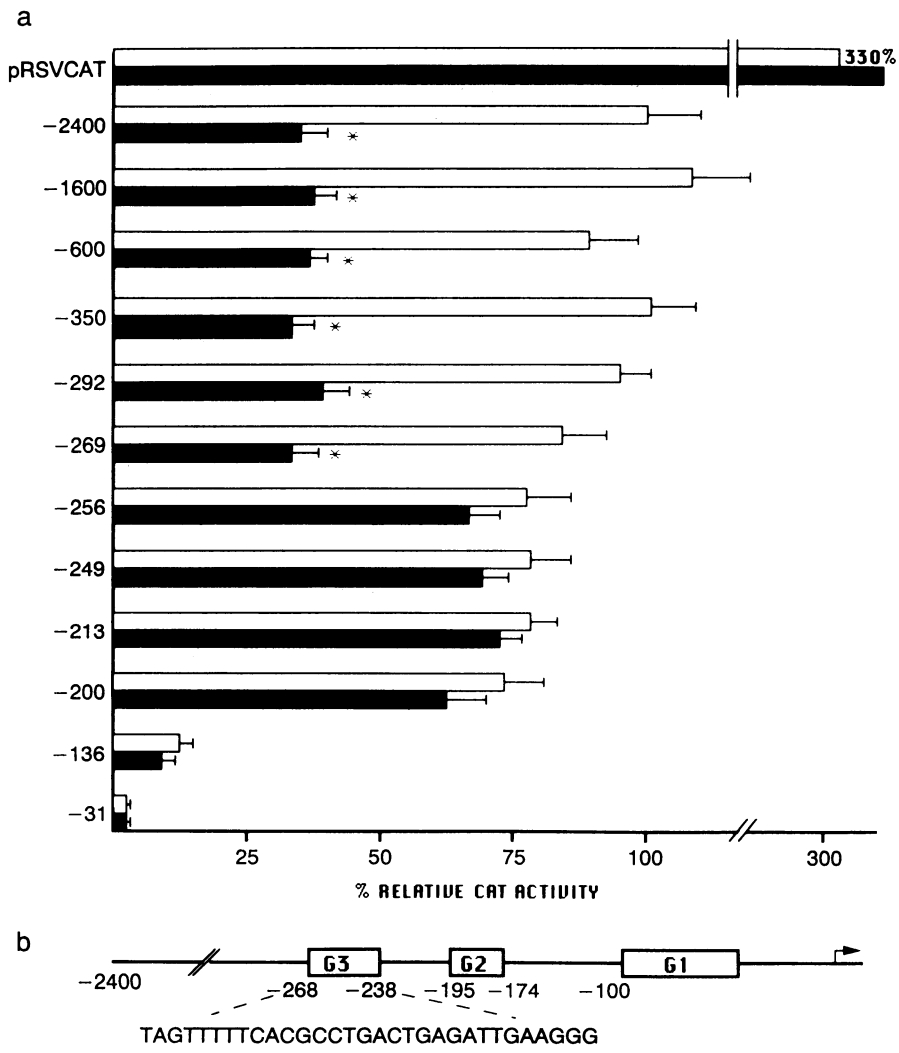

FIG. 1. (*a*) Insulin regulation of glucagon–CAT fusion genes. In-R1-G9 cells were transfected with indicator plasmid and control plasmids pxGH5 and pSV$_2$Apap to monitor transfection efficiency. Twenty-four hours after transfection, cells were incubated in RPMI 1640 containing 0.5% bovine serum albumin with (solid bars) or without (open bars) 10 nM insulin for 24 hr. The transcriptional start site was found at the expected site. Results are expressed as a percentage of the CAT activity measured in cells transfected with −2400 CAT and represent the mean ± SEM of four to seven experiments. The asterisks indicate statistically significant differences ($P < 0.05$). (*b*) The rat glucagon gene promoter is schematically represented by the solid line. DNA control elements are indicated by boxes with the nucleotide numbers of their 5′ and 3′ borders. The DNA sequence of G$_3$ is shown, and the transcriptional start site is at the arrow.

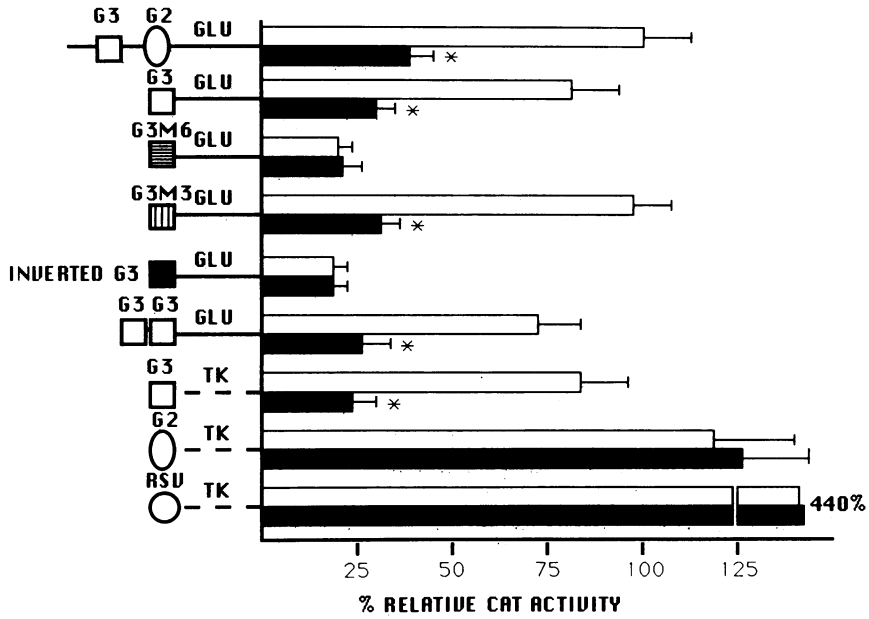

FIG. 2. G_3 mediates insulin responsiveness to the glucagon and HSV TK promoters. In-R1-G9 cells were transfected with both indicator and control plasmids, as indicated in Fig. 1. Cells were incubated with 0.5% bovine serum albumin with (solid bars) or without (open bars) 10 nM insulin for 24 hr. Results are expressed as a percentage of CAT activity measured in cells transfected with −350 CAT (containing both G_2 and G_3) and represent the mean ± SEM of five experiments. The asterisks indicate statistically significant differences ($P < 0.05$). Squares represent wild-type, mutated, or inverted G_3 (bp −274 to −234); ovals represent G_2 (bp −200 to −165); and circles represent the Rous sarcoma virus enhancer. GLU indicates the glucagon promoter from bp −136 to +51, and TK indicates the HSV TK promoter from bp −196 to +53.

wild-type and mutated G_3-containing plasmids was compared. Since our deletional analysis indicated that insulin responsiveness was lost at −256 CAT, I chose to mutate G_3 between bp −255 and −258 (G_3M_6). This mutant G_3 when inserted upstream of the first 136 bp of the glucagon gene promoter was not only unable to activate basal transcription but also to mediate insulin responsiveness (Fig. 2). By contrast, mutation of 4 bp at the proximal end of G_3 (G_3M_3), from bp −244 to −247, was without any effect on either basal transcriptional activity or insulin responsiveness. These results show that the distal part of G_3 is, in contrast to its proximal half, critical for both basal transcriptional activity and insulin responsiveness.

We then examined by electrophoretic mobility shift assays whether insulin could induce a specific DNA-binding activity on G_3. Nuclear extracts from both control and insulin-treated In-R1-G9 cells were prepared and incubated with a ^{32}P-labeled probe containing G_3. Two bands of retarded mobility were observed and specifically blocked by competition by a 25-fold excess of unlabeled G_3 but only slightly blocked by a 100-fold excess of unlabeled G_2 (Fig. 3*a*) [this slight competition with G_2 was not observed in the DNase I footprinting assay (10) and did not increase with a 1000-fold excess of unlabeled G_2]. There was, however, no difference in the band pattern between nuclear extracts from control or insulin-treated cells (Fig. 3*b*). This is consistent with previous data indicating a rapid effect of insulin on glucagon gene expression (9) (still present when protein synthesis is inhibited) and suggests that insulin action might result in the modification of a preexisting protein. Whether such a modification (like a phosphorylation or dephosphorylation) is maintained during the isolation of nuclear extracts is unknown. Gel retardation

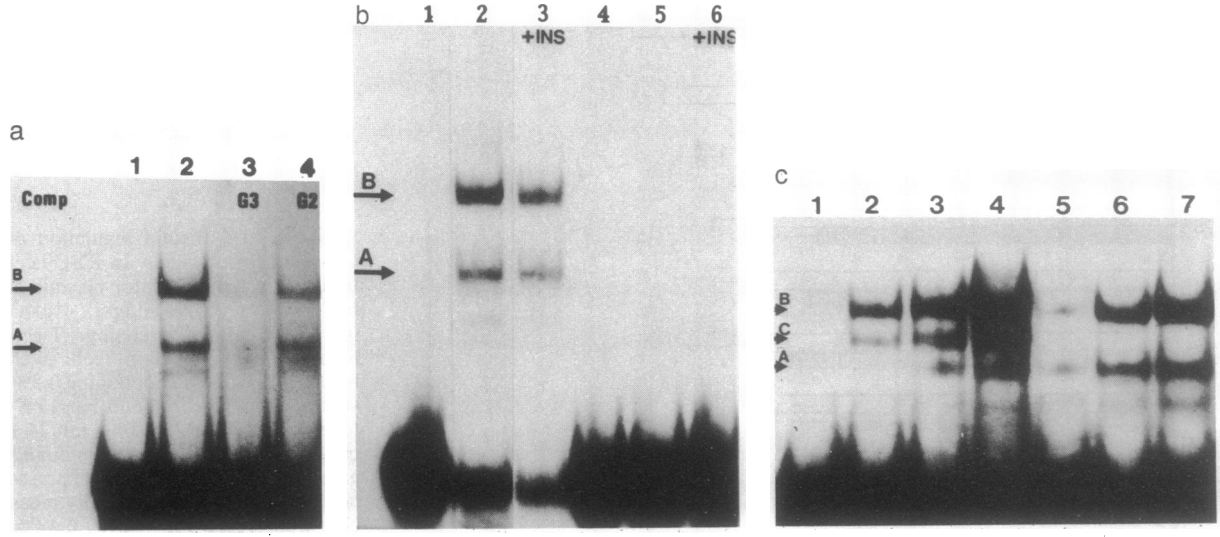

FIG. 3. Protein binding to G_3. Gel retardation assays were performed with a ^{32}P-labeled probe (−274 to −234) containing wild-type or mutated G_3 (G_3M_6) and incubated with In-R1-G9 nuclear extracts (*a*), In-R1-G9 nuclear extracts from control cells and 2-hr-insulin-treated cells (*b*), or In-R1-G9 and HeLa nuclear extracts (*c*). The samples were loaded on a 5% nondenaturing gel. The same data were obtained with nuclear or whole-cell extracts. (*a*) Lane 1, no nuclear extracts; lanes 2–4, 10 μg of In-R1-G9 nuclear extracts. Comp, competitor DNA (25-fold excess unlabeled G_3 in lane 3 and 100-fold excess unlabeled G_2 in lane 4). (*b*) Wild-type labeled G_3 was incubated without (lane 1) or with 12 μg of nuclear extracts from control (lane 2) or insulin-treated cells (lane 3). Mutated labeled G_3 (G_3M_6) was incubated without (lane 4) or with 12 μg of nuclear extracts from control (lane 5) or insulin-treated cells (lane 6). +INS designates nuclear extracts obtained from insulin-treated cells. (*c*) Lane 1, no nuclear extracts; lanes 2–4, 5, 10, and 15 μg of nuclear extracts from HeLa cells; lanes 5–7, 5, 10, and 15 μg of nuclear extracts from control In-R1-G9 cells. Arrows indicate retarded complexes: A, lower complex; B, upper complex; C, middle complex.

assays were also performed with a G_3 mutant (G_3M_6) to investigate whether the loss of basal transcriptional activity and insulin responsiveness were associated with modifications in binding activities. As shown in Fig. 3*b*, neither band A nor band B was detected in the presence of the distally mutated G_3 (lanes 4–6). We conclude that mutations within the distal half of G_3 (bp −255 to −258) lead to a loss of functional activity by preventing protein–DNA interactions.

To investigate the cell-specific distribution of these binding activities in nonislet cells, nuclear proteins were extracted from HeLa and JEG-3 cells; both binding activities were present in these cell lines (Fig. 3*c* and data not shown). An additional middle band (band C in Fig. 3*c*), which was not detected in islet nuclear or whole-cell extracts, was observed in nonislet cells. The significance of this additional band in nonislet cell extracts is presently unknown.

How insulin, after binding to its receptor, exerts its effects is still unknown; some of them are mediated through a phosphorylation–dephosphorylation cascade from the autophosphorylation of the receptor itself (1). Phosphorylation–dephosphorylation of trans-acting factors is the only posttranscriptional modification so far known to correlate with functional changes; it is thus conceivable that the rapid insulin effects on gene transcription are mediated by such a mechanism. The results of our gel retardation assays are compatible with this hypothesis inasmuch as the phosphorylation state of other trans-acting factors may not modify their electrophoretic mobility (21). Insulin also induces, however, new binding activities on the promoter sequences of positively regulated genes (encoding GH and GAPDH) (6, 24). Whether these binding activities result from synthesis of new proteins or posttranscriptional activation of preexisting proteins remains unknown.

The structural requirements of G_3 to act as an enhancer have recently been analyzed (23); G_3 can be subdivided into two domains, A (roughly between bp −258 and −252) and B (between bp −247 and −234); the mutations disrupting the A but not the B domain abolish transcriptional activity. Our results indicate that the A domain is also necessary for insulin responsiveness (see −256 CAT and G_3M_6). In contrast, domain B has no known functional relevance and, although others have detected protein binding to the B domain by the gel retardation assay (23), we have not. It is thus still unclear why the DNase I-protected G_3 element extends so proximally to bp −238. The DNA sequence of the core of domain A, $\overset{G}{C}GCCT\overset{G}{G}$, matches very closely the consensus sequence CGCCTC present in various genes regulated by insulin, including c-*myc* and the genes encoding GH, ornithine decarboxylase, tyrosine aminotransferase, P33, and GAPDH (6, 22, 24–27). In the latter, two IREs appear to be present; in one of them, the minimal sequence contacted by an insulin-regulated DNA binding protein is CCCGCCTC, which has 6 of its 8 nucleotides homologous to the A domain of G_3: CACGCCTG (6). It remains to be determined, however, whether such sequence homology is functionally significant in the context of other genes and whether the same sequences are involved in the positive and negative regulation of gene expression by insulin. In this regard, the characterization of IREs within the phospho*enol*pyruvate carboxykinase (TGGTGGGGTACAAC) and the amylase gene promoters (GCGTGAGAGTTTCTAAAA), which mediate negative and positive regulation by insulin, respectively, and which share no sequence homology with the glucagon IRE, indicates that different DNA sequence can confer insulin responsiveness (4, 5).

A persistent but minimal insulin-mediated inhibition of CAT activity is observed for the plasmids containing fragments of the glucagon 5′ flanking region smaller than 256 bp. It is thus possible that a second IRE is present proximally; interestingly, a 45-kDa protein has been shown to interact with domain A of both G_3 and G_1 (23).

The physiological relevance of the insulin-mediated inhibition of glucagon gene expression is indicated by the high plasma glucagon levels that characterize untreated type I or type II diabetes mellitus; tonic inhibition of glucagon gene expression by insulin normally prevails in nondiabetics. Increases in glucagon gene transcription secondary to the relative or absolute insulin deficiency can be hypothesized to result from the impaired activation of a negatively acting transcription factor. Identification of IREs should lead to a better understanding of how insulin regulates gene expression.

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