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RESPONSE TO GLUCOSE OF TRANSCRIPTION FACTOR BINDING TO THE RAT INSULIN-1 GENE PROMOTER

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SUMMARY: The mechanism by which glucose stimulates insulin gene expression has been investigated by studying the binding of nuclear proteins to a putative glucose-sensitive element (GSE) in the rat insulin-I gene promoter. Gel retardation assays showed that a specific binding activity was present in four different ß-cell lines. The binding activity was increased by glucose only in those ß-cell lines which were shown to retain glucose-regulated insulin gene transcription. However, a similar binding activity was also shown to be present in an α-cell line. The protein factor binding to the GSE was estimated to have a molecular weight of 27kD. This protein may play a pivotal role in glucose-regulated transcription of the insulin gene.

Insulin biosynthesis is restricted to the ß-cells of the islets of Langerhans in adult mammals by control at the transcriptional level of insulin gene expression. The ß-cell-specific expression of the insulin gene has been shown to be conferred by *cis*-acting sequences located within 350bp upstream of the insulin gene transcriptional start site [1-3]. Many promoter and enhancer elements have been described within the 5' flanking sequences of the human insulin gene [4] and both alleles of the rodent insulin genes [5-7]. The roles and interactions of these active elements are complex with both positive and negative influences being shown to be exerted by specific sequences [8, 9]. However, in the rat insulin-I gene, two elements have been shown to be of particular importance to ß-cell-specific

Abbreviations: CAT, Chloramphenicol acetyltransferase; RSV-gal, Rous sarcoma virus promoted ß-galactosidase; GSE, glucose-sensitive element; ppImRNA, preproinsulin messenger RNA.

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expression; these are known as the NIR (or IEB1) box located at -104 and the FAR (or IEB2) box located at -233 [5]. These sequences have been shown to bind a single factor, IEF1, which is only present in cells of an endocrine nature [10, 11]. These E-boxes have also been shown to be important in the expression of the human and rat-II insulin genes [9, 12].

Insulin biosynthesis is also subject to short-term control in response to changes in ambient glucose and other nutrient concentrations which modulate translation [13, 14], RNA turnover [14, 15] and transcription (for review see [16]). Glucose-mediated transcriptional regulation of a reporter gene has been shown to be conferred by 327bp of the human insulin gene promoter [17] and by 346bp (-345 to +1) of the rat-I insulin gene by transfection into a clonal ß-cell line [18]. The element(s) responsible within the rat insulin-1 gene promoter were further mapped by transfection of fetal rat islets with rat insulin-I chimaeric genes [19]. Although the overall glucose response may involve multiple elements [20] a major glucose sensitive element has been mapped to the rat insulin-I FF-minienhancer (-196 to -247) [19],[20] and further to the sequences between -193 to -227 [21] containing the FLAT element, an AT-rich region adjacent to the FAR E-box. Electrophoretic mobility shift assays have shown that a number of complexes bind to this region and the binding activity of one complex has been shown to be modulated by pre-exposure of isolated rat islets to varying concentrations of extracellular glucose (2-20mM) [21]. However, it was not shown in that study (21) whether the glucose-regulated factor was specific for ß-cells which retained glucose-sensitive insulin gene transcription.

The aim of the present study was to correlate glucose-sensitive protein complex binding to the glucose-sensitive element of the rat insulin-I gene with glucose-sensitive native and exogenous insulin gene transcription.
A preliminary account of our findings has been published in abstract form [36].

MATERIALS AND METHODS

Cell culture - Monolayer cell cultures were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum and penicillin/streptomycin in a humidified atmosphere of 95% air/5% CO_2 as previously described [22]. Culture media was replaced every 48h and cells were passaged weekly following detachment by trypsin-EDTA(Gibco).
RNA extraction and Northern blot analysis - Cells were grown to 80% confluence, pre-incubated for 2h in the absence of glucose, and then incubated with zero or 11mM glucose for 4-6h. Total RNA was extracted from the monolayer cultures by the acid-phenol method [23]. Heat-denatured RNA (10µg) was size-fractionated by electrophoresis through a 1.2% formaldehyde/agarose gel and transferred to a nylon membrane (Amersham). Blots were baked at 80°C for 2h and pre-hybridised for 4h at 42°C. Hybridisations were carried out at the same temperature for 18h by adding random-primed oligonucleotide cDNA probes complementary to insulin mRNA and to the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase mRNA as previously described [18]. Resulting autoradiographs were quantitated by scanning densitometry.
Transfection and analysis of chimaeric gene expression - ß-Cells cultured in 60mm plates were co-transfected with 5µg of test plasmid and 5µg of

internal control plasmid by the calcium phosphate co-precipitation technique [24]. The test plasmid, pOK1, was derived from pUC18 and contains 346 bp (-345 to+1) of rat insulin-1 gene 5' flanking region upstream of the coding sequence of the bacterial chloramphenicol acetyltransferase gene (CAT) as previously described [5, 18]. pRSVß-Gal was used as the control plasmid. Cells were allowed to recover in propagation media for 24h and then incubated in RPMI 1640 containing 1 or 11mM glucose (Gibco) for a further 24h. Cell extracts were prepared and analysed for appropriate enzyme activities. Extracts (100µg of protein) were used for CAT activity using a fluorescent chloramphenicol substrate (FluoCAT from Molecular Probes Ltd.) which was quantitated by fluorimetry after acetylation products were separated by thin layer chromatography. CAT activity measurements were performed in triplicate and were normalised against ß-galactosidase values.

Nuclear extract preparation - ß-Cells were harvested after incubation overnight in media containing 1mM or 11mM glucose. Nuclear proteins were extracted [25] in the presence of protease inhibitors (1mM PMSF, 1mM benzamidine and 5mg/ml leupeptin: Sigma). Total protein concentrations were determined by the Bradford method [26].

Gel retardation assay - Oligodeoxynucleotides were synthesised by Oswel, University of Edinburgh. The double stranded DNA probe (GSE) containing the sequence -206 to -227 of the rat insulin-I gene promoter [21] was labelled with [γ-^{32}P]-ATP and T4 polynucleotide kinase (Promega). Nuclear proteins (2µg) were incubated in 25µl of binding buffer (20mM Hepes (pH 7.9), 25% glycerol, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM EDTA and 0.5mM DTT) in the presence of 2µg of poly dI-dC at room temperature for 30 min. Labelled probe (1ng) was then added and incubated for a further 30 min. The DNA/protein complexes were analysed by electrophoresis through a native 10% polyacrylamide gel run with a high ionic strength buffer (25 mM Tris base: 250 mM glycine). The gels were dried under vacuum and exposed to autoradiography. Quantitation of individual bands was achieved by densitometry.

RESULTS AND DISCUSSION

The ß-cell line HIT-T15, cloned from hamster islets by SV40 transformation [27], retains glucose-responsive insulin secretion and biosynthesis [18, 22, 27]. Fig. 1 shows that incubation of HIT-T15 cells for 4h in 10mM glucose after 2h preincubation in glucose-free media increased preproinsulin mRNA (ppImRNA) levels 2 to 3-fold.

The X-ray induced rat insulinoma ß-cell line RINm5F [28] is known to contain insulin but this cell-line does not respond to glucose with increased insulin secretion. This inability to respond to glucose has been attributed to abnormal glucose metabolism in the RIN cell [29]. RINm5F cells did not respond to glucose with increased ppImRNA levels when incubated with 10mM glucose (Fig. 1), compared with a zero glucose control incubation.

More recently developed ß-cell lines have been isolated from targeted SV40 T-antigen-induced transgenic mouse tumours. The ß-cell line ßTC3 (obtained from Dr. D. Hanahan, University of California) contains much higher levels of insulin than the HIT and RIN ß-cell lines and responds to glucose with increased insulin secretion [30]. However the ßTC3 cells did not respond to incubation for 4h with 10mM glucose with increased ppImRNA levels (Fig. 1); indeed it was not possible to detect a difference in ppImRNA levels even after overnight incubation in zero glucose. This lack of response to glucose at the transcriptional level may be attributable to the high sensitivity of this cell line to glucose; maximal stimulation of secretion occurs at about 1mM glucose [30]. It has also been shown that the ßTC3

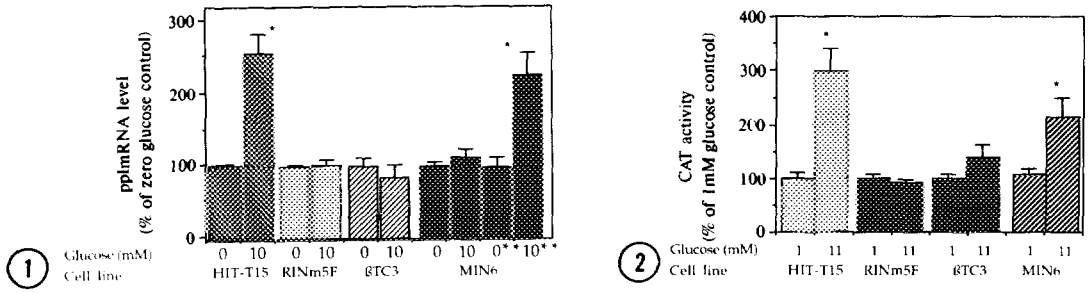

Fig.1. Effect of glucose on preproinsulin mRNA levels in ß-cell lines. ß-cells were incubated in the absence or presence of 10mM glucose for 4h (except for ** (12h)) after which the preproinsulin (ppImRNA) levels were measured by Northern blot analysis of total RNA and quantitated by autoradiography and densitometry. The values for ppImRNA are calculated relative to GAPDH mRNA values in the same extract and are expressed as a percentage of the zero glucose control. The data are plotted as means ±S.E.M. for three independent observations. *Significantly greater than value in the presence of zero glucose ($P<0.001$).

Fig. 2. Effect of glucose on insulin promoter-driven CAT expression in transfected ß-cell lines. ß-cell lines were co-transfected with a RSV-promoted ß-galactosidase expression plasmid and a rat insulin-I promoted (-345 to +1) CAT reporter gene expression plasmid. 24h after transfection the ß-cells were incubated at 1 or 11mM glucose for a further 24h before extraction and assay of enzyme activities. The values of CAT activity are expressed relative to ß-galactosidase activity in the same extract. The data are plotted as means±S.E.M. for three independent observations.
*Significantly greater than value at 1mM glucose ($P<0.001$).

cells need extended time periods (24-48h) for glucose-induced changes in insulin biosynthesis and ppImRNA levels to be detected [31]. However one study using a sensitive nuclear run-on assay [15] has shown a rapid-onset glucose-induced increase in βTC3 insulin gene transcription rates.

The MIN6 ß-cell line, also established from transgenic mice, retains sensitivity to glucose [32]. Although MIN6 ß-cells did not respond to high glucose with an increase of ppImRNA levels over 4-6h incubations, a change in ppImRNA level of 2-fold was detected when the cells were incubated with 10mM glucose for periods in excess of 12h (Fig. 1).

To further investigate the glucose-responsiveness of the insulin gene promoter in these four ß-cell lines, the cells were transfected with a vector containing a rat insulin-I promoter-driven reporter gene; expression of the reporter gene was monitored after incubation with 1 or 11mM glucose. The rat insulin-I gene promoter (-345bp to +1bp) was able to confer basal expression of the reporter gene in all ß-cell lines tested but the ability of these rat sequences to confer glucose-responsive gene expression was restricted to the HIT and the MIN6 ß-cell lines (Fig. 2). The βTC3 cell line appeared to express the reporter gene at higher levels than the other cell lines even under zero glucose conditions. This is consistent with the βTC3 cells being already stimulated at the low glucose control levels. Incubation of βTC3 cells at zero glucose for the periods of time needed for these studies was detrimental to the viability of the cultures. Thus these data demonstrate that the ß-

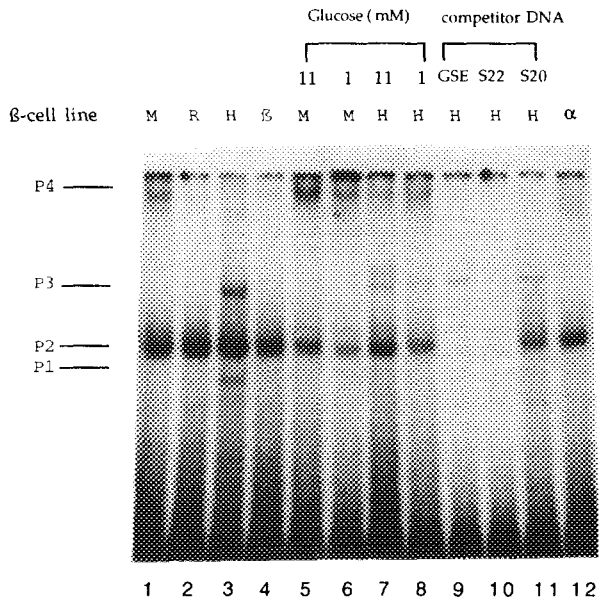

Fig. 3. Binding profile of ß-cell line nuclear factors to rat insulin-I GSE probe (-227 to -206). One example of a gel retardation assay using the GSE probe is shown. Assays were performed using ß-cell line nuclear extracts and labelled double-stranded GSE probe as described in the experimental section. M, MIN6 ß-cell line. R, RINm5F. H, HIT-T15. ß, βTC3. α, αTC1 cell line. All nuclear extracts were isolated from cells cultured for 16h in 11mM glucose except lanes 6 and 8 (1mM glucose for 16h). 2μg of nuclear proteins was used in all binding reactions except lane 3 in which 5μg was used. Competition assays were performed in the presence of 100-fold molar excess of unlabelled double-stranded DNA probes. GSE, glucose-sensitive element. S22, 365bp fragment containing block mutation at -233 to-241. S20, 365bp fragment containing block mutation at -211 to -222.

cell lines which retained a glucose response at the level of native ppImRNA transcription are also able to respond to glucose with increased expression of a reporter gene mediated by the exogenous rat insulin-I upstream sequences.

Gel retardation experiments were performed using nuclear proteins extracted from the same passages of the ß-cell lines used in the transcriptional studies in order to determine the extent to which the glucose-responsive pattern of gene expression was correlated with binding activities of proteins to the recently described "glucose-sensitive element" [21]. The double-stranded probe used in all binding studies contained the rat insulin-I promoter sequence between -206 and -227. Incubation of the labelled probe with nuclear protein extracts from ß-cell culture lines resulted in a characteristic binding profile revealed by gel retardation assay. One major band was prevalent in all ß-cell line extracts assayed (Fig. 3). Nuclear extracts from MIN6, RINm5F, HIT-T15 and βTC3 cells all formed a single major complex, P2 (lanes 1-4 respectively). Further complexes were revealed (P1 and P3) with all the ß-cell extracts but only when higher concentrations of nuclear proteins (5-8μg) were incubated with the probe, as exemplified in lane 3 (Fig. 3) with 5μg of HIT cell

extract. The specificity of complexes P1-P3 was assessed by competition with unlabeled probes. A 100-fold molar excess of unlabeled rat insulin-I probe (-206 to -227) competed for the binding of P1 and P2 complexes completely and P3 partially (lane 9). Fragments of rat insulin-I 5'-sequences containing block mutations were used in competition experiments to further characterise the binding specificity of the ß-cell extracts. A 366bp fragment containing mutation at -233 to -241 (S22) competed for all complex binding (Fig. 3, lane 10) whereas a 365bp fragment containing mutation at -211 to -222 (S20) did not compete (lane 11). A minor high molecular weight complex was often seen (P4) but its presence was not detected in all assays, suggesting that this complex was of a less specific nature. Complexes P1-P3 were not observed when nuclear proteins from HeLa and COS-7 culture cells or rat liver or rat spleen were incubated with the rat insulin-I probe (data not shown). However nuclear proteins isolated from the glucagon-producing αTC1 cell line [33] also bound the rat insulin-I probe to give the P2 complex (Fig. 3, lane 12). Therefore, although the nuclear protein(s) which binds as the major complex (P2) to the GSE appears to be islet-specific, it is not ß-cell-specific.

Nuclear proteins were extracted from ß-cells incubated in the presence or absence of glucose and the pattern of binding was assessed. A difference of intensity of binding was observed in MIN6 and HIT-T15 ß-cells when nuclear

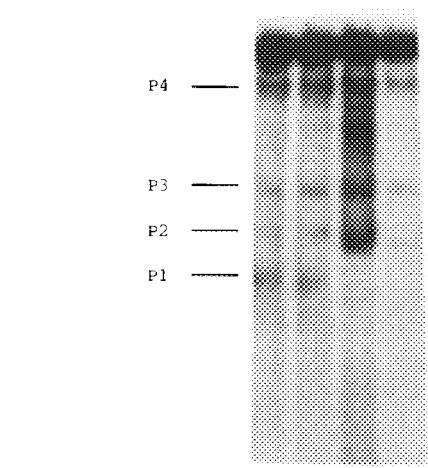

Fig. 4. Single-strand competition analysis of rat insulin-I probe binding. Specificity of probe binding was assessed by gel retardation assay using nuclear extracts from HIT-T15 ß-cells. Labelled GSE probe alone (lane 1). Binding reactions performed in the presence of 100-fold molar excess of unlabelled probes. ssU: single-stranded upper strand DNA probe (lane 2). ssL: single-stranded lower strand DNA probe (lane 3). GSE: double-stranded probe (lane 4)

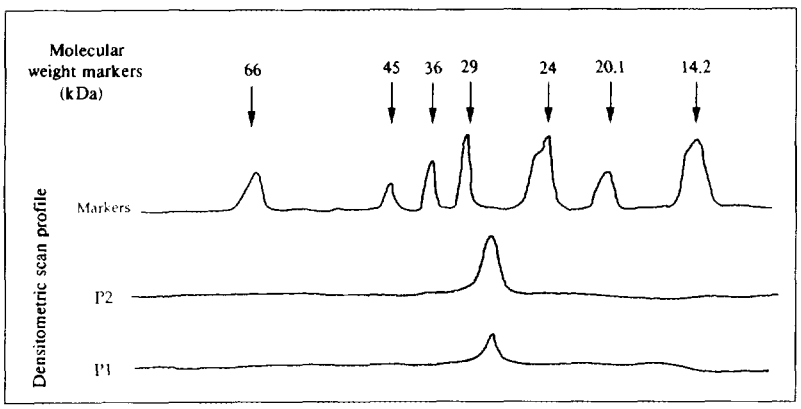

Fig. 5. SDS-PAGE analysis of P1 and P2 complex proteins.
Gel retardation assays were performed in native acrylamide gels. The resultant visualised complexes were excised from the wet gel, denatured in SDS-loading buffer and subjected to denaturing SDS-PAGE through a 10% gel by loading the gel slices into the denaturing gel wells. The proteins were visualised by silver-staining and size estimated by densitometric scanning. (P1 and P2 as denoted in fig. 3).

extracts were prepared after 16h incubation with 1mM or 11mm glucose-containing media (Fig. 3 lanes 5-8). The glucose-mediated increase in binding was restricted to the P2 complex with MIN6 cells (mean increase 2.42±0.35-fold) and with HIT cells (mean increase 2.37±.0.35-fold) when three independently isolated extracts were tested. These increases were significant ($P<0.001$ assessed by Student's t-test) although less marked than reported in intact rat islet studies [21]. No increase was elicited by glucose in RINm5F or βTC3 cells (data not shown).

To further characterise the composition of the DNA:protein complexes, the visualised bands were excised from retardation gels and subjected to electrophoresis on denaturing SDS-PAGE [34]. The proteins were no longer bound to the DNA probe under these conditions and silver staining [35] provided information on the number and molecular mass of proteins involved in a complex. The P1 and P2 complexes both migrated as a single band with an estimated M_r of 27kD (Fig. 5). This observation itself confirmed that the silver-stained proteins had been resolved from DNA:protein complexes rather than representing unbound soluble proteins which co-migrate with the DNA complexes at that position. The possibility that the P1 and P2 complexes contained the same protein was investigated by competition experiments using single-stranded probes. Incubation of HIT-T15 nuclear proteins with the labelled rat insulin-I double-stranded probe in the presence of 100-fold excess unlabeled single-stranded probes suggested that the P1 complex was formed by the P2 complex protein binding to residual lower single-stranded probe (Fig. 4) which remained after the annealing of the double-stranded probe.

The binding pattern and the specificity of the binding of nuclear extracts from the four ß-cell lines to the rat insulin-I probe is broadly in agreement with

earlier data[21] but it is not fully clear whether the P1-P3 complexes are the equivalent of C1-C3 according to the previous authors' nomenclature [21].

In conclusion we have shown that the P2 complex is the major band seen using ß-cell line extracts in high ionic strength gel retardation assays. The P2 complex protein is also present in α-cells but may be islet-specific. The binding of this P2 complex appears to be glucose-sensitive and the ability of extracellular glucose to increase the intensity of binding parallels the ability of glucose to mediate transcriptional regulation in the ß-cell. Interestingly, the glucose-sensitivity of P2 binding was lost in HIT-T15 ß-cells which no longer showed insulin secretion and transcriptional glucose responses due to prolonged passage (data not shown). The P2 complex protein may well play a pivotal role in glucose-regulated transcriptional events as it binds to a region which has been shown to confer glucose regulation upon heterologous genes [19, 21] although it may not be the only glucose-sensitive binding involved in the full physiological expression of the insulin gene. Further investigation of glucose-mediated regulation of DNA binding of any of the large number of insulin gene regulatory factors remains to be carried out.

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