Volume 247, number 1, 41–45 FEB 06990 April 1989

Negative regulation of human insulin gene expression by the 5′-flanking region in non-pancreatic cells

Jun Takeda*+, Shunsuke Ishii, Yutaka Seino+, Fumio Imamoto and Hiroo Imura*

*Second Department of Internal Medicine and +Division of Metabolism and Clinical Nutrition, Kyoto University School of Medicine, Kyoto and Laboratory of Molecular Genetics, Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), Ibaraki, Japan

Received 4 February 1989

We have examined the effect of the 5′-flanking region of the human insulin gene on its expression in non-pancreatic cells. The presence of the region containing the insulin gene enhancer (−339 to −169 bp) markedly repressed the promoter activity of the insulin gene. This suppressive phenomenon was restored by the addition of forskolin or dibutyryl cAMP, suggesting that this region alone is not sufficient to repress completely insulin gene expression in the presence of extracellular stimuli which increase the intracellular cAMP level. The hypervariable region (HVR) located at −365 bp also repressed the promoter activity. These results show negative regulation of human insulin gene expression in non-pancreatic cells by these regions.

Negative regulation; Insulin gene; cyclic AMP

1. INTRODUCTION

The insulin gene is expressed in pancreatic β cells in a tissue-specific manner and activated in response to extracellular inducers such as glucose and cAMP [1–4]. The 5′-flanking region of the insulin gene contains two *cis*-acting elements, the enhancer and the promoter, which exert their effect on transcription by interacting with various *trans*-acting factors which restrict insulin gene expression to the endocrine pancreas [1,5–7].

Recently, Nir et al. [8] have shown the presence of a negative *trans*-acting factor(s) which interacts with the 5′-flanking region of the rat insulin I gene in non-pancreatic cells. Stein and Ziff [9] have shown also that adenovirus type 5 infection to the insulin producing cell line, HIT T-15, repressess rat insulin II gene transcription. These findings suggest that, in addition to being regulated in a positive fashion, insulin gene expression also can be negatively regulated.

A unique hypervariable region (HVR) linked to the human insulin gene, which consists of a varying number of 14 or 15 nucleotides in repetition, resides ~365 bp upstream from the cap site [10,11]. This characteristic feature of the HVR is observed only in humans and higher apes [12]. The heterogeneity of the HVR in length has been extensively studied as a possible genetic marker of susceptibility to diabetes mellitus [13]. However, the functional role of the HVR and its surrounding sequences in the regulation of human insulin gene expression is unknown. Since the sequence of the enhancer and its upstream region of the insulin gene in rats and humans is relatively divergent, compared to the promoter region [4], it seems possible that insulin gene expression by these regions is regulated differently in the two species.

Here, we have examined the effect of the human insulin gene enhancer and its upstream regions including the HVR on insulin gene expression in non-pancreatic cells, CV-1 and HeLa cells. We

Correspondence address: J. Takeda, Division of Metabolism and Clinical Nutrition, Kyoto University School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606, Japan

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

have also studied the effect of cAMP on insulin gene expression in these cells.

2. MATERIALS AND METHODS

2.1. *Plasmid construction*

The structures of a series of recombinant CAT plasmids used are schematically shown in fig.1. The *Bgl*II-*Pst*I fragment (−168 to +111 bp from the cap site) containing the promoter region of the human insulin gene was inserted into the *Hin*dIII site of pSVOCAT [14] using a *Hin*dIII linker, designated pHICAT. The *Pst*I fragment (−339 to +111 bp) containing both the human insulin promoter and the enhancer regions was inserted into the *Hin*dIII site of pSVOCAT to generate pHICAT-4. The HVR adjacent to the human insulin gene can be classified into three groups according to variation in length: class 1–3 [15]. Class 1 (700 bp) and class 3 (2 kb) fragments were prepared by *Nco*I and *Pst*I digestion and were placed adjacent to the insulin promoter region of the pHICAT using an *Xho*I linker, designated pHICAT-1 and pHICAT-3, respectively. The constructs with fragments of class 1 and 3 in opposite directions were also prepared, denoted as pHICAT-1r and pHICAT-3r, respectively.

2.2. *Cell cultures and DNA transfection*

The human epitheloid carcinoma cell line, HeLa, and the African green monkey kidney cell line, CV-1, were maintained in ES medium (Nissui Seiyaku, Japan) supplemented with 10% fetal bovine serum. Cells were plated at a density of 4×10^5 per 10 cm Nunc tissue culture plate. A mixture of 18 μg of each plasmid DNA and 2 μg of pRSV-β-gal plasmid DNA was transfected using the calcium-phosphate precipitation method [16]. The plasmid pRSV-β-gal, which carries the β-galactosidase gene linked to the Rous sarcoma virus LTR promoter, provided an internal control for measuring differences in transfection efficiency among the experiments [17]. Forskolin (10 μM), dibutyryl cAMP (0.1 mM), phorbol 12-myristate 13-acetate (PMA) (0.1 mM), or glucose at various concentrations (50, 100 or 200 mg/dl) were added to the medium 20 h after transfection.

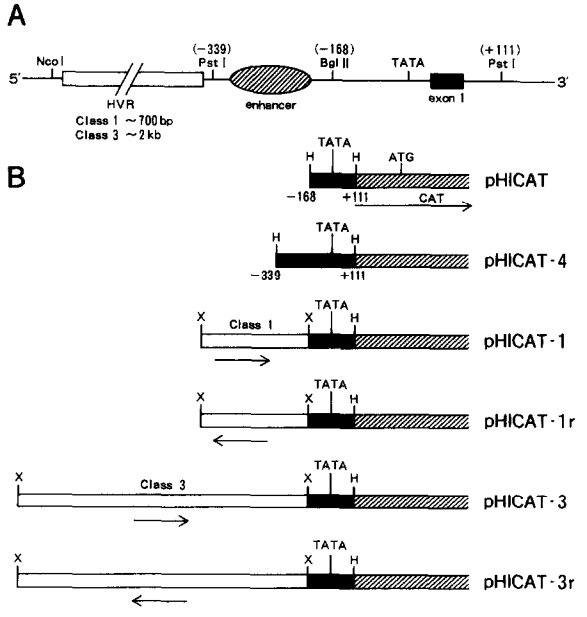

Fig.1. (A) Schematic representation of the 5′-flanking region of the human insulin gene. (B) Various CAT plasmid constructions. The hatched box represents a CAT gene. The closed and open boxes indicate the promoter region and the HVR, respectively. H, *Hin*dIII; X, *Xho*I.

2.3. *β-Galactosidase and CAT assays*

40 h after transfection, the cells were harvested in 150 μl of 0.25 M Tris-HCl (pH 7.8) and lysed by freeze-thawing and sonication to prepare extracts. For the β-galactosidase assay, 60-μl samples were incubated at 37°C for 1 h with 500 μl chlorophenol red–β-D-galactopyranoside solution (30.37 mg/ml in 2.5 mM $MgCl_2$) and the absorbance at 574 nm was measured. The amounts of cell extract used for CAT assays were normalized with respect to β-galactosidase activity. CAT enzyme reactions were performed as described by Gorman et al. [14] using 0.7 μCi [^{14}C]chloramphenicol. Acetylated reaction products were separated by thin-layer chromatography. Following autoradiography, the acetylated and unreacted forms were excised and counted in a scintillation counter.

2.4. *Preparation of nuclear extract and gel retardation assay*

A nuclear protein extract was prepared from HeLa cells as described by Wu [18]. The gel retardation assay was performed as described by Singh et al. [19]. The *Xho*I class 1 fragment was prepared from pHICAT-1 (fig.1B) and ^{32}P labeled at the 5′-end. After preincubation with poly(dI-dC):poly(dI-dC), HeLa nuclear extracts were incubated with the 5′-end-labeled class 1 fragment. The reaction products were separated by 4% polyacrylamide gel electrophoresis under low ionic conditions.

3. RESULTS

3.1. *Negative regulation of insulin gene expression by the enhancer-containing region in non-pancreatic cells*

The plasmid containing the human insulin gene promoter region (pHICAT) produced relatively high levels of CAT activity in both CV-1 and HeLa cells (fig.2). In contrast, the plasmid pHICAT-4 in which the enhancer region of the human insulin gene was placed adjacent to the promoter region decreased CAT activity to about 10 and 20% of that obtained by pHICAT in CV-1 and HeLa cells, respectively, suggesting that insulin gene expression is negatively regulated by the region from −339 to −169 bp in these cells.

Insulin gene expression is modulated by glucose in pancreatic β cells partly via cAMP-dependent protein kinase [3]. We have investigated,

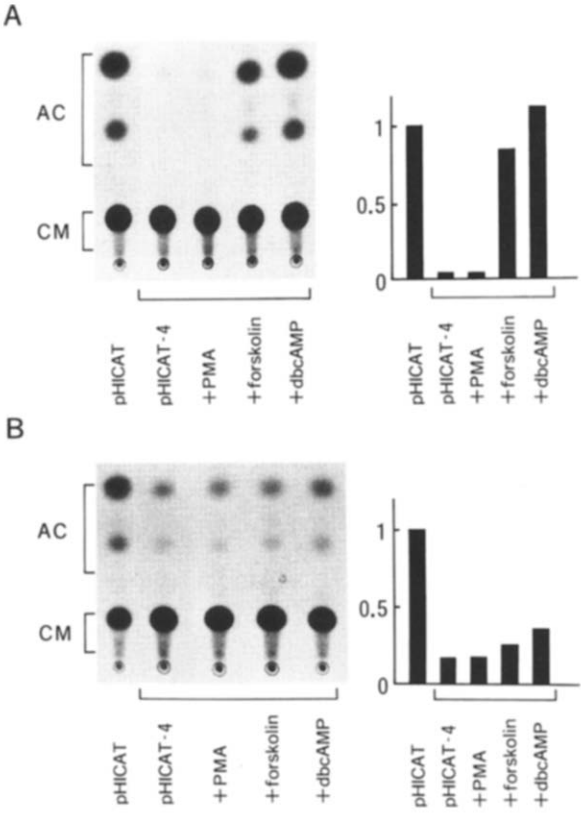

Fig.2. Effect of the 5′-flanking region of the human insulin gene on CAT gene expression. CV-1 (A) and HeLa (B) cells were transfected with a reporter plasmid, and then treated with PMA, forskolin, or dibutyryl cAMP. AC, acetylated derivatives of chloramphenicol; CM, unreacted species. CAT activities are shown on the right as a relative ratio to that of pHICAT.

therefore, whether the suppressive effect of the enhancer region can be modulated in response to an increased cAMP level in non-pancreatic cells. In fact, there is a motif in the 5′-flanking region of the human insulin gene similar to the cAMP responsive element (CRE), 5′-TGACGACCA-3′ (−181 to −174 bp), which is highly conserved in many other genes whose expression is regulated by cAMP [20]. The plasmid pHICAT-4 containing the insulin promoter and enhancer regions was transfected into CV-1 cells and the medium was subsequently treated with dibutyryl cAMP or forskolin to increase the intracellular cAMP level. The suppression of CAT activity by the plasmid pHICAT-4 was found to be restored to the level of that obtained by pHICAT in response to both inducers (fig.2A). However, when HeLa cells were transfected with the same plasmid, there was little noticeable increase in CAT activity in response to these reagents, as compared with CV-1 cells (fig.2B). It is therefore likely that the CRE functions with a certain cell specificity. We also investigated the effect of two other insulin secretagogues, glucose and PMA [21,22]. No increase in CAT activity by these inducers was observed in HeLa nor CV-1 cells (figs 2,3). To exclude the possibility that increased glucose was not taken up by the cells, Northern blot analysis was performed using the Hep G2-type glucose transporter cDNA probe [23]. An accumulation of glucose transporter mRNA proportional to the extracellular glucose concentrations in CV-1 cells showed that the glucose signal had been introduced into the cells (fig.3).

3.2. *Negative regulation of insulin gene expression by HVR*

The unique HVR class 1 (700 bp) or class 3 (2 kb) fragment was placed adjacent to the promoter region of the insulin gene to study its effect on insulin gene expression. Regardless of the orientation or length variation of the HVR, the level of CAT activity was reduced to about 20% of that obtained by the promoter region alone (fig.4), in-

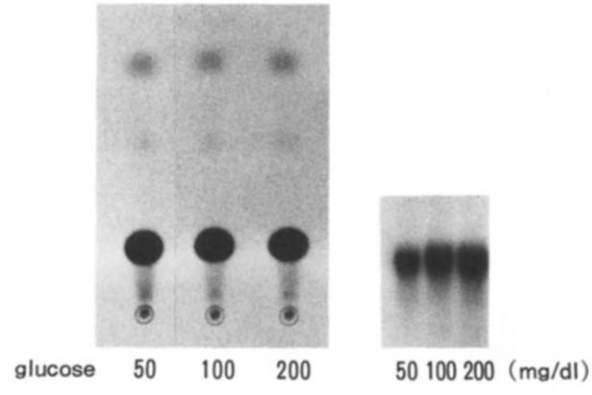

Fig.3. Effect of glucose concentration on CAT gene expression. CV-1 cells were transfected with pHICAT-4 and treated with various concentrations of glucose. On the right are shown 2.9 kb mRNA species which hybridized to the HepG2-type glucose transporter cDNA probe [23]. 15 μg total RNA were applied in each lane. Northern blot analysis was performed as in [24].

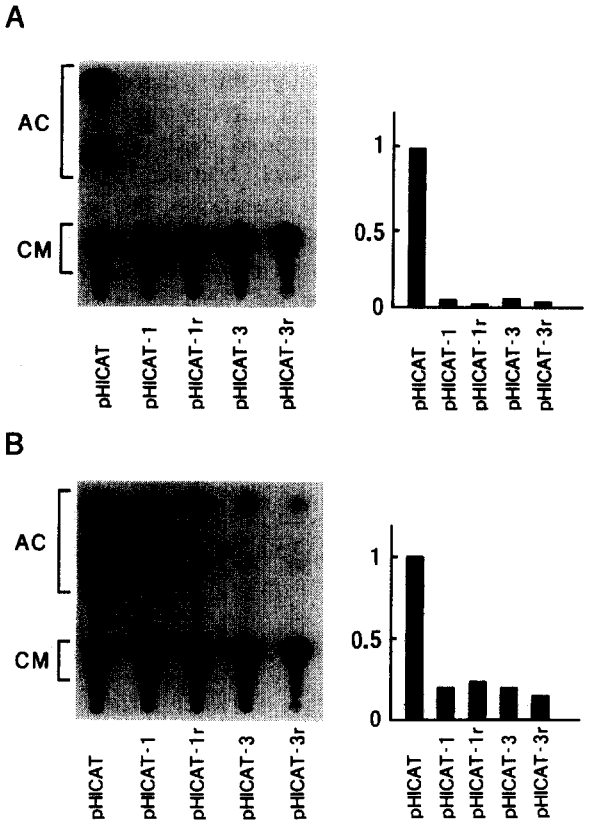

Fig.4. Effect of the HVR on CAT gene expression. CV-1 (A) and HeLa (B) cells were transfected with a reporter plasmid harboring class 1 or 3 fragments. CAT activities are expressed as a relative ratio to that of pHICAT.

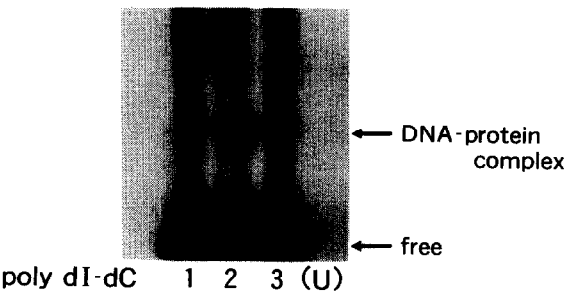

Fig.5. Gel retardation assay using a 5′-end labeled class 1 fragment and a crude nuclear extract from HeLa cells. The band corresponding to the DNA-protein complex is shown. To prevent nonspecific binding, poly(dI·dC) was used as a carrier.

dicating that the HVR has a suppressive effect on insulin gene expression in nonpancreatic cells. Treatment of cells with glucose, PMA, or dibutyryl cAMP failed to elevate CAT activity (not shown). The latter two inducers had been expected to modulate the effect of the HVR, since the HVR contains a consensus sequence, 5′-TCCC-CAGGCC-3′ [11], for the AP-2 binding site in the anti-sense strand, recently characterized as a final transmitter operating through a complex pathway of the effects of phorbol ester and/or cAMP [20]. Thus, the suppressive effect of the HVR on the transcription was not affected by these extracellular modulators, which suggests a mechanism different from that of the enhancer region.

Furthermore, gel retardation assay reveals a band corresponding to the DNA-protein complex (fig.5), suggesting the presence of a possible *trans*-acting protein(s) bound to the HVR in HeLa cells, although whether this factor is responsible for the suppressive function of the HVR is unknown at present.

4. DISCUSSION

The 5′-flanking region of the rat insulin 1 gene, from −219 to +51 bp from the transcription start site, has recently been suggested to be responsible for the negative regulation of gene transcription [8]. However, a mutational analysis by Karlsson et al. [7] failed to detect any key component sequence that would contribute to the negative control. This raises the possibility that multiple DNA regions are involved in repression of insulin gene transcription and also that a deletion of only one region does not interfere with the negative regulation. Furthermore, the previous finding that the sequence of the enhancer region and its upstream region of the insulin gene is relatively divergent between rats and humans [4] suggests that the negative regulation of insulin gene expression may differ between the two species.

We have demonstrated here that the region containing the human insulin gene enhancer also possesses a suppressive effect on transcription in non-pancreatic cells, as does the 5′-flanking region of the rat insulin I gene. Our results also show that an increase in intracellular cAMP level blocks the suppressive effect of the enhancer region on insulin gene expression in CV-1 cells, suggesting that this region alone is not sufficient to repress completely

insulin gene expression in response to various stimuli in non-pancreatic cells. It is likely, therefore, that the positive regulation of insulin gene expression by cAMP is exerted by the putative CRE, 5′-TGACGCCA-3′ (−181 to −174 bp), similar to the consensus sequence observed in other genes in which transcription is regulated by cAMP [20].

Extracellular glucose is the major physiological regulator of insulin biosynthesis in pancreatic β cells [21]; its effect is mediated in part by cAMP-dependent protein kinase. Unlike cAMP analogues, however, the addition of glucose failed to stimulate the activity of the human insulin promoter which is linked to the CRE region, suggesting that the signal-transduction pathway of glucose is not linked to the cAMP pathway in CV-1 cells. In pancreatic β cells, phorbol ester has also been shown to be a strong insulin secretagogue which activates the Ca^{2+}-phospholipid-dependent protein kinase [22]. Recently, the CRE of the human VIP gene was demonstrated to mediate transcriptional regulation by phorbol ester and cAMP in HeLa cells (Fink, I.S. et al., The Endocrine Society, 70th Annual Meeting, 1988). However, in the present study, PMA failed to induce CAT expression in CV-1 or HeLa cells, suggesting that there is no link between the phorbol ester and the cAMP pathway in insulin gene expression in non-pancreatic cells.

In this study, we have also shown that the HVR has a suppressive effect on insulin gene expression in non-pancreatic cells. The study of Walker et al. [1] using deletion analysis has indicated that the HVR does not influence gene expression in rodent insulin-producing cells. The discrepancy between the two experiments might be explained by the cell-specific difference in regulating insulin gene expression. An alternative explanation is that a *trans*-acting factor(s) which interacts with the HVR of humans is lacking in rodent cells. Although the HVR contains the consensus sequence for AP-2 binding [20] which mediates transcriptional activation in response to cAMP and/or phorbol ester, the suppressive effect of the HVR on insulin gene expression was not affected by dibutyryl cAMP nor PMA. It appears, therefore, that the negative regulation of insulin gene expression by the HVR is not modulated by the ATP-2 binding element; alternatively, the sequence, 5′-TCCCCAGGCC-3′ present on the anti-sense strand of the HVR may be incomplete for the AP-2 binding site.

Acknowledgements: We wish to express our appreciation to Dr G.I. Bell (Howard Hughes Medical Institute, University of Chicago) for having supplied us with the human insulin gene and HepG2-type glucose transporter cDNA clones and also to Dr S. Seino (Howard Hughes Medical Institute, University of Chicago) for critical reading of the manuscript.

REFERENCES

[1] Walker, M.D., Edlund, T., Boulet, A.M. and Rutter, W.J. (1983) Nature 306, 557–561.
[2] Hanahan, D. (1985) Nature 315, 115–122.
[3] Nielsen, D.A., Welsh, M., Casadaban, M.J. and Steiner, D.F. (1985) J. Biol. Chem. 260, 13585–13589.
[4] Steiner, D.F., Chan, S.J., Welsh, J.M. and Kwok, S.C.M. (1985) Annu. Rev. Genet. 19, 463–484.
[5] Karlsson, O., Edlund, T., Moss, J.B., Rutter, W.J. and Walker, M.D. (1987) Proc. Natl. Acad. Sci. USA 84, 8819–8823.
[6] Moss, L.G., Moss, J.B. and Rutter, W.J. (1988) Mol. Cell. Biol. 8, 2620–2627.
[7] Ohlsson, H., Karlsson, O. and Edlund, T. (1988) Proc. Natl. Acad. Sci. USA 85, 4228–4231.
[8] Nir, U., Walker, M.D. and Rutter, W.J. (1986) Proc. Natl. Acad. Sci. USA 83, 3180–3184.
[9] Stein, R.W. and Ziff, E.B. (1987) Mol. Cell. Biol. 7, 1164–1170.
[10] Bell, G.I., Karam, J.H. and Rutter, W.J. (1981) Proc. Natl. Acad. Sci. USA 78, 5759–5763.
[11] Bell, G.I., Sebby, M. and Rutter, W.J. (1982) Nature 295, 31–35.
[12] Seino, S., Steiner, D.F. and Bell, G.I. (1987) Proc. Natl. Acad. Sci. USA 84, 7423–7427.
[13] Leslie, R.D.G. and Pyke, D.A. (1987) in: The Diabetes Annual 3 (Alberti, K.G.M.M. and Krall, L.P. eds) pp.39–54, Elsevier, Amsterdam.
[14] Gorman, C.M., Moffat, L.F. and Haward, B.H. (1982) Mol. Cell. Biol. 2, 1044–1051.
[15] Bell, G.I., Horita, S. and Karam, J.H. (1984) Diabetes 33, 176–183.
[16] Graham, F. and Van der Eb, A. (1973) Virology 52, 456–457.
[17] Edlund, T., Walker, M.D., Barr, P.J. and Rutter, W.J. (1985) Science 230, 912–916.
[18] Wu, C. (1985) Nature 317, 84–87.
[19] Singh, H., Sen, R., Baltimore, D. and Sharp, P.A. (1986) Nature 319, 154–158.
[20] Roesler, W.J., Vandenbark, G.R. and Hanson, R.W. (1988) J. Biol. Chem. 263, 9063–9066.
[21] Hedeskov, C.J. (1980) Physiol. Rev. 60, 442–509.
[22] Zawalich, W.S. (1988) Diabetes 37, 137–141.
[23] Mueckler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard, J.E. and Lodish, H.F. (1985) Science 229, 941–945.
[24] Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201–5205.