Identification of a Glucose Response Element in the Promoter of the Rat Glucagon Receptor Gene*

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We cloned the 5′ upstream region of the rat glucagon receptor gene, demonstrating that the 5′ noncoding domain of the glucagon receptor mRNA contained two untranslated exons of 131 and 166 nucleotides (nt), respectively, separated by two introns of 0.6 and 3.2 kilobase pairs. We also observed an alternative splicing involving the 166-base pair exon. Cloning of up to 2 kilobase pairs of the newly identified genomic domain and transfection of various constructs driving a reporter gene, in pancreatic islet cell line INS-1, uncovered a strong glucose regulation of the promoter activity of plasmids containing up to nucleotide −868, or more, upstream from the transcriptional start point. This promoter activity displayed threshold-like behavior, with low activity of the promoter below 5 mM glucose, and maximal activation as of 10 mM glucose. This glucose regulation was abolished by mutation of the “E-boxes” CACGTG of this sequence located near −600 nt. Cloning of a fragment containing this palindrome and two additional nucleotides 5′ to it (CACGTG and CAGCTG) separated by 3 nt, a feature similar to the “L4 box” found in the pyruvate kinase L gene promoter. This is the first description of a G protein-coupled receptor gene promoter regulated by glucose.

The primary physiological role of glucagon, together with insulin, is maintenance of normal glycemia. The liver has a central role in handling absorbed nutrients and in the regulation of hepatic glycogen disposability; this requires high density of glucagon receptors in the liver (for review see Refs. 1 and 2). The glucagon receptor mRNA has also been detected at variable levels in other tissues such as heart, kidney, adrenal gland, and adipose tissue (3–5), as well as in pancreatic islets, especially in B cells (6, 7). The expression of the glucagon receptor mRNA is stimulated by glucagon and inhibited by cyclic AMP, both in liver (8) and in cultured endocrine cells (9). Consequently, the promoter of the glucagon receptor gene could contain regulatory elements for these factors. It has been recently suggested by Burrell et al. (10) that the glucose regulation of the glucagon receptor mRNA level differs from the glucose regulation of other genes such as Glut2, and might be mediated by triose metabolites, suggesting the existence of new enhancer sequences. To address this question, a prerequisite is the precise knowledge of the complete 5′ mRNA sequence. For the glucagon receptor mRNA, there was an ambiguity; the glucagon receptor cDNA has been first cloned (from rat) simultaneously by us (11, 12) and by Jelinek et al. (13). Our sequence (GenBank accession number L04796) was almost identical to that of Jelinek (GenBank accession number M96674) except for the 5′ end; the first 25 nt in our sequence (corresponding to positions −105 to −80 from the ATG codon) differed from the sequence published by Jelinek (13). The coding domain of the rat glucagon receptor gene is highly fragmented; it contains 12 introns with uneven splicing maturation (14) that may produce cloning artifacts resulting in the observed differences described for the 5′ sequences. Therefore, we first identified the correct organization of the 5′ end of the rat glucagon receptor mRNA. Thereafter, we cloned a fragment of genomic DNA located upstream from the transcription start point, and identified this fragment as a glucose activable promoter region using reporter gene studies. Finally, we localized a L4-like box sequence as the central motif of the glucose-induced gene stimulation.

MATERIALS AND METHODS

General Methods—Rat liver and heart fragments were homogenized in 4 M guanidine isothiocyanate, and total RNA was purified on CsCl gradient (15); poly(A) RNA was purified on oligo(dT)-cellulose (Invitrogen).

Reverse transcription was performed using Superscript II™ (Life Technologies, Inc.). Anchored PCR of the 5′ end was performed after single-strand ligation of phosphorylated oligonucleotide to cDNA using the 5′ RACE kit (CLONTECH). Subcloning of PCR product was carried out by TA ligation to the pCRII plasmid (Invitrogen).

Sequence of plasmids purified on Qiagen™ columns was performed with the Sequenase or Thermosequenase kit (U.S. Biochemical Corp.; Amersham). Polymerase chain reaction was performed as indicated in the legend of figures. Analysis of amplified DNA fragments on agarose gel, transferred to nitrocellulose filters and hybridized to 5′-32P-labeled oligodeoxynucleotide was, performed as detailed by Sambrook et al. (15).

Genomic Cloning and Construction of Reporter Gene Plasmids—Rat genomic DNA, partially digested with Sau3AI (15), was cloned in XhoI-digested and partially filled-A-FixI1 bacteriophage DNA (Stratagene), and packaged as recommended by the manufacturer. The resulting primary genomic library was screened at 40,000 plaque-forming units/plate.

* This work was supported in part by a grant from the Fonds de la Recherche Scientifique Médicale 3.4507.98, a Bekales grant, and a grant from the Association Belge du Diabète (Brussels, Belgium). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate that the costs of publication were defrayed in part by reason of the appearance of this advertisement in a journal published by the American Society for Biochemistry and Molecular Biology.

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‡ Recipient of a doctoral fellowship from the Fonds Pour la Formation à la Recherche Dans L’Industrie et Dans L’Agriculture (FRIA)-Belgium.

§ Recipient of a doctoral fellowship from the Interuniversity Poles of Attraction Program, Belgian State Prime Minister’s Office, Federal Office for Scientific, Technical and Cultural Affairs.

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1 The abbreviations used are: nt, nucleotide(s); PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; CDS, coding DNA sequence; GPCR, G protein-coupled receptor; GLP-1, glucagon-like peptide 1; PACAP, pituitary adenyl cyclase activating peptide; PKL, pyruvate kinase L; VIP, vasoactive intestinal peptide; bp, base pair(s); kb, kilobase pair(s); RACE, rapid amplification of cDNA ends; tk, thymidine kinase; HSV, herpes simplex virus; PTH, parathyroid hormone.
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145-mm plate. We initially screened the λ-FixII library with 32P-labeled oligonucleotides complementary to nt +147 to +128 and to nt +772 to +739 of the glucagon receptor CDS. One positive clone (P 10-C) was isolated. First, a 3.5-kb XbaI/PstI fragment was subcloned into pUC18. Second, a 1-kb XbaI/XmaI fragment located upstream from of the XbaI/PstI fragment was subcloned in pZErO™-1 (Invitrogen). The XbaI/PstI fragment contained a 2.5-kb sequence corresponding to the 5’ end of the coding domain of the glucagon receptor gene (Ref. 14; GenBank accession number L31574), and, in addition, a 1-kb sequence upstream from the previously described initiation codon. The 5’ end extended sequence is listed in GenBank under accession number U63021.

An oligonucleotide probe based on the 5’ end of this fragment was used as a probe to subclone a 2-kb PstI/SacI fragment in pBluescript SK+ resulting in clone “P.S.” The two subcloned fragments contain a 130-bp overlap with a unique XbaI site. We ligated a HindIII/XbaI fragment of clone P/S with a XbaI/XmaI fragment of the clone B/B in the pBlCat6 vector digested with HindIII/XbaI. The sequence of this constructed plasmid (called clone P/ctr/B) was deposited in GenBank under accession number U63022.

In a second screening we used 32P-labeled oligonucleotides J.for and S.for (described below). One positive λ-FixII clone (JS 3-A) was isolated, and a 1.3-kb BamHI/BamHI hybridizing fragment was subcloned first in p2ErO™-1 (Invitrogen) and then in the BamHI site of pBlCat6 vector (clone B/B).

An oligonucleotide probe based on the 5’ end of this fragment was used as a probe to subclone a 2-kb PstI/SacI fragment in pBluescript SK+ resulting in clone “P.S.” The two subcloned fragments contain a 130-bp overlap with a unique XbaI site. We ligated a HindIII/XbaI fragment of clone P/S with a XbaI/XmaI fragment of the clone B/B in the pBlCat6 vector digested with HindIII/XbaI. The sequence of this constructed plasmid (called clone P/ctr/B) was deposited in GenBank under accession number U63022.

In the first transfection experiments, we used clones B/B and P/ctr/B. These plasmids gave only a low reporter gene activity in transfected cells (see below); these plasmids bear approximately 1 kb of sequence situated downstream from the mRNA start point. This intervening sequence may prevent the efficient transcription of the reporter gene. We therefore prepared shortened plasmids by deletion of the sequence located immediately after the end of the first untranslated exon, using a unique Apal site.

The clone B/B was digested by Apal and XhoI, blunted and ligated to give clone “pCB.” The clone P/ctr/B was digested by Apal and NotI, blunted and ligated to give the clone “pCC.” The latter clone contained a XhoI site located 1 kb upstream from its 3’ end (ex-Apal site), and a second XhoI site in the polylinker, so that XhoI digestion excised a 1-kb insert, which was subcloned in XhoI site of pBlCat6 vector in both its normal and reverse orientation (clones pCX and pCXr).

The clone pCC was digested by BamHI, and a 0.5-kb fragment was removed; the plasmid was then recirculated by ligation. The first BamHI site is in position –287, and the second BamHI site is in the polylinker of pBlCat6, so BamHI digestion removed from the plasmid pCCX the 0.5-kb A/CCTTAT fragment equivalent to the pCB plasmid insert. The primer remaining primer domain (–2 kb to –287) was then excised by HindIII/BamHI and subcloned in the HindIII/BamHI sites of the pBlCat5 plasmid, i.e. upstream from the HSV thymidine kinase basal promoter. The resulting plasmid was called pTkCd.

Mutations—Mutations were introduced using the QuickChange site-directed mutagenesis kit from Stratagene according to manufacturer’s recommended procedure. Measurement of galactosidase activity was highly reproducible, with intra-assay variation of less than 25%.

Clonamphenicol acetyltransferase (CAT) activity of transfected cells was assayed by acylation of [14C]chloramphenicol (ICN) using β-butyryl-CoA (Promega) as an acetyl donor. An aliquot of the cell extract from individual wells was used for CAT assays and TLC analysis. Autoradiographic densities (measure of “volume” of absorption: integrated optical densities of the spot surface) were estimated using “Viber-Lourmat” image analysis apparatus. In control experiments we found a linear correlation between the amount of CAT in the assay and the “volume” of absorption measured in the range of 0–125 milliunits. Activity of our samples from transfected cells never exceeded 100 milliunits of CAT.

RESULTS

Amplification of cDNA Using Primers Based on the Conflicting Sequences—Analysis of the 5’ end sequence of the glucagon receptor mRNA was obtained by amplification of rat liver and heart cDNA using two forward primers J.for and S.for, based on the conflicting sequences reported in the literature. A forward primer based on an unambiguous sequence in the 5’ end was used as a control. Fig. 1A shows the amplified products electrophoretic pattern. The amplification specificity was confirmed by Southern blotting, using internal oligodeoxynucleotides as probes (data not shown).

The major products were subcloned and sequenced. Obtained sequences, confirmed later by sequencing products of anchored PCR and by sequencing cloned genomic DNA (see below), demonstrated that glucagon receptor mRNAs contain two untranslated exons, and that the second one could be spliced out. We called the first 5’ untranslated exon (described by Jelinek et al.; Ref. 13) U1, and the second alternatively spliced exon (described by Svoboda et al.; Ref. 11) US. These results are illustrated in Fig. 1B.

Anchored PCR of cDNA 5’ Ends (5’ RACE)—To identify the mRNAs transcription start site, and estimate the relative abundance of these polymorphic glucagon receptor mRNA populations, we performed 5’ end-anchored PCR.

We ligated liver and heart single-stranded cDNA to a phosphorylated anchor primer, and then used three different reverse primers to amplify this anchored cDNA. The procedure is detailed in the legend of Fig. 2. Hybridization using the internal labeled oligonucleotide probe (R-20) allowed us to visualize a major band with a smear for the liver and two separate bands for the heart (Fig. 2). The same pattern was observed in the three tested conditions. Shorter exposure clearly indicated that most of the DNA amplified from liver had the same mobility as the smaller fragment amplified from the heart. The smaller band corresponded to approximately 350 bp with R147, and about 160 bp with R-20 reverse primer. The size of the larger fragment amplified from the heart was in good agreement with the results expected in the presence of an additional 166-bp unspliced 5’ exon. These results indicated that the average size of the 5’ untranslated end of the glucagon receptor mRNA was approximately 180 bp + 180 + 166 bp.

We subcloned the products of the second step of the anchored PCR into the pCRII vector, and hybridized recombinant colonies with 5’-labeled J.for and S.for oligonucleotides. 90% of colonies originating from the liver hybridized with “J.for” only and did not contain the US exon (confirmed by sequencing). In contrast, among the 55 colonies originating from the heart, 21 hybridized with the “J.for” probe only, 31 (representing the longer form comprising the 166-bp US exon) hybridized with

μl of Doser reagent/35-mm diameter well. After 5 h, the transfection medium was replaced by RPMI 1640 medium, with variable glucose concentrations.

In several experiments, the transfection yield was estimated by cotransfection with 0.5 μg of pcDNA3.1/his/lacZ (Invitrogen). Galactosidase activity was quantified on 2% of the cell extract using o-nitrophenyl-β-D-galactopyranoside (Invitrogen) according to the manufacturer’s recommended procedure. Measurement of galactosidase activity was highly reproducible, with intra-assay variation of less than 25%.

Clonamphenicol acetyltransferase (CAT) activity of transfected cells was assayed by acylation of [14C]chloramphenicol (ICN) using β-butyryl-CoA (Promega) as an acetyl donor. An aliquot of the cell extract from individual wells was used for CAT assays and TLC analysis. Autoradiographic densities (measure of “volume” of absorption: integrated optical densities of the spot surface) were estimated using “Viber-Lourmat” image analysis apparatus. In control experiments we found a linear correlation between the amount of CAT in the assay and the “volume” of absorption measured in the range of 0–125 milliunits. Activity of our samples from transfected cells never exceeded 100 milliunits of CAT.
both “J.for” and “S.for” probes, and 3 colonies hybridized with “S.for” only and contained truncated US exon sequences. This result suggested that all transcripts originated from a single promoter located upstream UJ exon, rather than from two separate promoters found upstream from each untranslated exon. The reason for a tissue-specific use of the second US exon remains unclear.

To identify the transcription start point, we subcloned the products of the second anchored PCR step in the pCRII vector, and sequenced the 5’ end of 30 clones originating from both liver and heart. All the inserts yielded the same sequence, but starting at various points ranging from 73 to 131 nt upstream from the 3’ end of exon UJ (see Fig. 3). These sizes were in good agreement with the average size of the 5’ RACE amplified fragments shown in Fig. 2.

A supplement verification of the 5’ mRNA length was performed by two additional independent anchored PCR. Primers were located on exon UJ, in order to favor the longest 5’ transcripts. In a population of 20 clones, we did not find a sequence exceeding the point indicated in Fig. 3B by “start exon UJ,” strongly suggesting that we had indeed identified the actual mRNA 5’ end.

Screening of Rat Genomic Library and Genomic Organization—We constructed and screened a bacteriophage λ-FixII rat genomic DNA library. The first genomic clone obtained contained the complete glucagon receptor coding domain, and 1.5-kb sequence upstream from the ATG initiation codon. The genomic DNA sequence upstream from position nt 279 (GenBank accession number U63021) diverged from both published cDNA 5’ end sequences and represented thus obviously an intron. This sequence showed 83% identity (over 992 nt upstream from the ATG) with the reported putative mouse glucagon receptor gene promoter (GenBank accession number L38612) (17).

In the second screening, we obtained DNA fragments located up to 5 kb upstream from the initiation codon (GenBank accession number U63022). The genomic organization of the whole glucagon receptor gene deduced from these data is illustrated in Fig. 3A. The comparison of the rat glucagon receptor genomic sequence with published cDNA sequences confirmed the existence of two exons in the 5’ untranslated domain. Even our longest 5’-cDNA clones sequences were identical to the genomic DNA sequence. These clones indicate the putative
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Fig. 2. Southern blot of anchored PCR of the 5' end of glucagon receptor cDNA (5' RACE) from liver and heart. The liver and heart mRNA were reverse transcribed using the R426 reverse primer (complementary to nt +426 to 408) of the glucagon receptor CDS. The reaction product was purified and ligated to a phosphorylated anchor primer as recommended by the manufacturer (CLONTECH 5’ RACE kit). In the first amplification step, the ligated cDNA was amplified using the PCR anchor primer (CLONTECH) together with R356 (based on nt +356 to +339, samples 1–4) or R147 (based on nt +147 to +128, samples 5 and 6) reverse primers. The product of the first PCR was diluted 1000 times and reamplified using the same PCR anchor primer, and reverse primers R147 (lanes 1 and 2) or R-20 (based on nt −20 to −39, lanes 3–6). The product of the second PCR was separated on 1.2% agarose gel, transferred to nitrocellulose membrane, hybridized using R-20-labeled oligonucleotide as a probe, and autoradiographed.

transcription start site (Fig. 3B). The experimentally determined size of 5’ domain of cDNA (Fig. 2) is in good agreement with this position of the start point.

The sequence neighboring the putative start site was in relative agreement with the consensus sequence of promoters lacking TATA boxes. The genomic sequence upstream from this putative start site was highly (G + C)-rich and could correspond to the basal gene promoter. Moreover, we found at position −527 to −545 upstream from the transcription start point, highly palindromic sequence of 19 nucleotides containing a canonical E-box “CACGTG”, followed by three nucleotides “tga” and another palindromic E-box “CAGCTG” (Fig. 3B). This feature is very similar to the “4L box” found in pyruvate kinase L or Spot14 promoter, in which it is thought to be the glucose response element (GIRE or ChoRE; see “Discussion”). We call this nucleotide stretch the “G-box”.

Reporter Gene Study of the Promoter—We subcloned the putative promoter domain of the glucagon receptor gene in the pBlCat6 plasmid as illustrated in Fig. 4A. For the transfection studies, we deleted all sequences downstream from the first exon (UJ). Transfections were performed in INS-1 cells: a cell line established (16) and kindly provided by Prof. Wollheim. These cells contain the glucagon receptor mRNA and express functional glucagon receptors (18). Glucose regulated expression of the glucagon receptor in these cells was demonstrated by the adenyl cyclase stimulation: the activity was higher (225 pmol of cAMP/min/mg of protein; 4.2-fold stimulation) using membranes from cells grown in the presence of 20 mM glucose, then with the membranes from cells grown in the presence of 5 mM glucose (100 pmol of cAMP/min/mg of protein; 2-fold stimulation). An INS-1 subclone that expresses low levels of the glucagon receptor and a RIN cell line that does not express the glucagon receptor were used as controls of transfection specificity (data not shown).

We studied the glucagon receptor promoter activity and its regulation by glucose in the INS-1 cells expressing greatly glucagon receptors. We constructed CAT plasmid variants containing various putative promoter sequences. The DNA fragments tested are described in Fig. 4. We observed that the transfection with the short pCB (−236 to +156) plasmid gave the same, relatively large CAT activity in cells grown in medium containing either 5 or 20 mM glucose (Fig. 5).

In contrast, cells transfected by the longer plasmids pCX (−869 to +156) and pCC (−2 kb to +156) expressed a low CAT activity in the presence of glucose 5 mM and a spectacularly higher CAT activity in the presence of 20 mM glucose (Fig. 5). Transfection realized with plasmid pCX (−869 to +156) and plasmid pCC (−2 kb to +156), gave similar results, suggesting that clone pCX (−869 to +156) contained the DNA fragment responsible for the glucose responsiveness. This enhancer should be located upstream from the plasmid pCB insert sequence (i.e. between position −236 to −869 upstream from the transcription start site). Results obtained with the short pCB plasmid shown that these regulatory elements inhibited gene transcription at low, 5 mM glucose and activated the transcription at high, 20 mM glucose concentrations.

The shortened plasmid pCP (−541 to +156) gave a lower activity, with reduced glucose stimulation. The deleted plasmid pCD (−541 to +156) possessed a low activity that remained glucose regulated. Plasmid pCB (−156 to −236) possessed a low activity that was no regulated by glucose (Fig. 6). As the putative glucose enhancer sequence is highly palindromic, it is not surprising that glucose stimulation was also observed in reverse orientation of plasmid pCXi. Absolute activity value of reverse plasmids was lower, which was expected, as in this orientation the normal basal promoter was also in the reverse orientation. This was confirmed by the low activity of the reversed plasmid pCB containing only the putative basal promoter (i.e. GC-rich domain) (Fig. 6).

We also tested the promoter activity of plasmids bearing inserts in the reversed orientation (Fig. 6). Plasmid pCXii (−156 to −869) possessed a low activity that remained glucose regulated. Plasmid pCBii (−156 to −236) possessed a low activity that was no regulated by glucose (Fig. 6). As the putative glucose enhancer sequence is highly palindromic, it is not surprising that glucose stimulation was also observed in reverse orientation of plasmid pCXi. Absolute activity value of reverse plasmids was lower, which was expected, as in this orientation the normal basal promoter was also in the reverse orientation. This was confirmed by the low activity of the reversed plasmid pCB containing only the putative basal promoter (i.e. GC-rich domain) (Fig. 6).

We removed the basal promoter (insert of the clone pCB) by BanHI digestion of the whole promoter domain (plasmid pCC). We subcloned this genomic fragment (−2 kb to −287) upstream from the HSV thymidine kinase basal promoter in the pBlCat5 plasmid. Cells transfected with this plasmid, called pTkCd, which contains the reporter gene driven by the recombinant glucagon receptor enhancer and the tk basal promoter, displayed much higher activities that pCX plasmid. The pTkCd plasmid retained the same level of sensitivity that the pCX plasmid to glucose activation (Fig. 7). Therefore, this plasmid will be used as a model for further studies of the glucose enhancer.

We realized a glucose activation dose response curve. Average results are shown in Fig. 8. Most of activity variation was observed between 5 and 10 mM glucose, with about half-maximal activity observed at an average of 7.5 mM glucose. These glucose concentrations are within physiological glycemia range. In addition, the glucose stimulation of the gene transcription suggests a threshold-like behavior. Indeed, analysis of individual results indicates that the majority of experimental points offer a low activity up to 5 mM glucose, and high activity as of 10 mM glucose. Three dose-response experiments were performed with pCX plasmid (pBlCat5 derivative) Fig. 8, and one, with similar relative results, with pTkCd plasmid (pBlCat5 derivative including HSV tk promoter).

We used the Quick Change site-directed mutagenesis kit for
FIG. 3. Structure of the 5' end domain of the rat glucagon receptor gene. Panel A, schematic description of the whole rat glucagon receptor gene. Exons, closed bars; introns, single line; promoter, open box. The splicing pattern of the 5' untranslated domain is indicated by the dashed lines. The top line presents a partial glucagon receptor gene restriction map: A, ApaI; B, BamHI; P, PstI; S, SacI; Xb, XbaI; Xh, XhoI restriction sites. Panel B, sequence of the 5' end of the rat glucagon receptor gene: promoter domain and exon UJ (clone pCX). Genomic DNA sequences were obtained by sequencing the subcloned &-FixII genomic clones as indicated under "Materials and Methods." Uppercase letters, promoter domain; lowercase letters, exon UJ; italics, first intron; arrow, the position of 5' most transcription start site; bold, the 2 E-boxes, forming the L4-like box (underlined). The BamHI, PstI, and DraIII restriction enzyme-cut sites, which allowed the construction of the plasmids used for the transfection studies, are indicated over the sequence.
the preparation of several mutated clones. First, we deleted the G-box from the pCX clone, yielding the pCX \( D \) clone that lacks a 15-nucleotide domain that includes the two E-boxes. This deletion produced a total suppression of the glucose stimulation of the CAT activity in the transfected cells (Fig. 9A). In addition, two mutations were introduced in the G-box present in the pTkCd plasmid. In the first clone pTkCd-m1, we mutated the second E-box CAG \( C \) TG to CAG \( T \) TG. In the second mutated clone pTkCd-m2, we changed CA of the two E-boxes to TG. Transfections of INS-1 cells with the mutated plasmids showed that, whereas the C \( \rightarrow \) T point mutation of pTkCd-m1 did not impair glucose stimulation of the reporter gene expression, the CA \( \rightarrow \) TG mutations of pTkCd-m2 almost totally suppressed glucose activation of the reporter gene. These results demonstrate the key role of the G-box in the glucose activation of the rat glucagon receptor gene expression.

**DISCUSSION**

We have cloned the 5' upstream domain of the glucagon receptor gene, we demonstrated that it contains a functional glucose-regulated promoter, and we located its glucose regulatory element. This is the first time that a G protein-coupled receptor gene promoter regulated by glucose has been cloned.

**Discussion of Gene Organization**—The glucagon receptor belongs to the type B G protein-coupled receptor family (GPCRs; see GPCR data base (Ref. 19)). Several genes of this receptor family have been described, and share a similar coding domain, i.e. exon/intron organization pattern (14, 20–27). This pattern...
is distinct from the exon/intron organization of the other GPCR families.

Two types of 5' untranslated domains, with or without introns, have been described in this B type receptor family. In other words, the gene promoter could be located either immediately upstream of the ATG initiation site, or at a more distal position, such as the promoter of the porcine calcitonin receptor gene located 20 kb from the ATG initiation site and separated from this site by two 5' untranslated exons and two introns (21). The rat PTH receptor possesses two promoters, located upstream from the first or the third untranslated exon respectively, with different tissue specificities (22, 23). An alternative splicing of at least four untranslated exons was described for the human PACAP receptor (24).

In contrast, the gene promoter is close to the ATG initiation site in both the human and the rat VPAC1 receptor genes (formerly VIP1 receptor) (25, 26), as well as in the rat GLP-1 receptor gene (27).

Conflicting data have been published for the 5' end of glucagon receptor gene: Buggy et al. (28) reported the sequence of a putative human glucagon receptor promoter, separated from the initiation codon by an undescribed 5-kb intron (21). In contrast, Burcelin et al. (17) reported the sequence of a putative mouse glucagon receptor promoter adjacent to the initiation codon. However, no experimental data demonstrating the promoter activity were provided. Our results suggest that sequences upstream from the ATG of the mouse glucagon receptor belong to an intron (85% homology with our rat intronic sequence). A comparison of our experimentally active rat promoter with the sequence of the putative human glucagon receptor promoter indicates that the best significant homology (65% over 120 nt) is located in the vicinity of the mRNA start point. The size of the human 5' untranslated domain, estimated by primer extension assay, was larger (475 nt) than in rat (376 nt). The complete 5' sequence of human gene has not yet been reported (28), but comparison of the rat and the human glucagon receptor cDNAs shows a high (82%) homology in the coding domain (29, 30).

Fig. 6. CAT activity of INS-1 cell extract transfected with plasmids containing the glucagon receptor promoter in both orientations. Left panel, schematic representation of the pBlCat6 plasmids with inserts in the normal orientation (plasmids pCX (+689 to +156) and pCB (+236 to +156)), and in the reverse orientation (plasmid pCX (-156 to -689) and pCB, (-156 to -236)). Right panel, results of transfection experiments performed as in Fig. 5 using a culture medium containing 5 mM glucose (hatched bars) or 20 mM glucose (open bars). Results represented the mean of three separate paired experiments performed in duplicates ± S.E.

Fig. 7. Increasing CAT activity in INS-1 cells transfected with plasmids containing the glucagon receptor promoter subcloned upstream of a heterologous tk promoter. Cells were transfected by control pBlCat6 plasmid, reference pCX (+689 to +156) plasmid, or pTkCd plasmid. In pTkCd plasmid, the promoter domain containing the glucose regulatory element (~2 kb to ~287) was subcloned upstream from the HSV tk promoter of the pBlCat5 plasmid. Transfected cells were grown in either 5 mM glucose or 20 mM glucose and CAT activity of cell extracts was assayed as described under "Material and Methods." Figure shows an autoradiography of thin layer chromatography of a representative experiment made in duplicates (duplicates were migrated beside each other).
for the PTH receptor gene; Ref. 23), and the G-box would be located on the first intron. Such a location of a glucose regulatory element in the first intron has been described for the fatty acid synthase gene (see below).

Discussion of Glucose Regulation—Several gene promoters are up-regulated by increasing of extracellular glucose concentration. Glucose activation of two genes expressed in the liver, pyruvate kinase L (PKL) and Spot14 (a lipogenesis-associated protein) have been extensively studied. It appears that glucose stimulates the transcription of these two genes via similar motifs present in the promoter. These carbohydrate response elements were called GIRE (34) or ChoRE (35) for the PKL or Spot14 genes, respectively. The core motif in these elements is the so called E-box, constituted by the palindromic sequence CACGTG, the consensus recognition sequence of transcription factors belonging to the c-Myc family. Glucose regulation of PKL gene uses two imperfect E-boxes, separated by 5 nt, forming a perfect palindrome coined L4 box: CACGGGn5CCCGTG (36). A similar feature is required for glucose activation of the Spot14 gene: CACGTGn5CCCGTG (37). The spacing distance of 5 nt between these two E-boxes was shown to be essential for its activity (37). It is noteworthy that the L4 box found in the pyruvate kinase L gene is active in INS-1 cell line, whereas neither the proinsulin I nor the glucokinase mRNA levels are increased under the same conditions (38).

These glucose regulatory elements have different locations in the different promoters: located only at −168 to −144 nt from the start point of the pyruvate kinase L gene, whereas in the Spot14 gene it is found at nt −1448 to −1431. The gene of fatty acid synthase, another glucose-regulated gene, also possesses a sequence feature closely related to L4 box. However, this glucose regulatory element is located in the first intron (+283 to +303) (39).

We observed that the sequence motif CACGTGtgaCACGTG ca in the promoter domain of the glucagon receptor located at −545 to −527 is very similar to the motif of the regulatory elements described above. This highly palindromic (underlined), 19-nucleotide sequence contained two perfect, 6-nucleotide palindromes (uppercase). The first one represents a canonical E-box sequence. Results observed with different constructs obtained by with restriction digestion (Fig. 5) suggested that the glucose regulation is centered on this domain. We confirmed the essential role of this motif, the coined G-box, by the mutational studies (Fig. 9), where the deletion or mutation of this motif suppressed glucose stimulation.

The E-boxes found in our G-box motif are more palindromic than in the L4 box and in the L4-like glucose regulatory elements described previously. The palindromic nature of the E-boxes seems to be important for the glucose regulation; mutations that increase the palindromic nature of the second E-box of the L4 box in the Spot14 promoter strongly increase its glucose-stimulated enhancer activity (37).

As this enhancer is palindromic, it is not surprising that the glucose stimulation was also observed in the reverse orientation (Fig. 6). Absolute activity of the reversed plasmids is lower, which is expected, as in this orientation the normal basal promoter is also in the reverse orientation. The transcription start sites were not investigated for reverse orientation plasmids. Consequently, we did not determine which domain acts as a basal promoter in these plasmids. However, a GC-rich domain may act as a promoter in the reverse orientation (40), and a similar glucose regulatory element may act as enhancer downstream from the transcription start site (39).

Recently, USF-2 (upstream stimulatory factor 2) was shown to be directly involved in the glucose-induced stimulation of PKL and Spot14 gene transcription (41, 42). USF-2 is a mem-
A Deletion of G-Box

| X | B | A |
|---|---|---|
| pCX | | |

3.5m Glucose

20 mM Glucose

B Mutations in G-Box

| X | B | A |
|---|---|---|
| TGCACGTGTGACAGCTGCA | pTkCd |
| TGCACGTGTGACAGCTGCA | pTkCd-m1 |
| TGCACGTGTGACAGCTGCA | pTkCd-m2 |

FIG. 9. Effect of deletion (panel A) or mutations (panel B) of G-box on the CAT activity in transfected INS-1 cells. Panel A, Ins-1 cells were transfected by pCX (∼869 to +156) plasmid, or the G-box deleted plasmid pCXΔA prepared by QuickChange site-directed mutagenesis kit (Stratagene). Transfected cells were grown in either 5 mM glucose or 20 mM glucose, and the CAT activity of cell extracts was assayed as described under "Material and Methods." The mean ± S.E. of three (for pCXΔA) or five (for pCX) independent experiments, performed in duplicate, is represented. Panel B, INS-1 cells were transfected by pTkCd plasmid (pBlCat5, tk basal promoter with fragment −2 kb to −287) and two mutants of the G-box prepared by QuickChange site-directed mutagenesis kit (Stratagene) as described under "Materials and Methods." Mutations are indicated in bold and underlined. Transfected cells were grown in either 5 mM glucose or 20 mM glucose, and the CAT activity of cell extracts was assayed as described under "Material and Methods." The mean of two independent experiments, performed in duplicate, is represented.

Acknowledgments—We are grateful to Prof. Wollheim (University Medical Center, Geneva, Switzerland) for the grant of the INS-1 cell line. We thank Drs. P. Robberecht and M. Waelbroeck for the manuscript revision, M. Stievenart for the graphical representation of the results, and N. De Gendt-Pechot for secretarial assistance.

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