Insulin Responsiveness of the Glucagon Gene Conferred by Interactions between Proximal Promoter and More Distal Enhancer-like Elements Involving the Paired-domain Transcription Factor Pax6*

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Rafal Grzeskowiak‡, Jasmin Amin‡, Elke Oetjen, and Willhart Knepel§
From the Department of Molecular Pharmacology, University of Göttingen, 37070 Göttingen, Germany

Regulation of gene transcription is an important aspect of insulin's action. However, the mechanisms involved are poorly understood. Insulin inhibits glucagon gene transcription, and insulin deficiency is associated with hyperglucagonemia that contributes to hyperglycemia in diabetes mellitus. Transfecting glucagon-reporter fusion genes into a glucagon-producing pancreatic islet cell line, a 5×, 3′-, and internal deletion analysis, and oligonucleotide cassette insertions failed in the present study to identify a single insulin-responsive element in the glucagon gene. They rather indicate that insulin responsiveness depends on the presence of both proximal promoter elements and more distal enhancer-like elements. When the paired domain transcription factor Pax6 binding sites within the proximal promoter element G1 and the enhancer-like element G3 were mutated into GAL4 binding sites, the expression of GAL4-Pax6 and GAL4-VP16 restored basal activity, whereas only GAL4-Pax6 restored also insulin responsiveness. Likewise, GAL4-CBP activity was inhibited by insulin within the glucagon promoter context. The results suggest that insulin responsiveness is conferred to the glucagon gene by the synergistic interaction of proximal promoter and more distal enhancer-like elements, with Pax6 and its potential coactivator the CREB-binding protein being critical components. These data thereby support concepts of insulin-responsive element-independent mechanisms of insulin action to inhibit gene transcription.

The regulation of gene transcription by insulin is an important facet of this hormone's action. Insulin has been shown to stimulate or inhibit the transcription of a great number of genes (1). Based on the hormone response element paradigm, there has long been speculation that the effects of insulin are mediated through a common insulin-responsive element (IRE)1 and binding transcription factor (2–4). IREs have been characterized in a number of genes but, unlike cAMP, which regulates gene transcription predominantly through one cis-acting element, the CRE (5), it became apparent that a single consensus IRE does not exist (6, 1). Likewise, diverse transcription factors have been suggested to mediate the insulin response, including APF (7), FKHRL1 (8), FKHR (9), GABP (10), Fra-2/ Jun D (10, 11), Egr-1 (12), NF-1 (13), USF (14), IRE-ABP (2), and SRF (15). On the other hand, it has also been suggested that insulin may act independently of an IRE; insulin may rather target arrays of interacting transcription factors at the coactivator level (16–19).

The genes that are negatively regulated by insulin include the one encoding glucagon (20). This pancreatic islet hormone is a biologic antagonist of insulin and stimulates hepatic glucose production (21–23). In most species, the glucagon-secreting α-cells are located at the periphery of the islets of Langerhans and are exposed to high concentrations of insulin released from the more centrally located β-cells (21–23). Thus, inhibition of glucagon synthesis and secretion by intraislet insulin is thought to be important for the coordinated synthesis and secretion of the biologically antagonistic islet hormones (20–23). Consequently, insulin deficiency in diabetes mellitus is associated with hyperglucagonemia (20–23). The elevated glucagon levels in diabetes contribute to increased hepatic glucose output and hyperglycemia (20–26). However, the molecular mechanism of inhibition of glucagon gene transcription by insulin is poorly understood. It has been proposed that an enhancer-like element of the glucagon gene, the G3 element, functions as an IRE of the glucagon gene (27). The present study expands those experiments by demonstrating that not only the G3 element but also other enhancer-like elements of the glucagon gene can confer insulin responsiveness to the nonresponsive truncated glucagon gene promoter in a glucagon-producing pancreatic islet cell line. The results of the present study suggest that insulin responsiveness is conferred to the glucagon gene by the synergistic interaction between proximal promoter and more distal enhancer-like elements. The paired domain transcription factor Pax6, which binds to the proximal promoter element G1 and the enhancer-like element G3, and its potential coactivator CBP appear as essential components of this synergistic interaction. The data thereby support suggestions of IRE-independent mechanisms of insulin action to inhibit gene transcription.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Plasmids pT81Luc (28), −350GluLuc (29), 5xGal4/E1B/Luc (30), −292GluLuc, −238GluLuc, −200GluLuc, −169GluLuc, −60GluLuc, −350−48GluLuc, −350−91GluLuc, −350/ −150GluLuc, −350−210GluLuc, −350−210/−136GluLuc, 4xG2/T81-
Luc (31), 4xG3A/T81Luc (32), pGAL-CBP8 (33), and pGAL-VP16 (34) have been described previously. The plasmid pCMV-GFPp2m was purchased from Canberra-Packard, Dreieich, Germany. For −136GlucLuc, 3xGluCRE−136GlucLuc, 4xG3A−136GlucLuc, 4xG2−136GlucLuc, the plasmids pT81Luc, 3xGluCRE/T81Luc (35), 4xG3A/T81Luc, and 4xG2−(T81)Luc were digested with BglII, blunt-ended by Klenow fill-in reaction, and digested with SstII to remove the thymidine kinase promoter; the promoter fragment (from −138 to +58), which was obtained from −136Luc (32) as a SalI/HindIII (blunt-ended by Klenow fill-in reaction) fragment, was then ligated. The construct −350(−150−91)GlucLuc, containing an internal deletion from −149 to −92, was prepared by PCR from −350GlucLuc replacing the deleted bases by a single SacII site. In the constructs −350(mutG1)GluLuc, −350(mutG3)GluLuc, and −350(mutG1/G3)GluLuc the Pax6-binding PISCES motifs within G1, G3, or G1 plus G3 are mutated into a GAL4 binding site. The following primers, containing a restriction site at their 5′ ends, were used for the preparation of these constructs (the restriction site is underlined, the GAL4 binding site in lowercase): primer 1 (XhoI) 5′-CTGATCTCAGA-TGGCCTAATACGATACGG-3′; primer 2 (BglII) 5′-GTAGATCTCA-GAGGTCATCCCTTGTGG-3′; primer 3 (XhoI) 5′-CAGGCTAGCC-TTCAGCTCTGAAATGTGAATTTG-3′; primer 4 (XhoI) 5′-CAGGCTAGC-CTAGactgtcctccgTTGAAGGGTGTATTTCAAAC-3′; primer 5 (EcORI) 5′-CGAATTCgagagtactgtcctccgATTGTCAGCGTAATATCTG-3′; primer 6 (EcORI) 5′-GGAATTCgctggagtactgtcctccgTTTAGGCTATATATCTGCG-3′. The constructs were prepared by PCR from −350GlucLuc. For −350(mutG1/G3)GluLuc, two PCR fragments were generated with the primer pairs 6/2 and 1/5; after digest with the appropriate enzymes, the fragments were ligated into the XhoI/BglII sites of pXP2. For −350(mutG3)GluLuc, two PCR fragments were generated with the primer pairs 1/3 and 4/2; after digest with the appropriate enzymes, the fragments were ligated into the XhoI/BglII sites of pXP2. For −350(mutG1/G3)GluLuc, three PCR fragments were generated with the primer pairs 1/3, 4/5, and 6/2; after digest with the appropriate enzymes, the fragments were ligated into the XhoI/BglII sites of pXP2. An expression vector encoding GAL4-Pax6 fusion protein was prepared as follows. The RatHinII-KpnI fragment of the plasmid Pax6−135 (obtained from P. Gruss, Göttingen, Germany), containing full-length Pax6 cDNA, was cloned into the BamHI-KpnI sites of pSG424 (36); the HinIII/EcoRI fragment of this plasmid, containing the GAL4-Pax6 fusion protein, was cloned into the HinIII/EcoRV sites of the cytomegalovirus-driven eukaryotic expression vector pBAT14 (obtained from M. German, San Francisco, CA). For preparation of an expression vector encoding the Pax6 paired domain (amino acids 1–248), Pax6-PD) the plasmid pBAT14.m.Pax6 (obtained from M. German,) was digested with BglII and HinIII, blunt-ended by Klenow fill-in reaction, and religated. All construct were sequenced by enzymatic cycle sequencing to confirm the identity and the orientation of the inserts.

Cell Culture and Transfection of DNA—The glucagon-producing pancreatic islet cell line InR1-G9 (37) was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were trypsinized and transfected in suspension by the DEAE-dextran method (29) with 2 μg of indicator plasmid/6-cm dish, unless noted otherwise. Rous sarcoma virus-chloramphenicol acetyltransferase plasmid (0.4 μg/6-cm dish) or cytomegalovirus-green fluorescent protein (GFP) (plasmid pCMV-GFPp2m, 0.5 μg/6-cm dish) were added as second reporters to check for transfection efficiency. When indicated, expression vectors were cotransfected. These cotransfections were carried out with a constant amount of DNA, which was maintained by adding Bluescript (Stratagene, La Jolla). Twenty-four hours after transfection, cells were incubated in RPMI 1640 containing 0.5% bovine serum albumin and antibiotics as described above. When indicated cells were treated with insulin (10 nM) for 23 h before harvest, or with forskolin (10 μM), KCl (45 mM), or both for 6 h before harvest. Cell extracts (29) were prepared 48 h after transfection. The luciferase assay and chloramphenicol acetyltransferase assay were performed as described previously (29). Thin layer chromatography plates were analyzed with a Fuji PhosphorImager. GFP was measured in the cell extracts using the FluoroCount™ microplate fluorometer (Packard).

Materials—A stock solution of forskolin (100 mM) was prepared in dimethyl sulfoxide and further diluted in cell culture medium. Insulin was from Serva (Heidelberg, Germany), and a stock solution (10 μM) was prepared in 0.9% saline containing 2 mg/ml bovine serum albumin. Controls received the solvent only.

**RESULTS**

**Similar Inhibition of Glucagon Gene Transcription by Insulin under Basal Conditions and After Stimulation by cAMP or Forskolin**—Glucagon gene transcription is negatively regulated by insulin (20), and it has been shown that 350 base pairs of the glucagon gene promoter are sufficient to confer insulin responsiveness (27). This is confirmed in Fig. 1, demonstrating that a maximally effective concentration of insulin (10 nM) inhibited the transcriptional activity of a luciferase reporter gene under the control of 350 base pairs of the 5′-flanking region of the rat glucagon gene by about 50–60% after transfection into the glucagon-producing pancreatic islet cell line InR1-G9 (see also Fig. 2, A–C). The concentration of insulin that inhibited glucagon gene transcription by about 50% of the maximum effect was 0.5 nM (data not shown). Glucagon gene transcription is stimulated by cAMP- and membrane depolarization-induced signaling pathways (29, 38–41). After stimulation by high potassium-induced membrane depolarization, by the adenylyl cyclase activator forskolin, or both glucagon gene transcription was inhibited by insulin (10 nM) to a similar degree as under basal conditions (Fig. 1). Thus, the -fold stimulation by membrane depolarization and/or cAMP in the absence or presence of insulin was similar, suggesting that insulin does not interfere with the mechanisms that activate glucagon gene transcription in response to these stimuli.

**Insulin Responsiveness of the Glucagon Gene Depends on the Presence of a Glucagon Gene Enhancer-like Element**—The rat glucagon gene promoter contains the enhancer-like elements G2 and G3 (31, 38, 42–44) as well as a CRE (29, 39). The truncated glucagon gene promoter (136 base pairs) containing the proximal promoter elements G1 and G4 (44, 45) exhibits low transcriptional activity but is essential for proper enhancer function (44). An unconfirmed report proposed that the G3 element (from −268 to −238) is an IRE of the glucagon gene (27). However, it became clear meanwhile that the G3-binding transcription factor is Pax6 (46), and that Pax6 also binds the G1 element (47, 48), raising new questions about the role of Pax6 in glucagon gene transcription.
Expression of 5′-deleted mutant plasmids in InR1-G9 cells revealed that the insulin responsiveness of the glucagon gene 5′-flanking region was reduced when the 5′ end was shortened from −350 to −292 (Fig. 2A). Insulin inhibited the transcriptional activity of −350GluLuc and −292GluLuc by 62% and 22% (p < 0.01, Student’s t test), respectively (Fig. 2A). However, progressive deletions to −238 and −200 largely restored the effect of insulin (inhibition by 46% of −200GluLuc) (Fig. 2A). Noteworthy, the construct −200GluLuc does not contain the G3 element, indicating that it is not essential for negative regulation of glucagon gene transcription by insulin. This result is in some contrast to data obtained in a previous study (27). Truncation from −200 to −169 abolished insulin responsiveness (Fig. 2A). This deletion eliminates the G2 element (Fig. 2A). Further deletion to −60 had no effect (Fig. 2A). To further examine the role of the G2 element, this element was internally deleted (construct −350(−210/−136)GluLuc; Fig. 2B). As shown in Fig. 2B, insulin inhibited glucagon gene transcription also after deletion of the G2 element, although somewhat less effectively than the wild type (inhibition by 43% and 66%, respectively). When compared with −350GluLuc (100 ± 3%), the basal activity of −350(−210/−136)GluLuc was 5 ± 1% (n = 9). These results indicate that also the G2 element is not essential for negative regulation of glucagon gene transcription by insulin. When taken together with the results of the 5′-deletion analysis, the data suggest that each of the glucagon gene enhancer-like elements (CRE, G3, G2) is dispensable for the effect of insulin; however, insulin responsiveness of the glucagon gene seems to depend on the presence of one or more glucagon gene enhancer-like elements.

To examine this further, three or four copies of the glucagon gene enhancer-like elements CRE, G3A, or G2 were placed in front of the truncated glucagon promoter (−136GluLuc). −350GluLuc was included as a positive control. G3A contains the domain A of G3 (from −262 to −247), which provides the Pax6 binding site and confers strong basal transcriptional activity in islet cell lines (32, 42, 43, 46). The truncated glucagon promoter was not responsive to insulin (Fig. 2C), as expected from the 5′-deletion analysis (Fig. 2A). However, insulin inhibited by 30–40% the transcriptional activity of 3xCRE-136GluLuc, 4xG3A-136GluLuc, and 4xG2-136GluLuc (Fig. 2C). When compared with −136GluLuc (100 ± 3%), basal activity of the constructs was 204 ± 15% (3xCRE-136GluLuc), 51,840 ± 4,244% (4xG3A-136GluLuc), and 1,075 ± 80% (4xG2-136GluLuc).

**Fig. 2.** Inhibition of glucagon gene transcription by insulin depends on the presence of an enhancer-like element of the glucagon gene. A, 5′-deletion analysis. The indicated constructs were transfected into InR1-G9 cells, and the cells were treated with insulin (10 nm) as indicated. Luciferase activity in the presence of insulin is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective controls (no treatment). Values are means ± S.E. of four independent experiments, each done in duplicate. Control elements in the 5′-flanking region of the glucagon gene are indicated (see the text for an explanation). B, internal deletion of the G2 element. Plasmid −350GluLuc or −350(−210/−136)GluLuc was transfected into InR1-G9 cells, and the cells were treated with insulin (10 nm) as indicated. Luciferase activity in the presence of insulin is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective controls (no treatment). Values are means ± S.E. of three independent experiments, each done in triplicate. C, the enhancer-like elements CRE, G3A, and G2 of the glucagon gene confer insulin responsiveness to the nonresponsive truncated glucagon gene promoter. Plasmids −350GluLuc, −136GluLuc, 3xGluCRE-136GluLuc, 4xG3A-136GluLuc, and 4xG2-136GluLuc were transfected into InR1-G9 cells, and the cells were treated with insulin (10 nm) as indicated. Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective control (no treatment). Values are means ± S.E. of three independent experiments, each done in triplicate.
Inhibition of glucagon gene transcription by insulin depends on the presence of a proximal promoter element of the glucagon gene. A, 3′-deletion analysis. The indicated constructs were transfected into InR1-G9 cells, and the cells were treated with insulin (10 nM) as indicated. Luciferase activity in the presence of insulin is transfected into InR1-G9 cells, and the cells were treated with insulin (10 nM) as indicated.

**Fig. 3.** Inhibition of glucagon gene transcription by insulin depends on the presence of a proximal promoter element of the glucagon gene. A, 3′-deletion analysis. The indicated constructs were transfected into InR1-G9 cells, and the cells were treated with insulin (10 nM) as indicated. Luciferase activity in the presence of insulin is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective control (no treatment). Values are means ± S.E. of three independent experiments, each done in duplicate. TK, thymidine kinase minimal promoter. B, internal deletion. Plasmid 350GluLuc or 350(-150/-91)GluLuc was transfected into InR1-G9 cells, and the cells were treated with insulin (10 nM) as indicated. 136GluLuc) (n = 9 each). Thus, consistent with a previous report (27), G3A can confer insulin responsiveness to the non-responsive truncated glucagon promoter; however, these results show in addition that also the CRE and the G2 element can confer insulin responsiveness to the homologous promoter. In contrast, insulin had no effect on activity conferred to the truncated glucagon promoter by four copies of the Y box element of the murine Eα gene; transcriptional activity was 102 ± 6% in the presence of insulin (10 nM) relative to the untreated controls (n = 8 each). This demonstrates the specificity of the insulin-repressive effect conferred upon the truncated glucagon promoter by the three glucagon gene enhancers. When taken together, the results of the 5′-deletion, internal deletion, and oligonucleotide cassette insertion analysis suggest that insulin responsiveness of the glucagon gene is not conferred by a particular enhancer-like element but rather depends on the presence of at least one of the glucagon gene enhancer-like elements G2, G3, or CRE.

**Insulin Responsiveness of Glucagon Gene Transcription Depends on the Presence of a Proximal Promoter Element—**To examine the role of proximal promoter elements, a 3′-deletion analysis was performed. Fragments of the glucagon promoter with deletions at their 3′ end were linked to the minimal thymidine kinase promoter (−81 to +52) of herpes simplex virus. This promoter does not respond to insulin (Fig. 3A, construct pT81Luc). The glucagon gene 5′-flanking DNA from −350 to −48 conferred insulin responsiveness (Fig. 3A). A similar insulin responsiveness was observed with further 3′ truncation to −91 (Fig. 3A). When only sequences from −350 to −150 were fused to the thymidine kinase promoter, insulin no longer inhibited gene transcription (Fig. 3A). When compared with pT81Luc (1.00 ± 0.07), the basal activity of the 3′-deleted constructs was 17.04 ± 0.52 (−350/−48GluLuc), 2.93 ± 0.30 (−350/−91GluLuc), 8.41 ± 0.56 (−350/−150GluLuc), and 0.93 ± 0.11 (−350/−210GluLuc) (n = 6 each). These data indicate that the enhancer-like elements within the −350/−150 fragment (G2, G3, CRE) are not sufficient to confer insulin responsiveness to the heterologous promoter; the data suggest that within a 3′-deletion analysis a DNA control element required for insulin responsiveness may have its 3′ boundary and reside between −91 and −150. This portion contains the G4 element.

To examine the role of this DNA region further, sequences between −150 and −91 were internally deleted (construct −350(−150/−91)GluLuc, Fig. 3B). As shown in Fig. 3B, insulin inhibited glucagon gene transcription after this deletion to a similar degree (inhibition by 64 ± 5%) as it did the wild-type promoter (inhibition by 55 ± 6%). When compared with −350GluLuc (100 ± 4%), the basal activity of −350(−150/−91)GluLuc was 64 ± 5%. These results indicate that the G4 element is dispensable for the effect of insulin. When taken together with the results of the 3′ deletion analysis, the data suggest that inhibition of glucagon gene transcription by insulin depends on the presence of one of the proximal promoter elements, G1 or G4.

**Insulin Can Inhibit Pax6 Transcriptional Activity in Glucagon-producing Pancreatic Islet InR1-G9 Cells—**It has been shown previously that, as a synthetic minienhancer in front of the minimal thymidine kinase promoter, four copies of G3A show strong functional interaction (32). This is confirmed in the present study where four copies of G3A raised the trans-
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Pax6 Is Required for Repression of Glucagon Gene Transcription by Insulin—As a first approach to study the role of Pax6 in the repression of glucagon gene transcription by insulin, we coexpressed a portion of the Pax6 protein (amino acids 1–246) that contains the paired domain (without exon 5a) but lacks the transactivation domain and most of the homeodomain. This splice variant of the Pax6 paired domain has been shown to confer insulin responsiveness to a heterologous promoter (see also Fig. 5). In contrast, three copies of the glucagon CRE or four copies of the G2 element did not confer insulin responsiveness to this heterologous promoter (data not shown). G3A contains the PISCES motif (32, 42, 48) binding the paired-domain transcription factor Pax6 (42, 46). Pax6 also binds to the PISCES motif within the proximal promoter element G1 (47–49). To examine more directly whether insulin regulates Pax6 transcriptional activity, the GAL4 system was used. An expression vector encoding full-length Pax6 fused to the DNA-binding domain of the yeast transcription factor GAL4 was transfected into InR1-G9 cells together with a luciferase reporter gene placed under the control of a minimal E1B promoter and multiple GAL4 DNA binding sites (5xGal4E1BLuc) (Fig. 4B). As shown in Fig. 4B, coexpression of the GAL4-Pax6 fusion protein raised transcriptional activity 68 ± 1-fold. Insulin inhibited GAL4-Pax6 transcriptional activity by 30% (Fig. 4B). This effect was specific because insulin did not inhibit the expression of the GAL4-Pax6 fusion protein as revealed by electrophoretic mobility shift assay (Fig. 4C) and Western blotting (data not shown); furthermore, insulin had no effect on the transcriptional activity conferred by the viral VP16 protein (Fig. 4B). Thus, although the extent of inhibition of GAL4-Pax6 transcriptional activity was less than that of −350GluLuc, these results show that insulin can inhibit Pax6 activity in pancreatic islet cells.

Fig. 4. Effect of insulin on Pax6 transcriptional activity. A, effect of insulin on the transcriptional activity of G3A containing a Pax6 binding site. The plasmids −350GluLuc, pT81Luc, and 4xG3A(T81)Luc were transfected into InR1-G9 cells, and the cells were treated with insulin (10 nM) as indicated. Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective control (no treatment). Values are means ± S.E. of three independent experiments, each done in duplicate. *p < 0.005 (Student’s t test). B, inhibition by insulin of Pax6 transcriptional activity as determined using a GAL4 system. Expression vectors encoding GAL4-Pax6 or GAL4-VP16 (1 or 0.5 µg/6-cm dish, respectively) were transfected into InR1-G9 cells together with the 5xGal4(E1B)Luc reporter gene, and the cells were treated with insulin (10 nM) as indicated. Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured after cotransfection of GAL4-Pax6 or GAL4-VP16 without insulin treatment. Values are means ± S.E. of three (GAL4-Pax6) or four (GAL4-VP16) independent experiments, each done in duplicate. Luc, luciferase; Control, no insulin treatment. C, lack of inhibition by insulin of GAL4-Pax6 expression as revealed by electrophoretic mobility shift assay. An expression vector encoding GAL4-Pax6 (1 µg/6-cm dish) was transfected into InR1-G9 cells, and the cells were treated with insulin (10 nM) for 12 or 18 h or were left untreated. Nuclear extracts were prepared and incubated with a labeled GAL4 DNA binding site as described previously (38). The retarded band is shown, which is recognized by an antisera directed against the DNA-binding domain of GAL4 (α-gal4) (Santa Cruz Biotechnology, Heidelberg, Germany). First lane to the left, no nuclear extract added (probe only).
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bind to the PISCES motif (42), the Pax6 binding motif within the G3A and G1 elements of the glucagon gene (42, 43, 48). Through competition for DNA binding, the expression of the Pax6 paired domain can be expected to prevent transactivation domain-dependent functions of endogenous Pax6. As a positive control, the construct 4xG3A(T81)Luc was included in this experiment, since it is driven by a synthetic minihancer built of four copies of the Pax6 binding site within G3A. As shown in Fig. 5, the expression of the Pax6 paired domain decreased basal activity of the construct 4xG3A(T81)Luc by 90% and abolished negative regulation by insulin. This suggests that the expression of the Pax6 paired domain efficiently prevents Pax6 functions. After transfection of −350GluLuc, the expression of the Pax6 paired domain decreased basal glucagon gene transcription by 70% (Fig. 5), confirming that Pax6 is important for glucagon promoter activity. Whereas insulin inhibited glucagon gene transcription in the controls by 65%, it failed to do so in the presence of the Pax6 paired domain (Fig. 5), consistent with the assumption that Pax6 is required for repression of glucagon gene transcription by insulin.

As shown above (Fig. 2 C), four copies of the G2 element confer insulin responsiveness to the insulin nonresponsive truncated glucagon promoter (−136GluLuc). The G2 element does not contain a Pax6 binding site, although the nonresponsive truncated glucagon promoter does (within G1) (42, 43, 47, 48). To examine the role of Pax6 under this condition, the effect of expression of the Pax6 paired domain on G2-driven transcriptional activity was studied. As shown in Fig. 5, the expression of the Pax6 paired domain did not alter basal transcriptional activity of the G2 element in front of the truncated glucagon promoter. However, the expression of the Pax6 paired domain completely abolished the inhibition of transcription by insulin (Fig. 5), suggesting that Pax6 binding to the proximal promoter element G1 is required for insulin responsiveness conferred to the truncated promoter by G2.

As a second approach to study the role of Pax6 in the repression of glucagon gene transcription by insulin, the Pax6 binding sites (PISCES motifs) of the glucagon promoter within G3A or G1 or both were mutated and thereby changed into binding sites of the yeast transcription factor GAL4 (Fig. 6 A). As shown in Fig. 6 B, the mutation of the Pax6 binding sites within G3, G1, and G3 plus G1 markedly decreased basal transcriptional activity of the glucagon promoter to 14.1%, 4.4%, and 1.8% of wild type, respectively, again confirming that Pax6 is important for basal glucagon promoter activity. The remaining low transcriptional activities of the mutant glucagon promoters were only slightly inhibited by insulin (Fig. 6 B). Due to potentially overlapping binding sites, the mutation of the PISCES motifs may not only abolish Pax6 binding but also affect the binding of additional transcription factors like cdx2/3 and brain-4 within G1 (47, 49–51). We therefore examined whether basal activity and insulin responsiveness of the glucagon promoter can be restored by Pax6 recruited to the double mutant glucagon promoter through the GAL4 binding sites. When an expression vector encoding a GAL4-Pax6 fusion protein was transfected together with −350(mutG1/G3)GluLuc, basal transcriptional activity of the doubly mutated glucagon promoter was raised to a level similar to that of the wild-type promoter (Fig. 6 C). The expression of GAL4-Pax6 also conferred insulin

InR1-G9 cells together with the −350(mutG1/G3)GluLuc reporter gene, and the cells were treated with insulin (10 nm) or left untreated (control); for comparison, the wild-type −350GluLuc construct was also transfected (−350). Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the GAL4-Pax6 or GAL4-VP16 controls. Values are means ± S.E. of three independent experiments, each performed in duplicate.
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Inhibition by insulin of GAL4-CBP activity in the context of the glucagon promoter. An expression vector encoding GAL4-CBP (pGAL-CBPS, 2 μg/6-cm dish) was transfected into INR1-G9 cells together with -350(mutG1/G3)GluLuc or 5xGal4(E1B)Luc reporter gene, and the cells were treated with insulin (10 nm) or left untreated (control). Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective GAL4-CBP control. Values are means ± S.E. of three independent experiments, each done in duplicate.

responsiveness (Fig. 6C). After cotransfection of -350(mutG1/G3)GluLuc and the GAL4-Pax6 expression vector, insulin inhibited transcription by 43 ± 1%; this is similar to the inhibition by insulin of the wild-type glucagon promoter activity (56 ± 3%) (Fig. 6C). This effect of GAL4-Pax6 seems to be specific and also not secondary to the restoration of basal activity, because the expression of GAL4-VP16 restored basal activity of the doubly mutated glucagon promoter but did not confer insulin responsiveness (Fig. 6C). When taken together, these results suggest that Pax6 is not sufficient but required for insulin responsiveness of the glucagon promoter.

The Coactivator CBP Can Confer Insulin Responsiveness to the Glucagon Promoter but Not to the Viral E1B Promoter—Evidence suggests that the p300/CBP proteins may function as coactivators of Pax6 (52). To test whether CBP, like Pax6, can confer insulin responsiveness to the glucagon promoter, CBP was fused to the DNA-binding domain of GAL4. When co-transfected together with the mutated glucagon reporter gene, in which both Pax6 binding sites within G1 and G3 had been mutated into GAL4 binding sites, GAL4-CBP conferred basal activity and insulin responsiveness (Fig. 7). In contrast, when co-transfected with a reporter construct, in which multiple GAL4 binding sites had been placed in front of the truncated viral E1B promoter, GAL4-CBP conferred transcriptional activity that was not inhibited by insulin (Fig. 7). These data are consistent with the assumption that GAL4-Pax6 may confer insulin responsiveness to the glucagon promoter through the recruitment of CBP.

**DISCUSSION**

The molecular mechanism of inhibition of glucagon gene transcription by insulin is poorly understood. The present study confirms (27) that 350 base pairs of the glucagon gene 5'-flanking region are sufficient for negative regulation by insulin in a glucagon-producing pancreatic islet cell line. Insulin seems to interfere with mechanisms that dictate basal transcriptional activity of the glucagon gene, since the -fold stimulation of glucagon gene transcription by cAMP or membrane depolarization and calcium influx was similar in the presence or absence of insulin. In pancreatic islets, basal glucagon gene transcription seems to be conferred by a unique combinatorial and spatial arrangement of synergizing control elements and interacting proteins, some of which have now been identified (53). This view is confirmed in the present study, where an internal mutation or deletion of the G1, G2, or G3 element all decreased basal transcription by more than 80%. However, using 5', 3', and internal deletions, the present study failed to localize a single IRE in the glucagon gene 5'-flanking region. Insulin responsiveness rather requires proximal promoter elements (G1, G4) as well as more distal enhancer-like elements (G2, G3, CRE). According to a current view of enhancer function, specific interactions between enhancer-binding proteins and factors that bind proximal promoter elements are important to achieve enhancer-promoter selectivity (54). This may hold true also for the glucagon promoter, since internal deletions of the proximal promoter element G1 decrease glucagon gene transcription by about 95%, although the truncated glucagon promoter, including the proximal promoter elements G1 and G4, possesses only very low transcriptional activity (Ref. 44 and this study). We thus conclude that insulin responsiveness of the glucagon gene is conferred by an interaction between proximal promoter elements and more distal enhancer-like elements. The results of the present study raise the possibility that insulin may interfere with the function of a promoter-specific nucleoprotein coactivator complex, which integrates the activities of the transcription factors bound to the glucagon gene 5'-flanking region and establishes productive enhancer-promoter interactions.

This conclusion implies that, more or less, any DNA control element and transcription factor that takes part in the recruitment and positioning of the promoter-specific nucleoprotein complex contributes to both full basal activity and insulin responsiveness. The paired-domain transcription factor Pax6 may be of particular importance. One of the two Pax6 splice variants expressed in mature pancreatic islets and islet cell lines (42) binds through the PISCES motif to an enhancer-like element (G3A) and, possibly together with cdx2/3 or brain-4 (50, 51), to a proximal promoter element (G1) of the glucagon gene (42, 43, 46–49). The binding of Pax6 to both elements is critical for basal activity as indicated by the effects of internal deletions (44, this study). The mere presence of a Pax6 binding site is not sufficient for insulin responsiveness since reporter genes containing the truncated glucagon promoter (−136GluLuc) or the -deleted glucagon promoter fragment from −350 to −150 include a Pax6 binding site but are not negatively regulated by insulin. However, Pax6 seems to be required for the glucagon gene response to insulin. First, the activity of an artificial minienhancer consisting of synergizing Pax6 binding sites (G3A) in front of a heterologous promoter as well as Pax6 activity when assessed using a GAL4/viral E1B system, were inhibited by insulin in the islet cell line, although less than glucagon gene transcription. Second, the overexpression of the transactivation incompetent DNA-binding paired domain of Pax6 as well as the mutation of the Pax6 binding sites markedly decreased or abolished both basal activity and insulin responsiveness of the glucagon gene. Third, the overexpression of the Pax6 paired domain abolished the insulin responsiveness without changing the basal activity of a G2 minienhancer in front of the truncated glucagon promoter. Finally, when the Pax6 binding sites of the glucagon gene 5'-flanking region were mutated into GAL4 binding sites, the expression of GAL4-Pax6 restored basal activity and conferred negative regulation by insulin. Pax6 seems to exert a specific function, since insulin responsiveness was not conferred when basal activity was restored by the expression of GAL4-VP16. The herpes simplex virus VP16 protein has an acidic activation domain and interacts with a TATA box-binding protein-associated factor of...
TFIID, like hTAFII31 (55) and histone acetyltransferase combinations of an IRE-independent mechanism of inhibition of gene transcription by insulin. Indeed, the transcriptional activity conferred by GAL4-CBP to doubly mutated glucagon promoter was inhibited by insulin. In contrast, insulin did not affect GAL4-CBP activity using a reporter gene with GAL4 binding sites in front of the minimal viral E1B promoter, indicating that the specific glucagon promoter context is required for the effect of insulin on CBP activity. If Pax6 functions through CBP recruitment, the fact that GAL4-Pax6 but not GAL4-CBP confers insulin responsiveness to the 5xGal4/E1B-Luc reporter gene may be explained by the assumption that CBP may assume a different conformation and may function differently when bound to the promoter through recruitment by Pax6 or through fusion with the DNA-binding domain of GAL4. CBP and the closely related protein p300 are modular proteins with multiple functional domains (58–61). In addition to Pax6, other transcription factors that bind to the glucagon gene 5’-flanking region can interact with CBP including Beta2/E47/E12 basic helix-loop-helix proteins (62–64), NFATp (38, 65), Ets-like transcription factors (31, 66), and cAMP-responsive element-binding protein (35, 39, 67). Interestingly, the functional domains of CBP are differentially utilized by different transcription factors, implying conformational differences in the CBP-based coactivator complex bound to different classes of transcription factors (59, 60). Thus, through multiple contacts with CBP or through recruiting other coactivators, the specific glucagon promoter context may induce the formation of a promoter-specific nucleoprotein complex. The results of the present study suggest that Pax6 and CBP may be essential components of such a complex, which integrates the activities of proximal promoter elements and more distal enhancer-like elements and the function of which is sensitive to insulin.

The −292 glucagon reporter gene construct was less inhibited by insulin than constructs containing longer (350 base pairs) or shorter (200 base pairs) fragments. The set of DNA control elements within 292 base pairs appears thus to induce the formation of a distinct protein complex that functions in a less insulin sensitive manner. Most of the effect of insulin was lost after 5’-deletion of the glucagon promoter to −256 or shorter fragments in a previous study (27), whereas in the present study insulin was able to inhibit glucagon gene transcription after a 5’-deletion to −200. The reason for this discrepancy is not clear. Noteworthy, qualitatively consistent with the present study, a small but persistent inhibition by insulin of chloramphenicol acetyltransferase activity was observed in the present study, though members of the Forkhead family of transcription factors like AFX, FKHR, and FKHRL1 have been shown to bind to this sequence motif and to be regulated by insulin (7–9), it remains unclear whether and how they interfere in the presence of glucocorticoid-induced PEPCK and IGFBP-1 gene transcription. In contrast to this model, the results of the present study, are more consistent with recent suggestions of an IRE-independent mechanism of inhibition of gene transcription by insulin (16–19). In glucocorticoid-induced 6-phosphofructo-2-kinase gene transcription (17, 18) and in cAMP-induced PEPCK gene transcription (19) it has been shown that in each case a combination of several DNA elements and interacting transcription factors, called glucocorticoid or cAMP response unit, respectively, are required for both full responsiveness to the respective stimulus and inhibition by insulin. Site-directed mutational analysis revealed that none of the factors seems to be individually involved in the inhibitory effect of insulin (17–19). This led to the conclusion that the unique array of factors may be recognized, or stabilized, by a specific coactivator complex and that inhibition of gene transcription by insulin may result from the disruption of this higher order complex (17–19). Evidence suggests that CBP is part of this complex on the PEPCK promoter (16, 19). The finding that the mitogen-regulated S6 kinase pp90RSK binds to the C/H3 region of CBP and regulates CBP function (69) gives an example how an intermediate in a signaling cascade is able to interact with CBP and control gene transcription. The results of the present study support such concepts of IRE-independent mechanisms of insulin action to inhibit gene transcription.

It has been reported recently that zebrafish Pax6 is phosphorylated at three sites within its transactivation domain by the mitogen-activated protein kinases extracellular signal-regulated kinase and p38 kinase (70). However, the mutation of all three of these sites did not affect the ability of insulin to inhibit Pax6 transcriptional activity in InR1-G9 cells. Whereas these findings do not support a role for these mitogen-activated protein kinase phosphorylation sites in insulin action on the glucagon gene, Pax6 contains putative phosphorylation sites for other kinases. The insulin-induced signaling pathway to the glucagon gene and its target transcription factor/coactivator(s) remains to be defined.

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REFERENCES

1. O’Brien, R. M., and Graner, D. K. (1996) Physiol. Rev. 76, 1109–1161
2. O’Brien, R. M., and Graner, D. K. (1991) Biochem. J. 278, 609–619
3. O’Brien, R. M., Lucas, P. C., Forest, C. D., Magnuson, M. A., and Graner, D. K. (1990) Science 249, 533–537
4. Meyer, T. E., and Habener, J. F. (1993) Endocr. Rev. 14, 289–290
5. Chapman, S. C., Ayala, J. E., Streeper, R. S., Cubert, A. B., Eaton, E. M., Svitak, C. A., Goldman, J. K., Tavare, J. M., and O’Brien, R. M. (1999) J. Biol. Chem. 274, 18625–18634
6. Kops, G. J. P. L., de Ruiter, N. D., De Vries-Smits, A. M. M., Powell, D. R., Bos J. L., and Burgering, B. M. T. (1999) Nature 398, 630–634
7. Benezet, A., Banni, A., Zigmond, M. J., Lin, M. X., Jin, P. J., Hu, L. S., Anderson, J. M., Arden, K. B., Bennis, C. J., and Greenberg, M. E. (1999) Cell 96, 857–868
8. Durham, S. K., Swansonchik, A., Scheimann, A. O., Yee, D., Jackson J. G., Durr, F. G., and Powell, D. R. (1999) Endocrinology 140, 3140–3146
9. Ouyang, L., Jacob, K. K., and Stanley, F. M. (1990) J. Biol. Chem. 271, 10425–10429
10. Streeper, R. S., Chapman, S. C., Ayala, J. E., Svitak, C. A., Goldman, J. K., Cave, A., and O’Brien, R. M. (1998) Mol. Endocrinol. 12, 1778–1791
11. Barroso, I., and Sanz, B. (1991) J. Biol. Chem. 266, 18494–18497
12. Wang, D., and Sul, H. S. (1997) J. Biol. Chem. 272, 26367–26374
13. Thompson, M. J., Roe, M. W., Malik, R. K., and Blackshear, P. J. (1994) J. Biol. Chem. 269, 21127–21133
14. Leahy, P., Crawford, D. R., Grossmann, G., Gronostajski, R. M., and Hanson, R. W. (1999) J. Biol. Chem. 274, 8813–8822
15. Pierschbacher, M. D., and Loew, E. C. (1999) Mol. Cell. Endocrinol. 147, 1–5

2 R. Grzeszkowiski and W. Kneple, unpublished observation.
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(1998) Mol. Endocrinol. 12, 1343–1354
19. Yeagley, D., Agati, J. M., and Quinn, P. G. (1998) J. Biol. Chem. 273, 1873–1874
20. Philippe, J. (1998) J. Clin. Invest. 85, 672–677
21. Lefebvre, P. J. (1995) Diabetes Care 18, 715–719
22. Unger, R. H., and Orci, L. (1981) N. Engl. J. Med. 304, 1518–1524
23. Unger, R. H., and Orsi, L. (1981) N. Engl. J. Med. 304, 1575–1580
24. Creutzfeldt, W. O. C., Kleine, N., Willms, B., Orskov, C., Holet, J. J., and Nauck, M. A. (1996) Diabetes Care 19, 580–586
25. Drucker, D. J. (1998) Diabetes 47, 159–169
26. Freyse, E. J., Becher, T., El-Hag, O., Knope, S., Goke, B., and Fischer, U. (1997) Diabetes 46, 824–828
27. Philippe, J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7224–7227
28. Nordeen, S. K. (1988) BioTechniques 6, 454–457
29. Schwaninger, M., Lux, G., Blume, R., Oetjen, E., Hidaka, H., and KnepeL, W. (1995) J. Biol. Chem. 268, 5168–5177
30. Krüger, M., Schwaninger, M., Blume, R., Oetjen, E., and KnepeL, W. (1997) Naunyn-Schmiedeberg's Arch. Pharmacol. 356, 433–440
31. Fürstenau, U., Schwaninger, M., Blume, R., Kennerknecht, I., and KnepeL, W. (1997) Mol. Cell. Biol. 17, 1805–1816
32. Wrez, A., Diedrich, T., Hochhut, C., and KnepeL, W. (1995) Gene Exp. 4, 205–216
33. Chrivia, J. C., Kwok, R. P. S., Lam, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) Nature 365, 855–859
34. Roberts, S. G. E., and Green, M. R. (1994) Nature 371, 717–719
35. Oetjen, E., Diedrich, T., Eggers, A., Eckert, B., and KnepeL, W. (1994) J. Biol. Chem. 269, 27036–27044
36. Sadowski, I., and Ptashne, M. (1989) Nucleic Acids Res. 17, 7539
37. Takaki, R., Ono, J., Nakamura, M., Yokogawa, Y., Kumae, S., Hiraoa, T., Yamauchi, T., Hamaguchi, K., and Uchida, S. (1986) In Vitro Cell Dev. Biol. 22, 120–126
38. Fürstenau, U., Schwaninger, M., Blume, R., Jendrusch, E. M., and KnepeL, W. (1999) J. Biol. Chem. 274, 5851–5860
39. KnepeL, W., Chafiz, J., and Habener, J. F. (1990) Mol. Endocrinol. 4, 6799–6804
40. Schwaninger, M., Blume, R., Oetjen, E., Lux, G., and KnepeL, W. (1993) J. Biol. Chem. 268, 22111–22115
41. Schwaninger, M., Blume, R., Krüger, M., Lux, G., Oetjen, E., and KnepeL, W. (1995) J. Biol. Chem. 270, 8860–8866
42. Beimensche, S., Neubauer, A., Herzig, S., Grzeskowiak, R., Diedrich, T., Cierney, I., Scholz, D., Alejel, T., and KnepeL, W. (1999) Mol. Endocrinol. 13, 718–728
43. KnepeL, W., Jepel, L., and Habener, J. F. (1990) J. Biol. Chem. 265, 8725–8732
44. Philippe, J., Drucker, D. J., KnepeL, W., Jepel, L., Misurlovin, Z., and Habener, J. F. (1988) Mol. Cell. Biol. 8, 4877–4888
45. Cordis: Bauan, M., Morel, C., and Philippe, J. (1995) Mol. Cell. Biol. 15, 3904–3916
46. Sander, M., Neubauer, A., Kalamaras, J., He, H. C., Martin, G. R., and German, M. S. (1997) Genes Dev. 11, 1662–1673
47. Anderson, P. G., Keller, R. S., Petersen, H. V., Jensen, J., Madsen, O. D., and Serup, P. (1999) FEBS Lett. 445, 306–310
48. KnepeL, W., Vallejo, M., Chafiz, J. A., and Habener, J. F. (1991) Mol. Endocrinol. 5, 1457–1466
49. Ritz-Laser, B., Estreicher, A., Klages, N., Saule, S., and Philippe, J. (1999) J. Biol. Chem. 274, 4124–4132
50. Hussain, M. A., Lee, J., Miller, C. P., and Habener, J. F. (1997) Mol. Cell. Biol. 17, 7196–7204
51. Jin, T., and Drucker, D. J. (1996) Mol. Cell. Biol. 16, 19–28
52. Hussain, M. A., and Habener, J. F. (1999) J. Biol. Chem. 274, 28955–28957
53. KnepeL, W. (2000) in Molecular Basis of Endocrine Pancreas Development and Function (Hussain, M. A., Miller, C. P., and Habener, J. F., eds) Kluwer Academic Publishers, Boston, in press
54. Blackwood, E. M., and Kadowaga, J. T. (1998) Science 281, 60–63
55. Uesugi, M., Nyanguile, O., Lo, H., Levine, A. J., and Verdone, G. L. (1997) Science 277, 1310–1313
56. Utley, R. T., Ikeda, K., Grant, P. A., Cote, J., Steger, D. J., Eberharter, A., John, S., and Workman, J. L. (1998) Nature 394, 498–502
57. Tang, H. K., Singh, S., and Saunders, G. F. (1998) J. Biol. Chem. 273, 7210–7221
58. Eckner, R. (1996) Biol. Chem. 377, 685–688
59. Korza, E., Torchiia, J., Rose, D. W., Xu, L., Kurokawa, R., McInerney, E. M., Mullen, T. M., Glass, C. K., and Rosenfield, M. G. (1998) Science 278, 703–707
60. Kurokawa, R., Kallafus, D., Ogliastro, M. H., Kioussis, C., Xu, L., Torchiia, J., Rosenfield, M. G., and Glass, C. K. (1998) Science 279, 700–703
61. Torchiia, J., Glass, C., and Rosenfield, M. G. (1998) Curr. Opin. Cell Biol. 10, 373–383
62. Dumontelle, E., Lasser, B., Constant, I., and Philippe, J. (1998) J. Biol. Chem. 273, 19945–19954
63. Mutoh, H., Naya, F. J., Tsai, M. J., and Leiter, A. B. (1998) Genes Dev. 12, 820–830
64. Qiu, Y., Sharma, A., and Stein, R. (1998) Mol. Cell. Biol. 18, 2957–2964
65. Garcia-Rodriguez, C., and Rao, A. (1998) J. Exp. Med. 187, 2031–2036
66. Yang, C., Shapiro, L. H., Rivera, M., Kumar, A., and Brindle, P. K. (1998) Mol. Cell. Biol. 18, 2218–2229
67. Kwok, R. P. S., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. E. G., Green, M. R., and Goodman, R. H. (1994) Nature 370, 223–226
68. O'Brien, R. M., Noisin, E. L., Suwanichkul, A., Yamazaki, T., Lucas, P. C., Wang, J. C., Powell, D. R., and Granner, D. K. (1995) Mol. Cell. Biol. 15, 279–288
69. Nakajima, T., Fukamiu, A., Takahashi, J., Gage, F. H., Fisher, T., Blenis, J., and Montminy, M. R. (1996) Cell 86, 465–474
70. Monina, I., Bruun, J., Björkøy, G., Holm, T., and Johansen, T. (1999) J. Biol. Chem. 274, 15115–15126