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Positive and negative regulatory elements are involved in transcriptional control of the rat glucokinase gene in the insulin producing cell line HIT M2.2.2

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Abstract

Nested deletion mutants of the 5′ flanking region of the β-cell transcription unit of the rat glucokinase gene (rβGK) were fused to the CAT-reporter gene. Transient expression studies in HIT M2.2.2 and BHK21 cells revealed a distal (upstream of −359) and a proximal promoter region (between −278/−49) harbouring positive and negative regulatory elements. DNaseI footprinting revealed three protected areas between nucleotides −190 and −60. DNA-elements playing a crucial role in transcriptional control of the insulin genes (IEB- and CT-motifs) have been detected within the proximal promoter region and contribute to β-cell specific gene regulation. 3′ deletion analysis revealed that DNA-elements located downstream from transcription initiation sites (up to +123) contribute to transcriptional regulation.

Key words: Glucokinase; Gene expression; Pancreatic β-Cell; DNA–protein interaction; Promoter

1. Introduction

The maintenance of glucose homeostasis is an essential requirement for optimal function of many tissues and in this way of vital importance in higher organisms. Precise regulation of blood glucose is achieved by a complex set of interactions mediated on the one hand by 'glucose sensors' (pancreatic α-cells and β-cells, hepatocytes), on the other hand by receptors for insulin and glucagon. Within this regulatory circuit glucokinase (ATP:D-hexose-6-phosphotransferase; EC 2.7.1.1) has been recognised as an essential part of the glucose sensing mechanism in pancreatic β-cells [1]. This enzyme catalyses the initial step in glucose utilisation, and appears to be involved in regulating insulin secretion by β-cells. Recent discovery of mutations in the glucokinase gene in families with the 'Maturity Onset Diabetes of the Young (MODY)' has shown the glucokinase gene to be a candidate gene for Type 2 (non-insulin-dependent) diabetes (for review see [2]). It has been shown previously that the single-copy glucokinase gene is differentially regulated and processed in hepatocytes and pancreatic β-cells. In these two tissues specific transcription control regions govern the use of alternative first exons and generate transcripts that differ in their 5′ ends [3,4]. Additional spliced RNAs of the glucokinase gene have been identified [3–5]. The upstream (β-cell specific) transcriptional control region is active in insulin-producing cell lines as well as in a pituitary derived cell line, but enzyme activity is restricted to insulin-producing cells, suggesting that the expression of this gene may be also regulated at the post-transcriptional level [6,7]. An analysis of the transcriptional control elements of rat insulin genes I and II [8–13] and human insulin gene [14–16] has been carried out so as to delineate the factors necessary for β-cell specific gene expression. The functional analysis of *cis*- and *trans*-acting elements revealed the presence of both ubiquitous and cell-specific factors involved in insulin gene reguClation. At least two different elements with consensus sequences 5′-GCCATCTG-3′ (IEB-motif [10]) and 5′-CTtAAT-3′ (CT-motif [16]) seem to play a crucial role in transcriptional control of these genes. The characterisation of additional genes, primarily expressed in pancreatic β-cells, such as amylin and glucokinase genes, will allow a more comprehensive analysis of the mechanisms responsible for β-cell specific gene expression. Only a first characterisation of the rat glucokinase upstream promoter has been carried out so far [17,18].

The aim of this paper was to analyse rβGK-promoter elements contributing to transcriptional control in insulin producing cells. In this study: (i) we were able to delineate three DNA-elements between nucleotides −190 and −60 by DNase I footprinting analysis; (ii) we could

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demonstrate the impact of two *cis*-elements in transcriptional control by site-directed mutagenesis in both transient expression studies and DNA/protein binding analysis; (iii) we could show that beside positive-, also negative-acting regulatory elements are involved in gene regulation; and (iv) we demonstrate that DNA-elements located downstream of the transcription initiation sites contribute to transcriptional control of the rat glucokinase gene (rβGK).

2. Materials and methods

2.1. Plasmids and construction of CAT expression vectors

A DNA fragment containing 990 bp of the 5'flanking region and 123 bp of the first exon of rβGK was amplified by PCR from genomic DNA (Wistar rat). Genomic DNA was prepared as described previously [19]. We introduced a *Pvu*II site by using as the upstream primer 5'-CTACAGACAGCTcAGGAGTT-3' (corresponding to bp−1000/−981). The downstream primer 5'-TCAGTGTTGACTGACTTTTCCT-3' corresponded to nucleotides +155/+176. After digestion with *Pvu*II and *Xba*I, the resulting DNA fragment was subcloned into pBluescript. 5' deletion mutants were generated using either restriction enzyme digestion or *Bal*31 nuclease digestion. Deletion fragments were subcloned into the promoterless expression vector pBSV0CAT [20] generating expression constructs pBrβGK-n/+123CAT. Chimeras pBrβGK-n/+14CAT were created by digesting rβGK fragments with *Sac*I. To test promoter- and enhancer-like properties of rβGK upstream sequences, appropriate DNA fragments were subcloned upstream of the HSV thymidine kinase promoter (−109/+56) of pBTKCAT [20]. To get a comparable plasmid background for the rat insulin I gene promoter, the −410/+1 fragment of pOK1 [12] was subcloned into pBSV0CAT. For DNase I footprinting a *Pst*I/*Xba*I fragment of rβGK (nucleotides −190 to +123) was subcloned into pBluescript generating pBrβGK-190/+123. pBrβGK-359Δ-161/-89CAT and pBrβGK-609Δ-161/-89CAT were created by digesting pBrβGK-359CAT and pBrβGK-609CAT, respectively, with *Bst*EII. All constructions have been verified by dideoxy DNA sequence analysis.

2.2. In vitro DNase I footprinting

Crude nuclear extracts were prepared as described by Dignam et al. [21] and Boam et al. [16]. DNase I footprinting was performed as described by Boam et al. [16] except that a 6% (w/v) polyacrylamide, 7 M urea sequencing gel was used. G + A ladder was generated by chemical sequencing reaction of the probe according to Maxam and Gilbert [22].

2.3. Cell transfections and CAT assay

Transfection of HIT M2.2.2 and BHK21 cells were performed by the calcium phosphate co-precipitation technique [23], using 20 μg CsCl-purified CAT expression vectors and 4 μg pRSVβgal [9] per 100 mm dish. Transfection was carried out in Dulbecco's modified Eagles medium containing 2 g/l glucose supplemented as above. Cells were harvested and protein extracts were prepared 40–48 h after transfection. Extracts were analysed for CAT and β-gal activities [24,25]. The β-gal values were used to normalise the individual test CAT levels.

3. Results and discussion

3.1. Positive and negative cis*-regulatory elements control the expression of the glucokinase gene*

In order to characterise the 5' regulatory region we constructed a set of nested 5' deletion mutants from position −990 to −49, upstream of the published transcription start site (+1; [3]) and investigated their ability to control expression of the reporter gene CAT in insulin-producing (HIT M2.2.2) and non-producing (BHK21) cells. To ascertain whether differences in CAT-activities of transfected 5' deletion mutants of rβGK/CAT-fusion vectors in HIT M2.2.2 were transcriptional in origin, the locations of the transcription initiation sites of three different vectors (pBrβGK-990CAT, pBrβGK-139CAT, and pBrβBK-49CAT) were determined by primer extension and S1-nuclease mapping. Coinciding transcription start points were found for all tested expression vectors (data not shown).

To localise the *cis*-acting DNA sequences of the rβGK

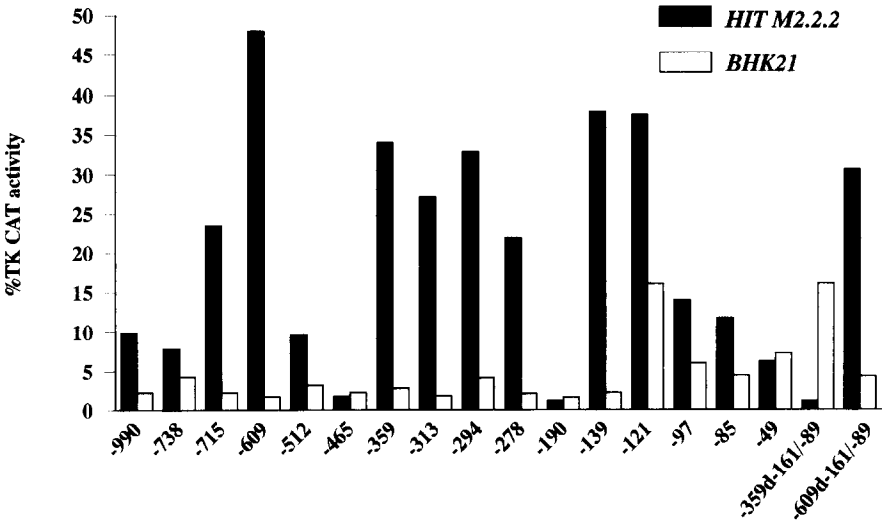

Fig. 1. Deletion analysis of the 5' flanking region of the rβGK transcription unit. 20 μg of each pBrβGK-n/+123CAT expression vector was co-transfected with 4 μg pRSVβgal into HIT M2.2.2 and BHK21 cells. CAT activities were normalised to β-gal activities. Values represent averages of at least three individual experiments and are expressed as percentages of the activities obtained with pBTKCAT. Constructs were shown as numbers corresponding to their 5' ends, −359d-161/−89 and −609d-161/−89 correspond to pBrβGK-359Δ-161/-89CAT and pBrβGK-609Δ-161/-89CAT, respectively.

transcription unit involved in transcriptional control in insulin-producing cells, expression vectors with different 5′ endpoints (pBrβGK-n/+123CAT) and/or with internal mutations were transfected into HIT M2.2.2 and BHK21 cells (Fig. 1). Highest CAT activity in HIT M2.2.2 cells was obtained with a −609 bp deletion mutant. The −990 bp chimera showed only 20% activity in comparison to that obtained by pBrβGK-609CAT. Deletion from −990 bp up to position −609 bp caused a stepwise increase in CAT activity followed by a 25-fold decrease when truncated from −609 to −465 bp. Deletion from −465 to −359 bp induced a 18-fold increase of CAT activity. Further truncation from −359 to −278 bp did not significantly change CAT activity, but did decrease activity more than 10-fold when shortened to position −190 bp. Further deletion to −139 bp caused a roughly 30-fold increase in CAT activity. Shortening from −139 bp to −121 bp did not change CAT activity in HIT cells, but did increase activity in BHK21 cells 6.7-fold. Further deletion from −121 to −49 bp resulted in a stepwise decrease of CAT activity. Data obtained from these transient expression studies showed that 5′ deletion of the rβGK upstream region from −465 to −359 bp, and from −190 to −139, caused a drastic increase in CAT expression in HIT cells and may reflect the presence of negative acting regulatory elements within these regions. Positive regulatory elements seem to be located between nucleotides −609 and −512, and −139 and −97. Activities of the −49 deletion mutants appeared to be identical in HIT M2.2.2 and BHK21 cells.

Sequence analysis revealed DNA-motifs very similar to reported IEB- and CT-boxes within the −139 promoter fragment. To test further the importance of these elements (also reported as to be a Far-FLAT-like element [26]) in transcriptional control we performed an internal deletion from −161 to −89 in two expression vectors (i.e. pBrβGK-609CAT and pBrβGK-359CAT) by digesting with *Bst*EII. Whereas the deletion eliminating the Far-FLAT-like element of pBrβGK-359CAT caused an almost complete loss of CAT activity in HIT M2.2.2 the same deletion in pBrβGK-609CAT reduced CAT activity only to 65%, relatively to the unmutated chimeras. In BHK21 cells the deletion resulted in a 6-fold increase of CAT activity for pBrβGK-359-Δ161/-89CAT, but only in a 2-fold increase for pBrβGK-609Δ-161/-89CAT, relatively to the unmutated chimeras. The dramatic decrease of CAT activity in HIT cells but not in BHK cells using pBrβGK-359Δ-161/-89CAT indicates the presence of *cis*-elements controlling transcriptional activity in a cell-type specific manner. The only moderate decrease of CAT expression in HIT cells and the only weak effect in BHK cells using pBrβGK-609Δ-161/-89CAT may reflect the presence of further cell type specific acting *cis*-elements upstream of −359 bp. These results and the data summarised in Fig. 1 indicate the presence of at least two distinct transcriptionally active control regions within the −990 bp flanking sequence of rβGK. The distal promoter region is localised upstream of position −359 bp, the proximal region could be positioned between −278 and −49 bp.

Some of our results differ greatly from the expression studies reported in [18], i.e. higher activity of −990 variant, lower activity of −140 and −120 constructs, as well as no decrease of luciferase activity in constructs deleted to −190. To determine whether these differences are the result of the different 3′ ends fused to the reporter genes, i.e. -n/+14 in Shelton's chimeras and -n/+123 in our fusion plasmids, we additionally generated three pBrβGK-n/+14CAT-fusion constructs (pBrβGK-990/+14CAT, pBrβGK-190/+14CAT, and pBrβGK-139/+14CAT). The comparison of the subsequent expression studies (Fig. 2) clearly demonstrated a different behaviour of the −990/+14 and −190/+14 variants from the −990/+123 and −190/+123 variants, respectively, but no significant differences in CAT activity in the −139 variants. In this way, these studies indicate the significance of the rβGK region +14/+123 in transcriptional control in HIT cells, but the different behaviour of our −139 constructs to that from Shelton remains unclear. A computer search of the region +14/+123 did not reveal any specific sequence elements known to be involved in the regulation of mammalian gene expression. While the location of promoter elements within intervening sequences have been reported [27,28] our studies suggest the presence of transcriptional control elements in the 5′ untranslated region of a gene. Nevertheless, further experiments are needed to demonstrate that the region +14/+123 is involved in the transcriptional control of rβGK.

3.2. *Multiple* trans-*acting factors bind to* cis-*regulatory elements identified in the β-cell specific 5′ region*

Several potential DNA/protein binding-sites have been detected by sequence analysis of the 5′ region of the β-cell specific transcription unit of the rat glucokinase

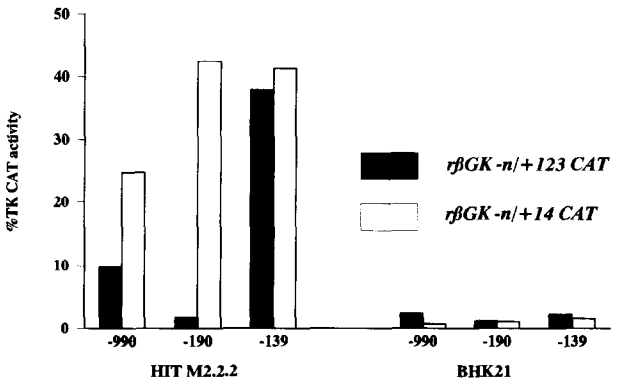

Fig. 2. Effect of 3′ end points of rβGK fragments in transient expression studies. Constructs were co-transfected with pRSVβgal into HIT M2.2.2 and BHK21 cells. CAT activities represent averages of at least three individual experiments and express relative activities to that obtained from pBTKCAT.

gene. A putative TG-element [29] is located between nucleotides −840/−814, a putative Sp1-binding site [30] is located between nucleotides −700/−631, potential CT-boxes (core sequences CTAATG or CTTAATT) are present at positions −941/−934, −734/−729, −521/−515, and −104/−97 bp, sequences similar to IEB-elements [10] and E-boxes [31,32] are found at −596/−601, −134/−126 bp. Short palindromes are present at −724/−715 (CTTTTAAAAG), −330/−322, −205/−197 (both CCAGGCTGG), −194/−181 (GTCACTGCAGTGAC), and −168/−153, −91/−81 bp (both containing TGGTCACCA).

In vitro DNase I footprinting and electrophoretic mobility shift assays (EMSAs) using crude nuclear extracts from insulin-producing cells (HIT M2.2.2) were used to search for DNA-binding protein factors interacting with the proximal rβGK regulatory region −190/−60. The footprints obtained from DNA/protein interactions delineate three protected areas within the region −190/−60 bp of the rβGK upstream sequence (Fig. 3). The most distal element, F3, extends from nucleotide −168 to −148 and from nucleotide −160 to −149 on the upper (normal, Fig. 3A) and lower (complem., Fig. 3B) strands, respectively, and contains the palindromic sequence motif TGGTcACCA. The middle footprint, F2, is located between nucleotides −105 and −94 and between nucleotides −105 and −91 (upper and lower strands, respectively) and covers the CT-like motif CTCTAATG. The most proximal element, F1, maps between −84 and −73 and between nucleotides −82 and −73 (upper and lower strands, respectively) and covers as well parts of a TGGT-motif as a part of a putative GAAA-motif (men-

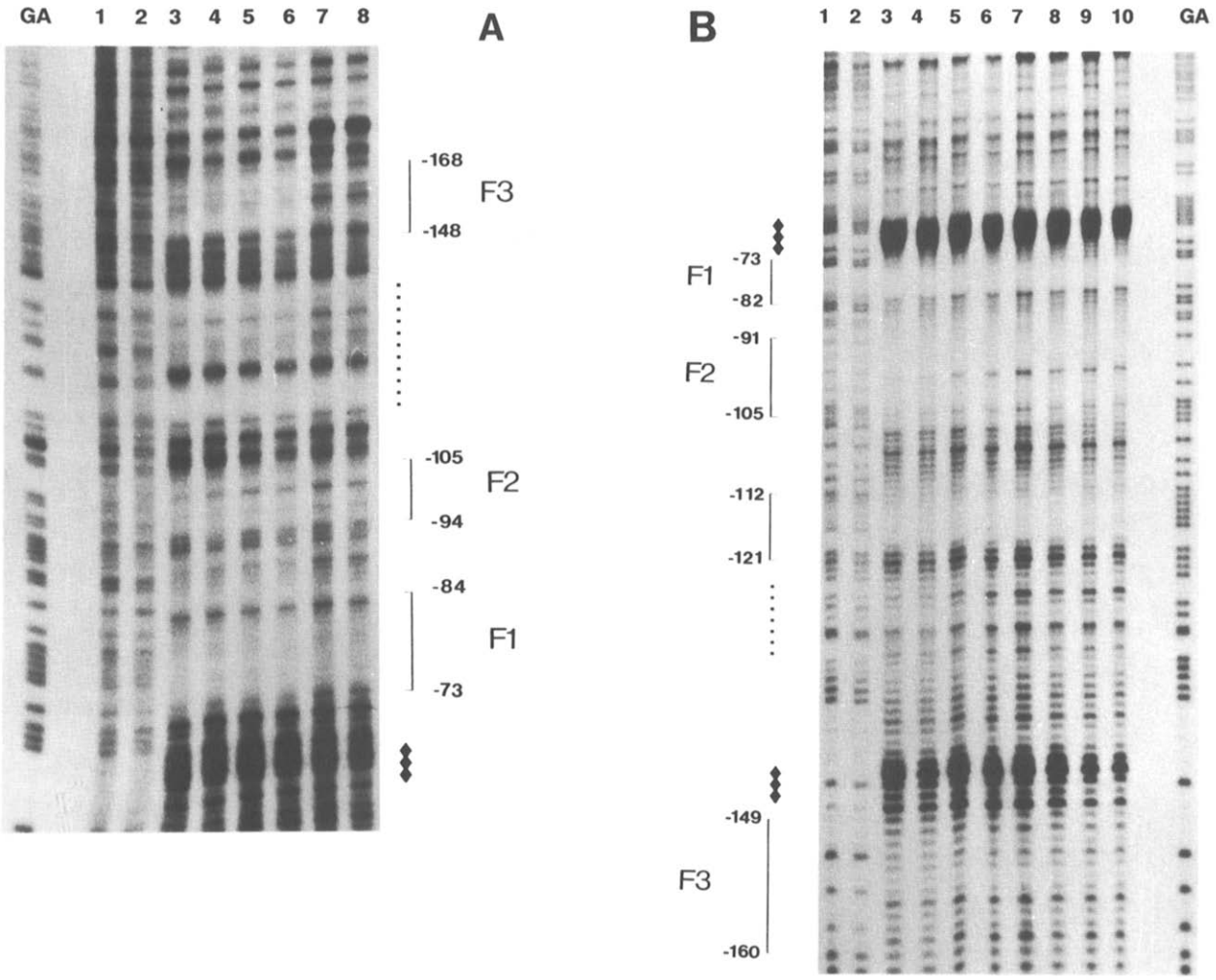

Fig. 3. In vitro DNase I footprinting analysis of the rβGK promoter. A *PstI*/*XbaI* fragment of rβGK containing nucleotides −190 to +123 was subcloned into pBluescript. Following digestion with *Eco*RI and *Not*I the resulting fragment was labelled at either end by Klenow fill-in reaction. The labelled probe was incubated for 20 min at 22°C with increasing amounts of nuclear extracts prepared from HIT M2.2.2 cells and treated with DNase I for 60 s at 22°C. The areas protected from DNase I digestion are bracketed. The corresponding nucleotide positions were determined by running of a G + A chemical sequencing ladder (GA). DNase hypersensitive sites are marked by ◆, small squares (■■■) delineate the position of the putative IEB-motif. (A) Upper strand, lanes 1 and 2: incubation with 30 μg bovine serum albumin, lanes 3 and 4: 75 μg nuclear extracts, lanes 5 and 6: 50 μg, lanes 7 and 8: 20 μg. (B) Lower strand, lanes 1 and 2: 30 μg bovine serum albumin, lanes 3 and 4: 75 μg nuclear extracts, lanes 5 and 6: 50 μg, lanes 7 and 8: 30 μg, lanes 9 and 10: 15 μg.

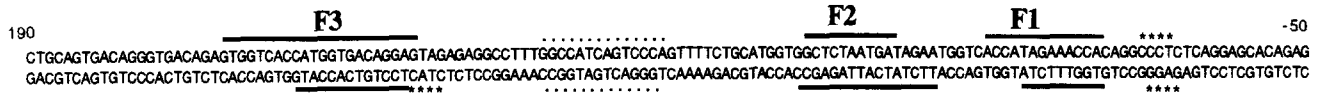

Fig. 4. Position of in vitro DNase I footprints between nucleotides −190 and −50 of the rβGK proximal promoter element. Areas protected from DNase I digestion (footprints F1, F2, and F3), DNase hypersensitive sites (*), and the location of the putative IEB-motif (■■■) are shown.

tioned as to be a GG-motif in the human insulin promoter [16]). DNase I hypersensitive sites are apparent 3′ flanking of F3 at −143 to −148 (lower strand), and 3′ flanking of F1 at nucleotides −68 to −65 and nucleotides −67 to −64 (upper and lower strands, respectively). Varying the nuclear extract concentrations in footprinting assays on both upper and lower strands showed that all three elements F1 to F3 behaved in a similar manner (Fig. 3A lanes 3–8, Fig. 3B lanes 3–10).

Analysis of the sequence obtained from footprint F2 revealed the protected sequence CTCTAATG. This sequence motif has previously been noted in promoters of rat insulin genes I and II, and in the human insulin gene regulatory 5′ region and described as CT-motifs [16] and FLAT-E-element [26]. Our studies demonstrate that the rβGK CT-motif is mutationally sensitive in both, protein-binding analysis and transient expression studies and is able to bind as well as compete for binding to similar – probably the same – nuclear factor(s) that bind to CT-motifs of insulin and amylin gene promoters (B. Leibiger at al., manuscript in preparation). To assess the cell type specificity of the interaction between the putative *cis*-elements mapped by in vitro DNase I footprinting we used a set of overlapping double-stranded oligonucleotides covering region F1, F2, and F3 (Fig. 4), in electrophoretic mobility shift assays. The DNA/protein complexes formed by all probes using nuclear extracts from HIT cells showed a different pattern in comparison with extracts isolated from BHK cells, and demonstrate the formation of HIT cell-specific as well as ubiquitous DNA/protein complexes (data not shown).

3.3. *A negative acting regulatory* cis*-element is located between nucleotides −190 and −140*

The 5′ deletional analysis of the rβGK upstream region revealed a roughly 30-fold increase of CAT activity in HIT M2.2.2 cells when truncating the promoter from nucleotides −190 to −139. To test whether the down-regulating effect of this region is cell-type specific we placed the fragment −190/−140 upstream of an heterologous promoter (TK) generating the expression vector pBnegTKCAT. After transfection it down-regulates CAT expression in both HIT M2.2.2 and BHK21 cells to approximately 40% of the activity obtained from pBTKCAT alone (Fig. 3). We noted that shortening from −190 to −139 did not result in an increase in CAT expression in BHK21, perhaps due to the action of cell-specific regulatory elements localised between −139 bp and −121 bp (Fig. 1). Furthermore, truncating the 3′ region from +123 to +14 of pBrβGK-190CAT caused a loss of down-regulating properties in HIT cells (Fig. 2). We suggest that for the down-regulating effect in the intact rβGK-promoter an element located within the region −190/−140 is necessary (pBnegTKCAT; Fig. 5) but not sufficient (pBrβGK-190/+14CAT versus pBrβGK-190/+123CAT; Fig. 2). Moreover, the effect of this element seems to be dependent on the action of further elements, located upstream of −190 (compensating the negative effect) and located between nucleotides +14 and +123 (enhancing the negative effect). Functional analysis of eukaryotic promoter/enhancer complexes demonstrates the involvement of both, positive and negative acting regulatory elements in transcriptional control.

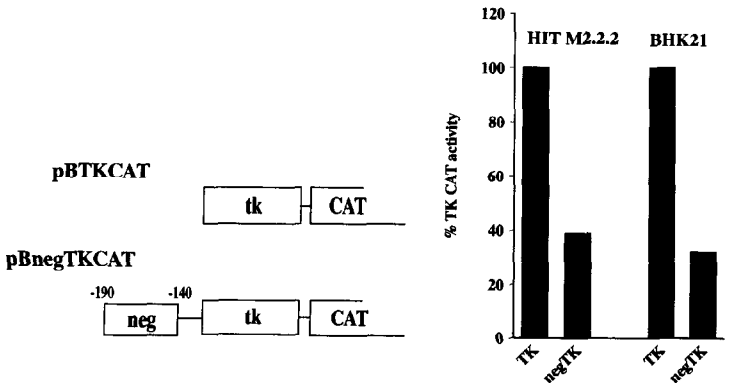

Fig. 5. Effect of rβGK promoter region −190/−140 on HSV thymidine kinase promoter of pBTKCAT. A fragment of rβGK containing nucleotides −190/−140 was cloned upstream of the TK promoter in the vector pBTKCAT [23]. pBnegTKCAT and pBTKCAT, respectively, were co-transfected with pRSVβgal into HIT M2.2.2 and BHK21 cells. Relative CAT activities were normalised to β-gal activities. Values represent averages of at least three individual experiments and are expressed as percentages of the activities obtained with pBTKCAT.

Studies on rat insulin genes I and II revealed negative control in non-β-cells [33,34]. Negative control in a β-cell specific manner was reported for the human insulin gene [16] and is assumed to 'fine-tune' transcriptional activity by interacting with positive regulatory elements. We observed a putative control element having down-regulating properties in both, insulin-producing and non-producing cells. This element lies in the proximal promoter region of rβGK between nucleotides −190 and −140 (Fig. 1). In DNA/protein interaction studies DNase I-footprinting revealed a protected area (footprint F3; Fig. 3) within the region that covered a TGGTcACCA palindromic sequence motif (TGGT-motif). Computer search of the region −190/−140 and especially of the DNase I-protected area F3 (Figs. 3 and 4) did not reveal any homology to known reported *cis*-elements. The possible involvement of the TGGT-motif in negative regulation remains to be elucidated further as well as the role of the second TGGT-motif between −90 and −83 bp.

In conclusion, the results obtained studying the rβGK upstream region revealed the presence of transcriptional control elements having positive and negative effects on gene expression. The identification of similar *cis*- and *trans*-acting elements involved in transcriptional control of insulin, amylin, and glucokinase genes, suggest common and co-ordinated mechanisms in the regulation of these genes. Considering that beside cell-specific, additionally metabolic signals take part in gene regulation a more complex view in understanding β-cell specific gene expression is required. Further studies are needed to explore the impact of unique *cis*-/*trans*-elements in the transcriptional control of the rβGK itself as well as in the transcriptional network of co-ordinately expressed genes in the pancreatic β-cell.

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