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Specific co-ordinated regulation of PC3 and PC2 gene expression with that of preproinsulin in insulin-producing βTC3 cells

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Short-term (less than 2 h) glucose stimulation of isolated pancreatic islets specifically increases the biosynthesis of proinsulin and its converting enzymes PC2 and PC3 at the translation level. To determine whether gene expression of PC2 and PC3 was also regulated by longer-term (more than 6 h) glucose stimulation along with that of preproinsulin, studies were performed with the βTC3 insulin-producing cell line. By Northern blot analysis, glucose maintained PC2 and PC3 mRNA levels in parallel with those of preproinsulin. After 48 h, mRNA levels of preproinsulin, PC2 and PC3 were, respectively, 2.9 ($P < 0.05$), 3.0 ($P < 0.005$) and 5.3 ($P < 0.001$) times greater in the presence of glucose than in βTC3 cells cultured in the absence of glucose. Glucose-regulated PC2 and PC3 gene expression, like that of preproinsulin, was maximal at glucose concentrations above 5.5 mM. Studies of mRNA stability showed that the half-lives of PC2 (9 h) and PC3 (5 h) mRNA were much shorter than that of preproinsulin mRNA (over 24 h), but little effect of glucose on stability of these mRNAs was observed. Nuclear run-off analysis indicated that transcription of preproinsulin, PC2 and PC3 was modestly induced after 1 h exposure to 16.7 mM glucose. Therefore preproinsulin, PC2 and PC3 mRNA levels in βTC3 cells were most probably maintained at the level of gene transcription. In contrast, elevation of cyclic AMP by forskolin had no effect on mRNA levels or gene transcription of preproinsulin, PC2 and PC3, despite a cyclic-AMP-induced phosphorylation of the cyclic AMP response element binding protein that correlated with a marked increase in c*Jun* and c*Fos* gene transcription in the same β-cells. These results suggest that preproinsulin, PC2 and PC3 gene transcription can be specifically glucose-regulated in a mechanism that is unlikely to involve a key role for cyclic AMP. The co-ordinate increase in PC2 and PC3 mRNA levels with that of preproinsulin mRNA in response to chronic glucose represents a long-term means of catering for an increased demand on proinsulin conversion.

INTRODUCTION

Insulin production in the pancreatic β-cell requires prior conversion of its precursor molecule, proinsulin, by limited endoproteolytic cleavage (see [1–4] for reviews). Essentially, conversion of proinsulin is catalysed by two distinct endopeptidase activities, PC3 (also known as PC1) and PC2. PC3 cleaves on the carboxyl side of Arg-31, Arg-32 at the human proinsulin B-chain/C-peptide junction, and PC2 cleaves C-terminal of Lys-64, Arg-65 of the C-peptide/A-chain junction. The exopeptidase carboxypeptidase-H (CP-H) removes Arg and Lys from the carboxyl terminals immediately after endoproteolytic cleavage. These converting enzymes are co-packaged with proinsulin into insulin secretory granules where they become fully activated, ensuring that most proinsulin conversion takes place in the organelle where insulin is stored before regulated secretion [5,6].

It has been postulated that regulation of prohormone conversion can be mediated by several factors including intra-organellar environment, control of endopeptidase activity, cell-specific expression of certain proprotein-processing endopeptidases, and co-ordinate control of endopeptidase biosynthesis with that of its proprotein substrate [1–4,6,7]. In particular, for pancreatic β-cells where proinsulin biosynthesis is highly regulated [3,8], maintenance of intracellular proprotein-converting endopeptidase levels seems to be crucial for efficient proinsulin processing. Retarded proinsulin conversion in an insulin-producing cell line has been associated with diminished PC3 levels [9]. Furthermore, short-term (less than 2 h) glucose regulation of proinsulin biosynthesis at the translational level [10,11] is co-ordinately regulated with that of PC2 and PC3 biosynthesis in isolated islets from normal and obese rodents [12,13]. For longer-term exposure to elevated glucose concentrations (over 6 h), there is also glucose regulation of preproinsulin gene transcription in cultured islets [14], as well as in states of hyperglycaemia [15,16] and refeeding after a period of fasting [17]. Recently, we have made some preliminary observations in islets isolated from rats exposed *in vivo* to 48 h chronic hyperglycaemia that suggest PC3 and preproinsulin mRNA levels were specifically increased, whereas PC2 and CP-H mRNA levels were unchanged [18].

The mechanism by which glucose regulates preproinsulin gene transcription is not particularly well understood (see [19,20] for review). However, it is possible that cyclic AMP might be a candidate for facilitating glucose-induced insulin gene transcription in pancreatic β-cells. Glucose has been shown to increase cyclic AMP levels in β-cells [21,22] and under some circumstances it has been shown that elevation of intracellular cyclic AMP can potentiate long-term glucose-stimulated preproinsulin gene expression [23–26]. Induction of preproinsulin gene transcription by cyclic AMP has been localized to specific regions in the rat preproinsulin-1 [27] and human preproinsulin [28] gene promoters. These regions contained sequence similarity to a con-

Abbreviations used: CP-H, carboxypeptidase-H; CRE, cyclic AMP response element; CREB, cyclic AMP response element binding protein; P-CREB, Ser-133-phosphorylated CREB; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PKA, cyclic-AMP-dependent protein kinase; TBS, Tris-buffered saline.

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sensus sequence for a response element found in a wide variety of other genes known as the cyclic AMP response element (CRE), which mediates transcriptional effects of cyclic AMP [29]. Activation of gene transcription through a CRE is facilitated by several structurally related protein transcription factors, of which the best characterized is the ubiquitously expressed cyclic AMP responsive element binding protein (CREB, [29]). CREB binds the CRE as a homo- or heterodimer that activates transcription when phosphorylated at Ser-133 by cyclic-AMP-dependent protein kinase (PKA) [30]. Sequence analysis of human PC2 [31] and mouse PC3 [32,33] gene promoter regions has revealed that CRE consensus sequences are present, raising the possibility that cyclic AMP may be involved in the potential of co-ordinating gene expression of PC2 and PC3 with that of preproinsulin.

In this study we have examined the chronic effect that elevated glucose levels have on regulating gene expression of preproinsulin and the proinsulin-processing endopeptidases PC2 and PC3 in pancreatic β-cells. Owing to the difficulty in obtaining adequate amounts of islet tissue for gene expression studies and interfering contributions made from non-β-cells of the islet, the βTC3 insulin-producing cell line was used [34]. βTC3 cells represent a reasonable experimental model for gene expression studies especially because it has previously been shown that preproinsulin gene transcription is regulated by glucose in these cells [35,36]. The role that cyclic AMP might have in regulating glucose-induced preproinsulin, PC2 and PC3 gene expression via phosphorylated-CREB bound to CREs was assessed by measuring cyclic-AMP-induced Ser-133 CREB phosphorylation in βTC3 cells [37]. The effects of cyclic AMP on gene expression of preproinsulin, PC2 and PC3 were measured in parallel with reference to cFos and cJun gene expression in the same βTC3 cells. The cFos and cJun oncogenes were used as positive controls because it is well characterized that they are cyclic-AMP-responsive genes whose expression is mediated via CREB/CRE interaction [29,30].

EXPERIMENTAL

Chemicals and reagents

Forskolin was purchased from Calbiochem (La Jolla, CA, U.S.A.). BSA was purchased from United States Biochemical Corp. (Cleveland, OH, U.S.A.). Alkaline phosphatase colour reagents Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Promega (Madison, WI, U.S.A.). Guanidinium thiocyanate was purchased from Fluka Biochemical (Buchs, Switzerland). Phenol was purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). Radiolabelled nucleotides were purchased from Du Pont NEN (Boston, MA, U.S.A.). Biotrans nylon membrane was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA, U.S.A.). Prime-it II random primer labelling kit was purchased from Stratagene (La Jolla, CA, U.S.A.). All other biochemicals were from either Sigma (St. Louis, MO, U.S.A.) or Fisher (Pittsburgh, PA, U.S.A.), and were of the highest grade and purity available.

Rat preproinsulin-2 cDNA probe (424 bap), which also hybridizes with mouse preproinsulin-2, was obtained from Dr. Lydia Villa-Komaroff (Children's Hospital, Boston, MA, U.S.A.). PC3 (2511 bp) and PC2 (2200 bp) cDNA probes were obtained from Dr. Alwanaz Rehemtulla (University of Michigan Medical School, Ann Arbor, MI, U.S.A.). The 36B4 (800 bp) cDNA probe, encoding ribosomal phosphoprotein PO, was used as a negative control as previously described [38]. The c*Fos* (1500 bp) probe was generously given by Dr. J. G. Belasco and Dr. M. E. Greenberg (Harvard Medical School, Boston, MA, U.S.A.). The c*Jun* (2000 bp) probe originated in the laboratory of Dr. R. Tjian [39].

Goat anti-rabbit IgG alkaline phosphatase conjugate was from Promega (Madison, WI, U.S.A.). Rabbit anti-phosphoserine-133 phosphorylated CREB antiserum and rabbit anti-CREB antiserum, the latter of which does not differentiate between phosphorylated and unphosphorylated isoforms of CREB, were generously given by Dr. M. E. Greenberg [37].

Culture of βTC3 cells

Passage 40–60 βTC3 cultures (from Dr. Shimon Efrat, Albert Einstein College of Medicine, Bronx, NY, U.S.A.) were maintained in Dulbecco's modified Eagle's medium (DMEM; 5.5 mM glucose) containing 10 % (w/v) fetal bovine serum (FBS), on 60 mm diameter dishes, in a humidified atmosphere of 95 % air/5 % CO_2 at 37 °C and subcultured every 5–7 days. For glucose studies, when cells were 60–70 % confluent they were washed with Hanks balanced salt solution, pre-incubated for 2 h in DMEM containing 0.1 % BSA, without glucose. At the beginning of the treatment period, glucose concentration was increased in 'stimulated' dishes by the addition of glucose to a final concentration of 16.7 mM. In the absence of glucose (actually determined to be less than 0.1 mM glucose), βTC3 cells were provided with alternative nutrient sources such as pyruvate and amino acids present in the DMEM [35], and maintained a viable appearance throughout the incubation period. For mRNA stability studies, βTC3 cells were preincubated for 24 h in DMEM containing 0.1 % BSA and 16.7 mM glucose. The media was then replaced with fresh DMEM containing 10 μg/ml actinomycin D (an inhibitor of gene transcription [40]), 0.1 % BSA, with or without 16.7 mM glucose and incubated further for up to 24 h. For forskolin studies, βTC3 cells were serum-starved for 24 h in DMEM media containing 5.5 mM glucose with 0.5 % FBS and then stimulated with 10 μM forskolin (an activator of adenylate cyclase to raise intracellular cyclic AMP levels) in the continued presence of 5.5 mM glucose in DMEM.

Northern blot analysis

βTC3 cells were first washed with ice-cold Hanks balanced salt solution to remove any unattached cells. RNA was extracted with acid guanidinium thiocyanate/phenol/chloroform [41]. Total RNA, 10 μg per sample, was run on a 1 % (w/v) agarose/6 % (v/v) formaldehyde/Mops gel and transferred by capillary elution to Biotrans nylon membrane. Probes were labelled with [^{32}P]dCTP (3000 Ci/mmol) using Prime-it II random primer labelling kit. Pre-hybridization (1 h) and hybridization (overnight) reactions were carried out in a solution of 50 mM Pipes, pH 6.5, 100 mM NaCl, 50 mM sodium phosphate buffer, 1 mM EDTA, 5 % (w/v) SDS and 60 μg/ml denatured salmon sperm DNA at 65 °C in a rotating hybridization oven. Blots were rinsed in 5 % (w/v) SDS, 0.5 % SSC (3 M NaCl/0.3 M sodium citrate, pH 7) at room temperature and washed three times in 5 % SDS, 0.5 % SSC at 65 °C for 20, 30 and 40 min respectively. Each blot was stripped by washing three times for 3 min each with boiling 0.1 % SDS and then once with 2 × SSC. This stripping process enabled the same blot to be sequentially reprobed for detection of PC2, PC3, preproinsulin, 36B4, c*Jun* and/or c*Fos* mRNA levels in the same βTC3-cell sample. Northern blot autoradiographs were determined quantitatively with a Molecular Dynamics (Sunnyvale, CA, U.S.A.) densitometer and normalized for loading differences to corresponding

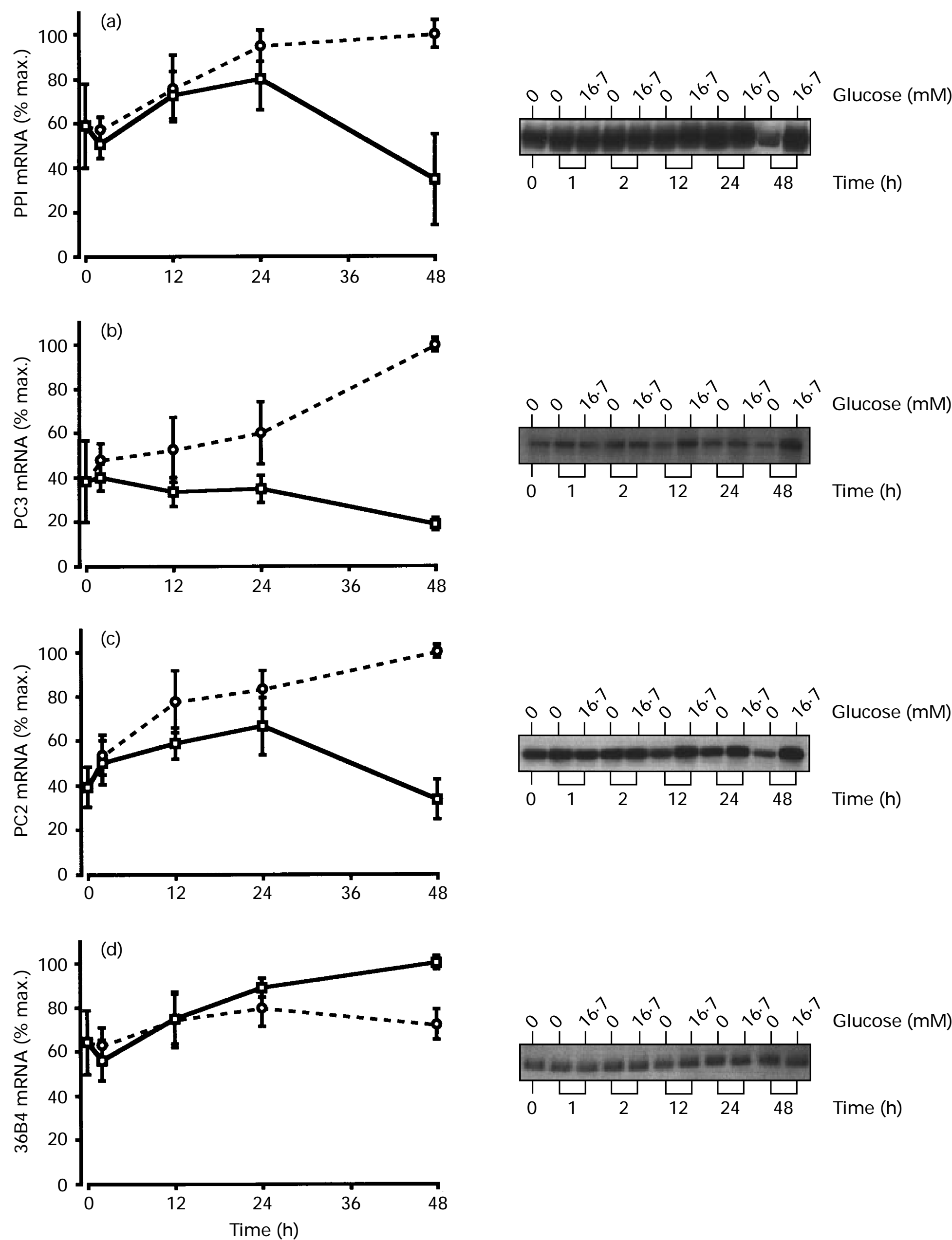

Figure 1 Glucose effects on mRNA levels of preproinsulin, PC2 and PC3 in βTC3 cells

βTC3 cells were preincubated for 2 h in medium containing 0.1% BSA in the absence of glucose. At time 0 the glucose concentration was subsequently adjusted to 16.7 mM (broken line) or maintained without glucose (solid line). At the indicated time RNA was processed for Northern blotting and each blot sequentially probed for preproinsulin (PPI) (**a**), PC3 (**b**), PC2 (**c**) and ribosomal phosphoprotein PO, designated 36B4 (**d**), mRNA as described in the Experimental section. Representative blots are depicted and results from densitometric scanning (means ± S.E.M.; $n = 3$) expressed as a percentage of the maximum.

ethidium bromide staining of the 28 S ribosomal subunit RNA in the appropriate agarose gel. The normalized data are indicated graphically, but not in example autoradiographs shown.

Nuclear run-off transcription analysis

βTC3 cells were maintained in DMEM/10% FBS on 150 mm diameter dishes. Each treatment consisted of five dishes of 50–60% confluent βTC3-cell cultures which were washed twice with ice-cold Hanks balanced salt solution and preincubated overnight in DMEM/0.1% BSA in the absence of glucose. The βTC3 cells were then incubated for 1 h at 37 °C in DMEM/0.1% BSA containing either no glucose, 16.7 mM glucose or 5.5 mM glucose plus 10 μM forskolin. Afterwards, nuclei were isolated and stored in liquid nitrogen, pending 30 min run-off reactions performed as previously described [42]. RNA was isolated by the acid guanidinium thiocyanate/phenol/chloroform method [41] and hybridized with 5 μg of plasmid restriction digest containing the probe of interest that had been dot–blotted onto Biotrans nylon membrane with the hybridization and wash conditions

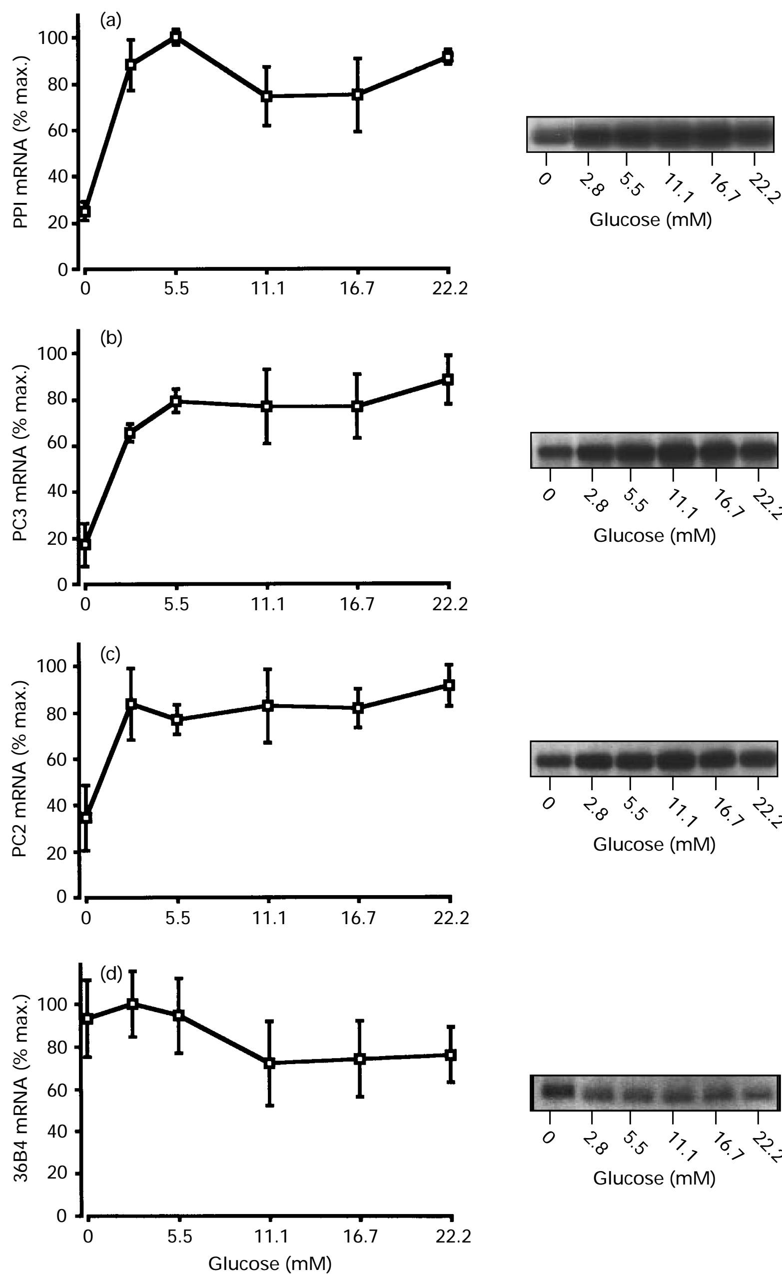

Figure 2 Glucose dose–response for gene expression of preproinsulin, PC2 and PC3 in βTC3 cells

βTC3 cells were preincubated for 2 h in medium containing 0.1 % BSA in the absence of glucose. The glucose concentration was subsequently adjusted to 2.8–22.2 mM for a 48 h incubation. RNA was processed for Northern blotting and each blot sequentially probed for preproinsulin (PPI) (**a**), PC3 (**b**), PC2 (**c**) and 36B4 (**d**) mRNA as described in the Experimental section. Representative blots are depicted and results from densitometric scanning (mean $\pm$ S.E.M.; $n = 3$) expressed as a percentage of the maximum.

previously described for Northern blot analysis. Dot–blot autoradiographs were measured by densitometric scanning with a Molecular Dynamics densitometer.

Immunoblot

For Ser-133 phosphorylated CREB (P-CREB) analysis, 60–70 % confluent cultures on 60 mm dishes were washed with PBS and scraped into boiling sample buffer [125 mM Tris, pH 6.8/2 % SDS/10 % (v/v) β-mercaptoethanol/20 % (v/v) glycerol/ Bromophenol Blue], boiled for 5 min and stored at −20 °C. Proteins were separated by SDS/PAGE (10 % gel). Gels were washed with transfer buffer [60 mM Tris, pH 9.2/1.63 mM SDS/48.8 mM glycine/20 % (v/v) methanol] and transferred to a nitrocellulose membrane by using a semi-dry blot transfer apparatus. Membranes were blocked for 1 h with Tris-buffered saline (TBS), pH 8/0.05 % Tween 20/1 % (w/v) goat serum/5 % (w/v) BSA, incubated overnight in TBS/0.05 % Tween 20/rabbit anti-P-CREB (1:20000 dilution) or rabbit anti-CREB (1:15000) at room temperature, then washed with TBS/0.05 % Tween 20. Specific P-CREB/CREB bands were detected with anti-rabbit IgG alkaline phosphatase conjugate as previously described [12]. Western blots were measures by densitometric scanning with a Molecular Dynamics densitometer.

Other analysis

Data were expressed as means ± S.E.M. and comparisons between groups were made with the appropriate Student's *t*-test. Gene sequence analysis was done with the Genetics Computer Group Inc. FindPatterns and Gap software.

RESULTS

Levels of preproinsulin, PC2 and PC3 mRNA from the same βTC3 cells maintained with or without glucose were measured over a 48 h period. Both PC2 and PC3 mRNA increased in βTC3 cells treated with 16.7 mM glucose and after 48 h each was 2.6-fold elevated ($P < 0.01$) compared with levels at time zero (Figures 1b and 1c). In contrast, in the absence of glucose there was a tendency for PC2 and PC3 mRNA levels to decrease, although this was not significant compared with levels at time zero. Nevertheless, by 48 h PC2 and PC3 mRNA levels were 3.0 ($P < 0.002$) and 5.3 ($P < 0.001$) times greater in the presence of glucose than in βTC3 cells maintained in the absence of glucose (Figures 1b and 1c). Glucose regulation of PC2 and PC3 mRNA

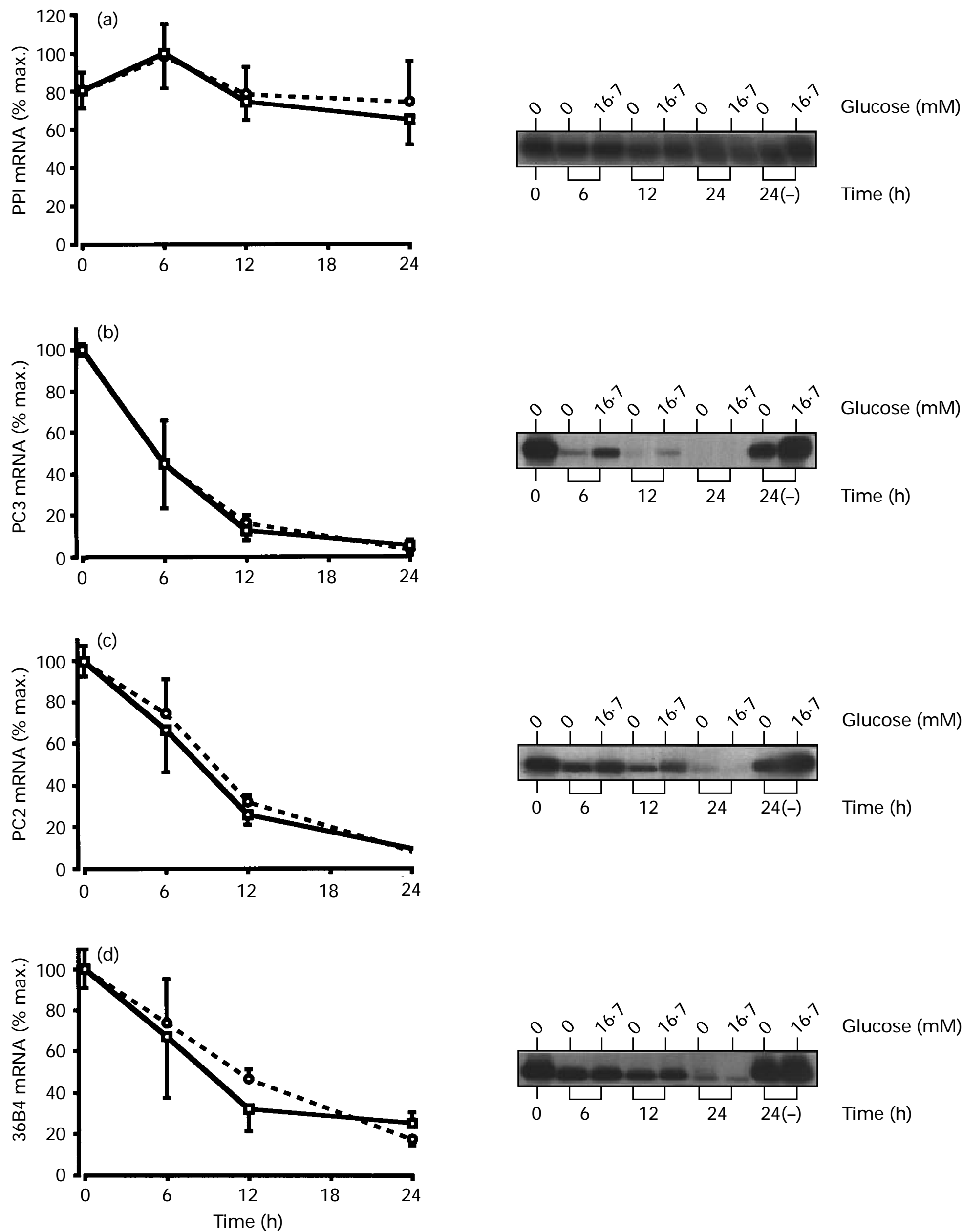

Figure 3 Glucose effects on stability of preproinsulin, PC2 and PC3 mRNA in βTC3 cells

βTC3 cells were stimulated for 24 h in medium containing 0.1% BSA and 16.7 mM glucose. Medium was then changed to include 10 μg/ml actinomycin D in the presence (16.7 mM; solid line) or absence (broken line) of glucose for the indicated incubation times. Control blots incubated for 24 h without actinomycin D are labelled 24(—). RNA was processed for Northern blotting and each blot sequentially probed for preproinsulin (PPI) (**a**), PC3 (**b**), PC2 (**c**) and 36B4 (**d**) mRNA as described in the Experimental section. Representative blots are depicted and results from densitometric scanning (means ± S.E.M.; $n = 3$) expressed as a percentage of the maximum.

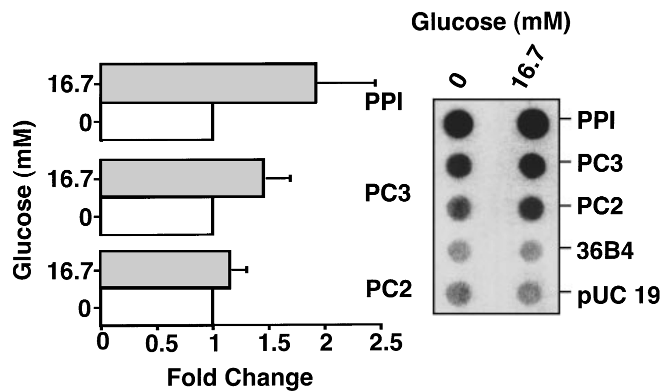

Figure 4 Glucose effects on transcriptional activity of preproinsulin, PC2 and PC3 genes in βTC3 cells

βTC3 cells were preincubated overnight in medium containing 0.1% BSA without glucose. Glucose either remained absent (open bars) or was increased to 16.7 mM (shaded bars) for 1 h at 37 °C. Nuclei were processed for a 30 min run-off reaction, RNA was isolated and hybridized with dot blots of preproinsulin (PPI), PC3, PC2 and 36B4 plasmid digest as well as a pUC vector control, as described in the Experimental section. Representative blots are depicted and results from densitometric scanning expressed as a means ± S.E.M. ($n = 3$) fold change from absence of glucose.

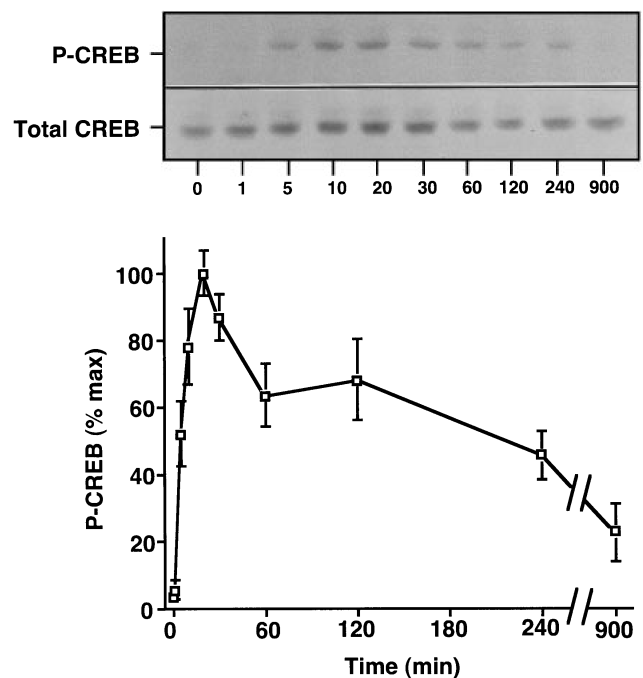

Figure 5 Forskolin stimulation of Ser-133 CREB phosphorylation in βTC3 cells

βTC3 cells were preincubated for 24 h in medium containing 0.5% FBS, then stimulated with 10 μM forskolin for the indicated times and processed for P-CREB and CREB immunoblotting as previously described in the Experimental section. Representative immunoblots are depicted. The upper, P-CREB, blot was obtained with anti-Ser-133 phosphorylated CREB antiserum and the lower, total CREB, blot was obtained by reacting aliquots of the same samples with a combination of anti-Ser-133 phosphorylated CREB antiserum and antiserum for CREB not directed against the phosphorylated form. The graph depicts densitometric scanning results, means ± S.E.M. ($n = 5$) expressed as a percentage of the maximum.

levels in βTC3 cells paralleled that of preproinsulin mRNA (Figures 1a, 1b and 1c), which was 3-fold ($P < 0.05$) greater in βTC3 cells exposed to glucose than in the absence of glucose. The glucose effects on preproinsulin, PC2 and PC3 mRNA levels were specific because mRNA levels of ribosomal phosphoprotein PO detected with the 36B4 probe were unchanged in the same βTC3 cells in the presence or absence of glucose (Figure 1d). Further characterization of glucose-regulated preproinsulin, PC2 and PC3 gene expression in βTC3 cells was performed for a 48 h period over a range of glucose concentrations. Preproinsulin, PC2 and PC3 mRNA levels were essentially maximal at glucose concentrations above 5.5 mM (Figures 2a, 2b and 2c). Again, no effect of glucose on ribosomal phosphoprotein PO (36B4 probe) mRNA levels was observed (Figure 2d).

To determine whether changes in mRNA stability contributed to the observed differences in mRNA levels of βTC3 cells in the presence or absence of glucose, the rate of mRNA degradation in the presence of the transcriptional inhibitor actinomycin-D was studied. Preproinsulin mRNA levels seemed to be quite stable in βTC3 cells in that there was only marginal degradation after 24 h (Figure 3a). This observation was consistent with previous studies in pancreatic β-cells where a half-life of 77 h for preproinsulin mRNA was estimated in the presence of glucose [40]. Within the 24 h period, glucose seemed to have little effect on preproinsulin mRNA stability (Figure 3a). However, this does not rule out an effect of glucose in influencing preproinsulin mRNA stability in β-cells at times over 24 h [40]. In contrast with preproinsulin mRNA stability, PC2 and PC3 had relatively short half-lives of 9 and 5 h respectively (Figures 3b and 3c), which were similar to the 9.5 h half-life of the control 36B4 (Figure 3d). Glucose had no significant effect on the stability of PC2, PC3 or 36B4 mRNAs.

Nuclear run-off studies were conducted to assess the role of transcription in glucose-regulated preproinsulin, PC2 and PC3 gene expression. After a 1 h stimulation with 16.7 mM glucose, gene transcription of preproinsulin, PC3 and PC2 was modestly elevated 1.8, 1.5 and 1.2 times respectively. Although these effects were not statistically significant compared with non-stimulated controls, glucose-induced preproinsulin, PC3 and PC2 gene transcription was a consistent observation in all three experiments (Figure 4). Low background levels of preproinsulin, PC3 and PC2 gene transcription were determined by using the pUC vector alone in control blots. The 36B4 cDNA was contained in a pSP64 vector and background measurements using pSP64 vector alone were undetecable. Although 36B4 gene transcription appeared to be somewhat low, no effect of glucose was observed.

Glucose has been shown to increase cyclic AMP levels in β-cells [21,22] which may in part play a secondary messenger role in long-term glucose-regulated preproinsulin gene expression [23–26]. To determine whether elevation of intracellular cyclic AMP levels could modulate CREB/CRE interaction in β-cells, levels of P-CREB were measured by immunoblot analysis of βTC3 cells treated with 10 μM forskolin in the presence of 5.5 mM glucose. Phosphorylation of CREB was detectable within 2 min and reached maximal levels by 20 min [26-fold stimulation (Figure 5); $P < 0.001$ compared with time zero]. Thereafter, levels of P-CREB gradually declined over a 15 h period, whereas total CREB levels remained stable for the duration of the experiment (Figure 5).

To determine whether cyclic-AMP-induced phosphorylation of CREB on a background of 5.5 mM glucose could correlate with any gene expression induced by cyclic AMP under the same conditions, preproinsulin, PC2 and PC3 mRNA levels were measured by Northern blot analysis in the same βTC3 cells.

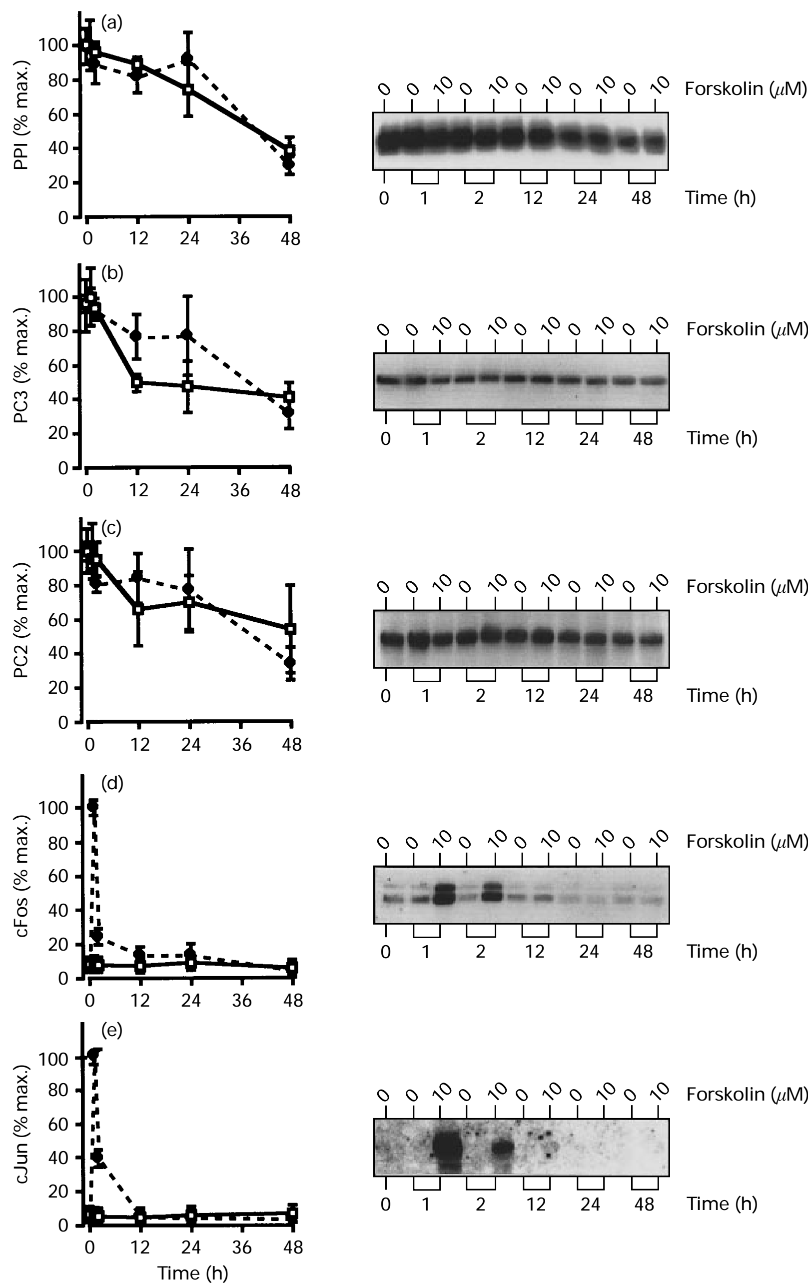

Figure 6 Forskolin effects on mRNA levels of preproinsulin, PC3, PC2, c*Fos* and c*Jun* in βTC3 cells

βTC3 cells were preincubated for 24 h in medium containing 0.5% FBS then stimulated with (broken lines) or without (solid lines) 10 μM forskolin for the indicated times. RNA was processed for Northern blotting and each blot sequentially probed for preproinsulin (PPI) (**a**), PC3 (**b**), PC2 (**c**), c*Fos* (**d**) and c*Jun* (**e**) mRNA as described in the Experimental section. Representative Northern blots are depicted and cumulative results (means ± S.E.M.; $n = 3$) generated from densitometric scanning analyses normalized to the 28 S ribosomal subunit and expressed as a percentage of the maximum.

However, no significant difference in levels of preproinsulin, PC2 or PC3 mRNA over a 48 h period were detected between cyclic-AMP-stimulated and control βTC3 cells (Figures 6a, 6b and 6c). Nevertheless, in contrast with preproinsulin, PC2 and PC3 mRNA levels, in the same βTC3 cells c*Fos* and c*Jun* mRNA levels were elevated 13-fold ($P < 0.001$) and 16-fold ($P < 0.001$) respectively (Figures 6d and 6e) after 1 h incubation with 10 μM forskolin in a manner that correlated with a prior increase in P-CREB levels (Figure 5). Changes in these mRNA levels seemed to be mediated at the transcriptional level. Nuclear run-off analysis indicated that a 1 h treatment of βTC3 cells with 10 μM forskolin on a background of 5.5 mM glucose did not influence transcription preproinsulin, PC2 or PC3 genes (Figure 7), but did specifically increase the transcription of c*Fos* (6-fold; $P < 0.05$) and c*Jun* (5-fold; $P < 0.02$) in the same βTC3 cells (Figure 7). Thus, despite a lack of cyclic-AMP-induced effect on preproinsulin, PC2 and PC3 mRNA levels or gene transcription, the transcriptional increase in c*Fos* and c*Jun* mRNA levels indicated that a cyclic AMP signalling pathway mediating effects on specific gene transcription was functional in βTC3 cells.

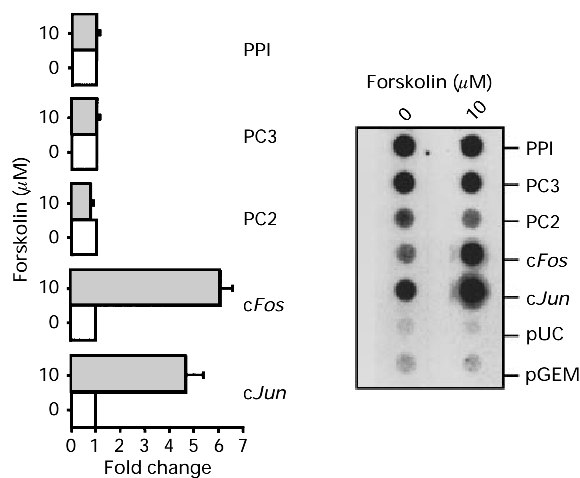

Figure 7 Effects of forskolin on transcriptional activity of preproinsulin, PC3, PC2, c*Fos* and c*Jun* genes in βTC3 cells

βTC3 cells were preincubated for 24 h in medium containing 0.5% FBS, then stimulated with (shaded bars) or without (open bars) 10 μM forskolin for 1 h. Nuclei were processed for a 30 min run-off reaction, RNA was isolated and hybridized with dot–blots of preproinsulin (PPI), PC3, PC2, c*Fos* and c*Jun* plasmid digest as well as pUC and pGEM vector controls as previously described in the Experimental section. Representative blots are depicted and graphs of cumulative results (means ± S.E.M.; $n = 3$) from densitometric scanning analyses expressed as fold change from βTC3 cells incubated in the absence of forskolin.

DISCUSSION

Chronic exposure (more than 6 h) of pancreatic β-cells to glucose induces an increase in preproinsulin gene transcription [23,35], which corresponds to increased preproinsulin mRNA levels in the pancreatic β-cell [19]. Thus there is a rise in the mRNA pool available for increased proinsulin biosynthesis that, in turn, could place an increased demand on the mechanism for convertion of proinsulin to insulin. In this study we have found that in βTC3 cells long-term exposure to glucose specifically increases mRNA levels of the proinsulin conversion endopeptidases, PC2 and PC3, in parallel with preproinsulin mRNA levels in βTC3 cells. Therefore the potential problem of an increased demand for proinsulin conversion made on the β-cell by glucose-induced preproinsulin mRNA levels is compensated for by a co-ordinate increase in PC2 and PC3 gene expression. Co-ordinate glucose stimulation of preproinsulin and PC3 mRNA levels in βTC3 cells was consistent with observations in pancreatic islets isolated from hyperglycaemic rats infused with glucose for 48 h, which also had elevated preproinsulin and PC3 mRNA levels [18]. However, in βTC3 cells PC2 mRNA levels were also increased in the presence of glucose, in contrast with a lack of effect by glucose on PC2 mRNA levels in isolated islets previously exposed to chronic hyperglycaemia [18]. This discrepancy was most probably due to differential expression of the converting enzymes in β-cells and non-β-cells of the islet. PC3 is expressed predominantly in islet β-cells and PC2 expression is much greater in islet non-β-cells compared with low PC2 levels in islet β-cells [43,44]. Non-β-cells are unresponsive to glucose regulation and thus the high background levels of PC2 mRNA contributed by non-β-cells could have obscured glucose-induced changes in β-cell PC2 mRNA levels in studies with isolated islets [18]. Because βTC3 cells are a homogenous β-cell population [34,35], PC2 expression in this cell line is not obscured by PC2 present in other cell types. This line of argument is complemented by similar observations made for glucose-regulated PC2 and PC3 biosynthesis at the translational level. In islets isolated from normal rodents, there is parallel glucose regulation of PC3 and proinsulin biosynthesis, but not that of PC2 [12,13]. However, in islets isolated from obese mice, where the proportion of β-cells per islet is markedly increased [13], glucose-regulated PC2 biosynthesis can be observed in parallel with that of PC3 and proinsulin [13]. Thus, as for glucose-regulated gene expression of PC2, it seems that in normal rodent islets glucose-regulated PC2 biosynthesis in normal islet β-cells is also masked by a higher level of glucose-unresponsive PC2 biosynthesis in non-β-cells, although a difference between dedifferentiated βTC3 cells and differentiated islet β-cells cannot be ruled out.

Short-term glucose stimulation (less than 2 h) of β-cells results in a co-ordinated 20–30-fold increase in proinsulin, PC2 and PC3 biosynthesis from translational regulation of pre-existing mRNAs [10–13]. Only for prolonged periods of glucose exposure to β-cells is additional control of proinsulin biosynthesis mediated by increased preproinsulin gene transcription and/or stability [23,40]. In this study we have found that glucose-regulated preproinsulin, PC2 and PC3 gene expression is rather modest, usually about a 2–3-fold increase, as found in other preproinsulin gene expression studies [23,40]. However, it should be considered that by polysome formation and re-initiation processes in the mRNA translation mechanism, many protein molecules can be produced by a single mRNA template [45]. Thus a doubling of preproinsulin, PC2 and PC3 mRNA levels by glucose could well translate into a much higher quantity of proinsulin, PC2 and PC3 protein synthesized. Nevertheless, this latter consideration implies that minute-to-minute control of proinsulin, PC2 and PC3 biosynthesis by glucose in the β-cell would be regulated mostly at the translational level [10–13]. Only for rather unusual situations of chronic exposure to glucose, such as prolonged hyperglycaemia [16,18] or fasting/refeeding [17], would glucose-regulated preproinsulin gene transcription (and that of PC2 and PC3) apply as a key additional factor in the control of proinsulin biosynthesis.

Glucose-induced changes in βTC3-cell mRNA levels of preproinsulin, PC2 and PC3 could be attributed to either changes in gene transcription and/or mRNA stability. Increasing glucose concentration has previously been shown to increase preproinsulin gene transcription in islets [23,26], βTC3 cells [35] and HIT-cells [24,46]. Lowering glucose levels has been shown to decrease preproinsulin gene transcription in hypoglycaemic clamped rat pancreas [47] and INS-1 cells [48]. In this study we found there was negligible influence of glucose on preproinsulin, PC2 and PC3 mRNA stability within a 24 h period. However, nuclear run-off analysis of βTC3 cells indicated that glucose exposure modestly increased preproinsulin, PC2 and PC3 gene transcription. Therefore the effect of glucose to uphold mRNA levels of preproinsulin, PC2 and PC3 in βTC3 cells was most probably mediated primarily at the transcriptional level rather than by preserving mRNA stability. Thus, in considering that PC2 and PC3 mRNAs have a relatively short life compared with preproinsulin mRNA, transcriptional regulation is probably the sole mechanism whereby glucose maintains levels of PC2 and PC3 mRNA. However, for preproinsulin mRNA an additional glucose effect on mRNA stability cannot be ruled out for periods greater than 24 h [40].

Glucose has been shown to exert stimulatory effects on the rat preproinsulin-1 gene promoter through a discrete region known as the Far Flat mini-enhancer [26] located between −196 and −247 bp from the transcription initiation site, which in turn has been shown to bind a variety of transcription factors [19,20,49]. Gene sequence analysis (Genetics Computer Group, Inc., Find Patterns and Gap software) of human PC2 [31] and mouse PC3

[32,33] promoter regions revealed no matches (over 50% similarity) to the entire 51 bp Far Flat mini-enhancer; however, regions of similarity (no more than 1 bp mismatch) to smaller elements (6–8 bp) within the Far Flat mini-enhancer, namely the Flat F, Flat E and CT box elements [19,20], were found in both PC2 and PC3 promoter regions. This analysis suggested that the promoter regions of PC2 and PC3 has elements in common with the preproinsulin promoter that facilitate co-ordinated transcription in response to glucose. However, these potential glucose-regulatory regions within the PC2 and PC3 promoter regions will have to be tested experimentally to establish their function.

The co-ordinate regulation of preproinsulin, PC2 and PC3 gene transcription in βTC3 cells by glucose could indicate a common regulatory mechanism. One common feature of the preproinsulin, PC2 and PC3 promoter regions is that consensus CREs are present [19,20,31–33]. Thus there is a possibility that glucose-induced co-ordinate gene transcription of preproinsulin, PC2 and PC3 could, in part, be mediated via cyclic AMP. In this light, glucose has been shown to increase cyclic AMP levels in β-cells [21,22] and elevation of intracellular cyclic AMP can potentiate glucose-stimulated preproinsulin gene expression [23–26]. However, it should be noted that the role of cyclic AMP in regulating preproinsulin mRNA expression in pancreatic β-cells has been controversial. Some previous studies have shown that cyclic AMP can modestly increase [23,27] or potentiate glucose-regulated preproinsulin gene expression [23–26], whereas others have indicated no effect of cyclic AMP on preproinsulin gene transcription, whether glucose was present or not [35]. The discrepancy between these observations could be simply due to species differences or the use of β-cell lines from various sources at differing passage numbers. Another consideration might have been that a cyclic AMP signalling system to regulate gene transcription was disabled in βTC3 cells [35]. In this study, forskolin treatment of βTC3 cells, in the presence of glucose, resulted in stimulated phosphorylation activation of the CREB transcription factor that was correlated with a transient increase in mRNA levels of c*Fos* and c*Jun*. Thus a cyclic AMP signalling mechanism for inducing gene transcription was present in βTC3 cells. However, in the very same βTC3 cells, cyclic AMP seemed to have no effect on preproinsulin, PC2 and PC3 mRNA levels or gene transcription, despite CREs being present in the promoter region of these genes [19,20,31–33]. Therefore cyclic AMP is not necessarily a key component in the mechanism behind co-ordinated glucose-induced transcription of preproinsulin, PC2 and PC3. It should be noted that in these studies forskolin treatment of βTC3 cells was used on a background of 5.5 mM glucose and thus a potentiating role of cyclic AMP at lower glucose concentrations cannot be ruled out [23,24]. Nevertheless, because cyclic AMP regulation of CREB/CRE interaction was sufficient for inducing c*Fos* and c*Jun* transcription in βTC3 cells, it is best conjectured that additional transcription factors and/or signals will act in concert with cyclic AMP if it is to have any role in mediating glucose-induced preproinsulin, PC2 and PC3 gene transcription.

Co-ordinated gene expression of a prohormone with its specific proprotein-processing endopeptidases is not unique to the pancreatic β-cell. In the intermediate lobe of the pituitary, gene expression of both PC2 and PC3 has been shown to be co-ordinately regulated by dopamine (via D2 dopamine receptors) with that of another of their prohormone substrates, pro-opiomelanocortin [50]. It is interesting to note that dopamine binding to D2 dopamine receptors, and subsequent coupling to heterotrimeric G-proteins, influences the activity of adenylate cyclase in pituitary cells [51]. Furthermore, increased intracellular cyclic AMP in pituitary cells is important in controlling pro-opiomelanocortin gene expression via CREB activation [52]. Thus cyclic AMP probably plays a prominent role in co-ordinated expression of PC2, PC3 and pro-opiomelanocortin in pituitary cells. Unlike in pituitary cells, cyclic-AMP-induced CREB/CRE interaction is not necessarily important in controlling co-ordinate preproinsulin, PC2 and PC3 gene transcription in pancreatic β-cells. Therefore it follows that the mechanism behind regulating PC2 and PC3 gene expression in parallel with that of a prohormone substrate will be particular to a given neuroendocrine cell type.

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