

## Insulin-like growth factor-II gene expression in a rat insulin-producing beta-cell line (INS-1) is regulated by glucose

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**Summary** A highly differentiated rat glucose-responsive insulin producing cell line INS-1 expresses high levels of insulin-like growth factor-II (IGF-II). Basal levels of IGF-II gene mRNA were expressed in cells cultured at 1–6 mmol/l glucose. At glucose concentrations of 10–20 mmol/l, IGF-II mRNA was increased more than threefold after 44 h of incubation. Levels of IGF-II mRNA in INS-1 cells incubated at 5.6 and 20 mmol/l glucose in the presence of 4 µg/ml actinomycin D are comparable and are not reduced during 20 h of treatment, indicating the high stability of IGF-II mRNA in this cell line. From the three rat IGF-II promoters, promoter 3 is by far the most active in INS-1 cells. The IGF-II promoter 3 activity and IGF-II mRNA production at high glucose concentrations increased threefold over their respective levels at low glucose concentration, suggesting that the glucose-induced IGF-II gene expression in this

beta-cell line might be transcriptionally controlled. The up-regulation of IGF-II mRNA by glucose was not due to the increased intracellular cyclic AMP levels or protein kinase C activation. A protein kinase C activator had no effect on IGF-II gene expression, and an adenylate cyclase activator (forskolin), suppressed the stimulatory effects of glucose on the IGF-II mRNA. Under all the experimental conditions examined, the IGF-II and insulin genes were differentially regulated in INS-1 cells. The IGF-II gene expression and DNA synthesis, however, were regulated in parallel, suggesting that these two cellular activities are closely associated. [Diabetologia (1995) 38: 927–935]

**Key words** Beta cells, insulin-like growth factor II, insulin, glucose, gene expression.

Insulin-like growth factors (IGF-I and -II) are regulatory peptides of about 7.5 kDa, involved in the proliferation and/or differentiation of numerous cell types [1–6]. IGFs are members of the larger family of insulin-related peptides (insulin, IGF-I and -II, and relaxin) exerting their activities mainly through

endocrine, autocrine or paracrine modes of action [7–9]. IGFs bind to IGF type-1, type-2 (IGF-II/cation-independent mannose 6-phosphate) and insulin receptors, which are present in most tissues. The major activities of both IGFs, however, are through the IGF type-1 receptor [1, 7, 10–12]. IGFs and insulin are believed to diverge from a common gene, and apart from structural similarities, they exert similar activities on different cell types [7, 13, 14]. While the biological role of IGF-I, after birth and during adulthood, is well-established, IGF-II is believed to be more effective during fetal development [15]. In rats, IGF-II gene expression is limited to the fetal life in most tissues, including liver. Among the exceptions are the choroid plexus, leptomeninges [16, 17], and islet beta cells of the endocrine pancreas [18, 19].

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*Abbreviations:* CM, Complete medium; IGF-II, insulin-like growth factor-II; PBS, phosphate buffered saline; SFM, serum-free medium; PMA, phorbol 12-myristate 13-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid; ACC, acetyl CoA carboxylase.

Islet cells produce IGF-I and -II and secrete the two peptides in the culture medium [20–24]. IGF-I has been reported to be present in human [21] and rat [25] fetal islet beta cells. In a recent report [19], using immunohistochemical techniques it has been demonstrated that IGF-II in the adult human, dog, and rat islets, is coexpressed with insulin, while IGF-I has been detected in glucagon- and/or somatostatin-producing cells [19, 25]. In a report using Northern blot techniques, no IGF-I mRNA was detected in rat islets [26]. The IGF-II gene, in humans and rodents, is closely linked to the loci for insulin [14, 27, 28]. These observations have raised the question as to whether the IGF-II and insulin genes are co-regulated. The fact that IGF-II is expressed in fetal and adult islet tissue suggests a possible important role for this peptide as a beta-cell autocrine growth and/or differentiation regulatory hormone. Its regulation, therefore, during intrauterine life and after birth, may have an impact on maturation and proper functioning of the islets after birth.

The goal of the present work was to investigate the regulation of the IGF-II gene expression in beta cells. As a model, we chose a highly differentiated rat insulin producing cell line (INS-1) which exhibits many properties of normal beta cells, including: response to glucose in the physiological range [29], expression of glucose transporter-2, glucokinase [30], and growth hormone and prolactin receptor genes [31] at levels comparable to those of normal islets. Here we have demonstrated that INS-1 cells express the IGF-II gene, which is regulated by glucose; and while variation in IGF-II gene expression parallels DNA synthesis, it markedly differs from that of insulin gene expression.

## Materials and methods

**Hormones and chemicals.** Recombinant IGF-II was a gift of Kabi Pharmacia (Stockholm, Sweden);  $^{125}\text{I}$ -IGF-II was purchased from Amersham (Les Ulis, France). Unless stated otherwise, all the chemicals and reagents were purchased from Sigma (St. Quentin Fallavier, France).

**Cell culture.** INS-1 cells were maintained in complete medium (CM), containing RPMI/1640, 2 mmol/l glutamine, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 1 mmol/l sodium pyruvate, 10% heat-inactivated fetal calf serum, 10 mmol/l HEPES, and 50  $\mu\text{mol}/\text{l}$  2-mercaptoethanol, at 37°C in a humidified atmosphere with 5%  $\text{CO}_2$  as previously described [29]. Specific culture conditions for each experiment are described in Results.

**RNA extraction and Northern blot analysis.** Cells were seeded into 100-mm tissue culture plates and at the end of the incubation period the plates were chilled on ice and washed once with phosphate-buffered saline (PBS). RNA was isolated in urea-lithium chloride [32]. For RNA preparation from rat liver and muscle the fresh tissues were washed in ice-cold PBS and cut into small pieces. About 300 mg of tissue was transferred into

15 ml of ice-cold urea-lithium chloride, homogenized, and RNA was then purified.

Total RNA (20  $\mu\text{g}$ ) was denatured in formamide/formaldehyde at 65°C for 10 min and fractionated on formaldehyde (2.2 mol/l)-1% agarose gel, blotted onto a nylon membrane (Hybond, Amersham) and UV-cross-linked. Blots were hybridized with  $\alpha^{32}\text{P}$ -labelled cDNA probes overnight at 42°C in a solution containing 5  $\times$  SSPE (sodium salt phosphate EDTA), 5  $\times$  Denhardt's solution, 0.1% sodium dodecyl sulphate (SDS), and 50% formamide. After hybridization the filters were washed twice in a solution of 2  $\times$  SSC, 0.1% SDS for 15 min at room temperature and once in the same solution at 45°C for 60 min. The filters were exposed to RX film (Fuji Medical X-ray film) at -70°C using intensifying screens. The mRNAs on autoradiograms were quantified by scanning densitometry (Shimadzu Dual-wavelength TLC Scanner CS-930, Kyoto, Japan).

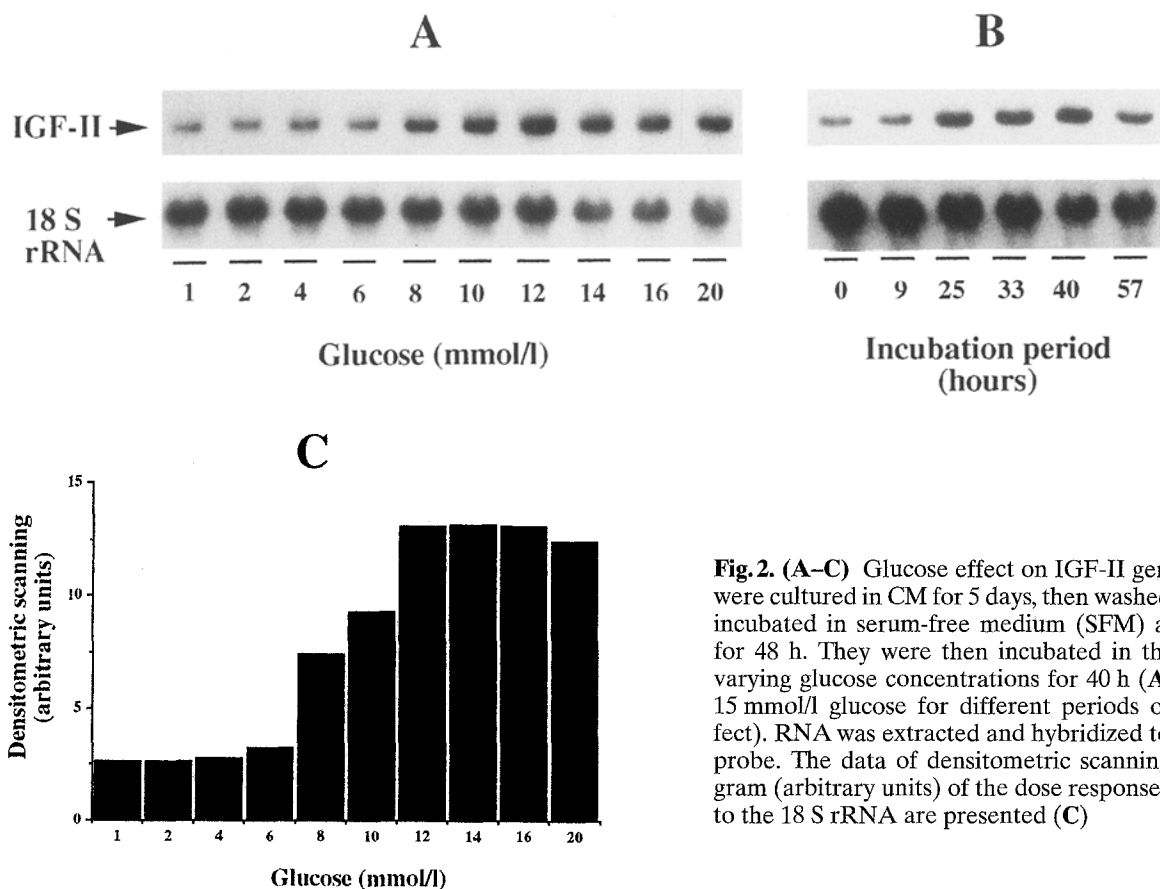
**cDNA probes.** The probes were human placental IGF-II cDNA insert (663-base pair) containing 5'-untranslated region, the entire coding region, and part of the 3'-untranslated region [33]; rat insulin I cDNA [34], and beta-actin [35]. For control of RNA loading, [ $\gamma^{32}\text{P}$ ]ATP-labelled oligonucleotides complementary to 18 S ribosomal RNA [36] were used.

**Rat IGF-II reporter gene constructs.** Rat IGF-II promoter fragments were cloned into the promoterless firefly luciferase gene containing vector pSLA3 [37]. The rat promoter P1 fragment (-499/+213, relative to the transcription initiation site), was subcloned as a SacI/SalI fragment into pSLA3, resulting in construct RAP1. The rat promoter P2 construct, RAP2, contained a 1304 nt long BamHI/SalI (-1164/+140), cloned directly into pSLA3. Rat promoter P3 was cloned by means of PCR, containing a 884 nt promoter P3 fragment (-809/+75), flanked by BamHI/SalI sites, resulting in construct RAP3.

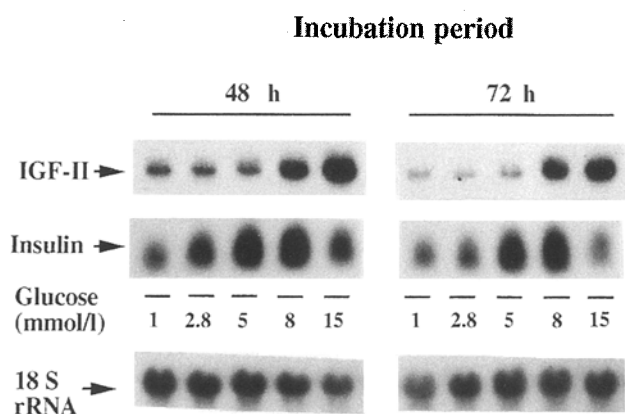
**Transient transfection and luciferase expression assay.** Cells were cultured in 12-well culture plates at  $0.5 \times 10^6$  cells  $\cdot$  ml $^{-1}$  per well and incubated for 72 h in CM. They were washed with PBS and preincubated at 5 mmol/l glucose, in pyruvate and serum-free-medium (SFM), for 41 h. They were then transfected using LipofectAMINE Reagent (GIBCO BRL, Paris, France) at 5 mmol/l glucose, for 7 h and incubated in fresh SFM (2 ml per well) at different glucose concentrations for 48 h. For each transfection assay 400 ng plasmid DNA per million cells was used. At the end of the incubation period cells were harvested in 250  $\mu\text{l}$  lysis buffer [38], from which 200  $\mu\text{l}$  was used for detection of luciferase activity (Luamt LB 9501 with injector, Berthold s.a.r.l., Elancourt, France), and 50  $\mu\text{l}$  for determination of protein content (Bio-Rad Dc Protein Assay Kit; Bio-Rad Laboratories, Richmond, Calif., USA). At each culture condition the luciferase activity was normalized for the total protein. All constructs were tested in triplicate for each culture condition.

**IGF-II assay.** INS-1 cells were seeded into 60-mm tissue culture plates in CM for 72 h. They were washed with PBS and preincubated in SFM and 5 mmol/l glucose for 48 h. The preincubation medium was changed with fresh SFM containing 8 mmol/l glucose and then incubated for a further 48 h. At the end of the incubation period, the released IGF-II in the culture medium and in the cell extract (acid-ethanol, 0.15 mol/l HCl in 75% ethanol at -20°C) were separated from the binding proteins as previously described [39]. Briefly, the lyophilized culture medium or the cell extract were separated from binding proteins in acidic solution (1 mol/l acetic acid, 0.15





**Fig. 2.** (A–C) Glucose effect on IGF-II gene expression. Cells were cultured in CM for 5 days, then washed with PBS and pre-incubated in serum-free medium (SFM) at 5 mmol/l glucose for 48 h. They were then incubated in the same medium at varying glucose concentrations for 40 h (A dose effect), or at 15 mmol/l glucose for different periods of time (B time effect). RNA was extracted and hybridized to the IGF-II cDNA probe. The data of densitometric scanning of the autoradiogram (arbitrary units) of the dose response effects, normalized to the 18 S rRNA are presented (C)



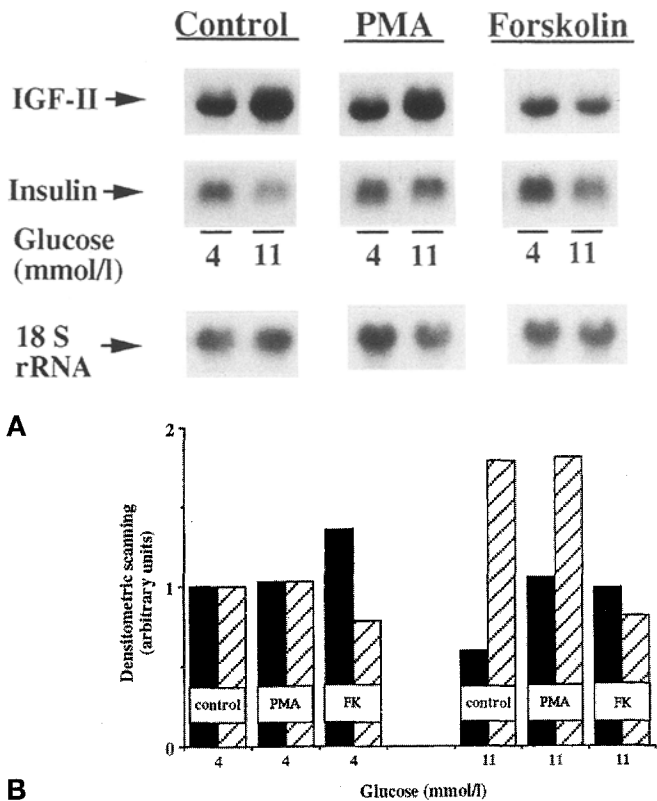
**Fig. 3.** Glucose effect on IGF-II and insulin gene expression. Cells were cultured in CM for 5 days, then washed and incubated in medium at varying glucose concentrations. RNA was extracted at 48 and 72 h after incubation and hybridized simultaneously to the IGF-II and insulin cDNA probes

ffects on insulin gene expression under identical conditions (Fig. 4). At high glucose concentrations insulin mRNA levels were increased in the presence of forskolin or PMA, and at low glucose, forskolin was still effective in insulin mRNA up-regulation.

*Effects of glucose, PMA and forskolin on cell proliferation.* IGF-II is an autocrine growth factor in many

cell types [7–9]. To determine whether a correlation between the regulation of the IGF-II gene expression and cell proliferation could be detected, INS-1 cell proliferation was determined under the same conditions as used for the determination of IGF-II mRNA regulation. As an indication of cell proliferation, we assessed the rate of  $^3\text{H}$ -thymidine uptake. As is illustrated in Figure 5A, B, glucose increased the thymidine uptake of INS-1 cells in a dose- and time-dependent manner. The maximal thymidine incorporation was reached at 15 mmol/l glucose and the significant thymidine uptake above basal level (5 mmol/l glucose) was observed after 26 h incubation at high glucose. The thymidine uptake at various glucose concentrations in the presence of PMA was identical to that of the control, but it was markedly reduced in the presence of 10  $\mu\text{mol/l}$  forskolin at high glucose concentrations (Fig. 5B).

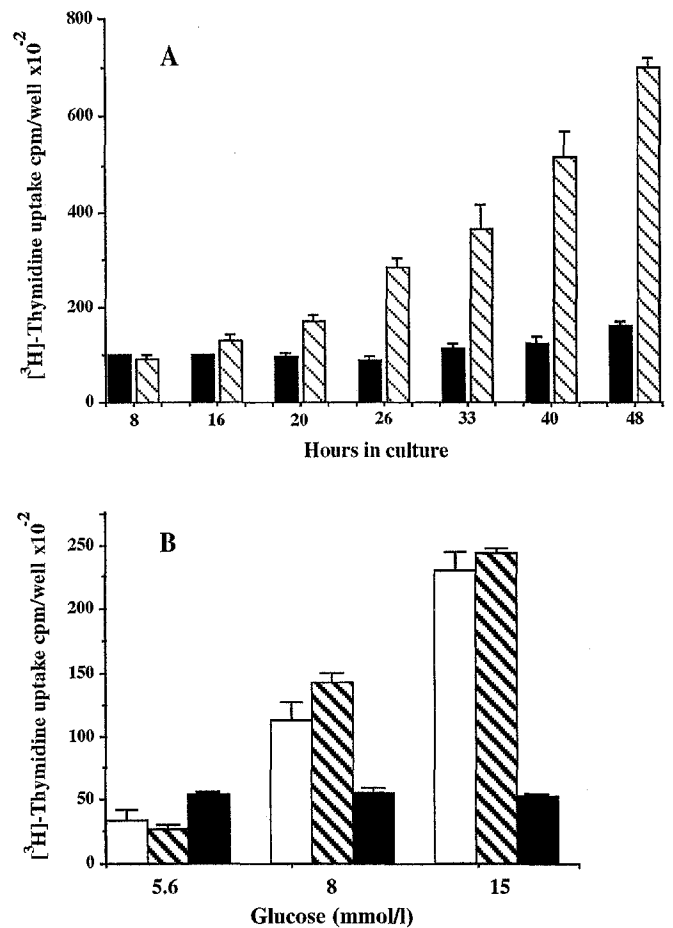
*Stability of IGF-II mRNA in INS-1 cells.* To determine the stability of IGF-II mRNA, cells were incubated at low (5 mmol/l) and high (20 mmol/l) glucose concentration in the presence of 4  $\mu\text{g/ml}$  of the RNA synthesis inhibitor actinomycin D. RNA was extracted at different time intervals and hybridized to the cDNA probes of IGF-II and  $\beta$ -actin (as a control). It was found that the levels of IGF-II mRNAs were the same at both glucose concentrations and



**Fig. 4. (A, B)** Forskolin, and PMA effects on IGF-II and insulin gene expression. Cells were cultured and preincubated as explained in the legend of Figure 2. They were then incubated in SFM at varying glucose concentrations in the presence of PMA (0.1  $\mu\text{mol/l}$ ) or forskolin (10  $\mu\text{mol/l}$ ). **A** Northern blot of RNA extracted after 40 h incubation and hybridized to IGF-II and insulin cDNA probes. The glucose concentrations (4 and 11 mmol/l) reflect the actual concentrations at the time of RNA extraction, the starting concentrations were 5.6 and 15 mmol/l. **B** The scanning data of both insulin (■) and IGF-II (▨) mRNA normalized to the 18 S rRNA are presented. This is representative of three separate experiments

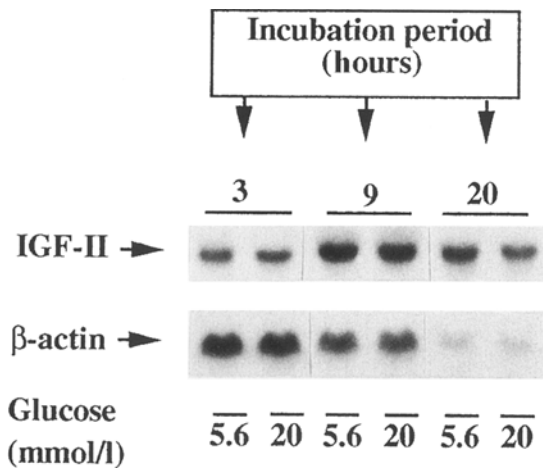
did not vary during 20 h of actinomycin D treatment (Fig. 6). However, expression of the control gene,  $\beta$ -actin mRNA, which has a half-life of less than 8 h [45] was reduced to about 50 and 20% at both glucose concentrations after 9 and 20 h incubation, respectively. This is consistent with a previous report on the high stability of IGF-II mRNA in BRL 3A cells [45]. The apparent slight increase of IGF-II mRNAs at 9 and 20 h incubation in comparison to 3 h is due to the difference in the total RNA loading (data not shown).

*Transient expression of IGF-II promoters.* The rat IGF-II gene is transcribed from three different promoters (P1, P2 and P3). In order to determine which of the three rat promoters could be activated in this beta-cell model, we transfected the INS-1 cells with plasmids containing the three rat IGF-II promoters fused to the luciferase reporter gene. Each construct was transfected in triplicate. As is illustrated in Fig-

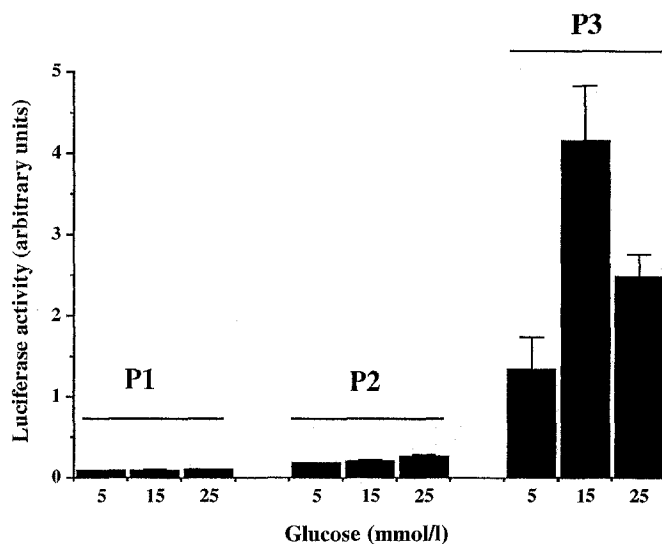


**Fig. 5. (A, B)** Glucose, forskolin and PMA effects on INS-1 cell DNA synthesis. Cells were cultured in 24-well culture plates and incubated for 72 h in CM. The cells were then washed with PBS and preincubated in SFM and 5 mmol/l glucose for 48 h. **A** The preincubation medium was replaced by SFM, containing 5 (■) or 15 (▨) mmol/l glucose. At different time intervals cells were pulsed with 2  $\mu\text{Ci}$   $^3\text{H}$ -thymidine per well (triplicate well for each culture condition) for 2 h (results of two independent experiments). **B** The preincubation medium was removed and cells were incubated in SFM containing varying glucose concentrations (control □) and 0.1  $\mu\text{mol/l}$  PMA (▨), or 10  $\mu\text{mol/l}$  forskolin (■) for 40 h. Cells were pulsed with 1  $\mu\text{Ci}$   $^3\text{H}$ -thymidine per well (triplicate well for each culture condition) for 7 h (results of four independent experiments)

ure 7, the rat IGF-II P3 is by far more active than the other two promoters, at all glucose concentrations, in this cell line, indicating that the 3.6 kb mRNA is transcribed from the IGF-II P3. The IGF-II P3 activity in transfected INS-1 cells incubated at 15 mmol/l glucose was  $3.13 \pm 0.38$  and  $1.6 \pm 0.21$  times higher than the levels found in cells incubated at 5 and 25 mmol/l glucose, respectively.



**Fig. 6.** IGF-II gene expression under actinomycin D treatment. Cells were cultured in CM for 5 days, then washed with PBS and incubated in medium containing 0.1% serum (to increase the cell survival), 4 μg/ml actinomycin D and 5.6 or 20 mmol/l glucose, respectively. RNA was extracted at different time intervals and hybridized to IGF-II and β-actin cDNA probes



**Fig. 7.** Transient transfection of the rat IGF-II promoter constructs. Cells were cultured in 12-well culture plates at  $0.5 \times 10^6$  cells. ml<sup>-1</sup> per well and transfected with plasmids carrying IGF-II promoters (P1, P2, P3) fused to firefly luciferase reporter gene (Materials and methods). The cells were then incubated in fresh medium at different glucose concentrations for 48 h. At each culture condition the luciferase activity was normalized with the total protein content and all the constructs were tested in triplicate experiments. The luciferase activities at each glucose concentration were normalized for the total protein at each culture condition. The values are mean ± SEM of two independent experiments (P1), and five independent experiments (P2 and P3)

## Discussion

Our data demonstrate that the rat beta-cell line, INS-1, produces high levels of IGF-II mRNA, the production of which is controlled by glucose. Multiple IGF-

II mRNAs are expressed in INS-1 cells with the predominant mRNA of about 3.6 kb. The IGF-II gene in humans and rodents comprises multiple transcription products resulting from alternative promoter usage, differential splicing, and multiple polyadenylation signals. In rodents the major transcripts are 3.8, 4.6, and 3.6 kb derived from the promoters 1, 2, and 3, respectively. In newborn rat heart and brain tissues the IGF-II mRNA is transcribed from P3, giving rise to a predominant species of 3.6 kb with a small transcript of 1.2 kb. Also, a 4.6 kb transcript derived from P2 is present in these tissues. In the rat hepatocyte cell line BRL3A IGF-II mRNA is derived from P1, P2, and P3 with a preference for P3. It could be said that in rat cell lines and tissues P3 is the most active promoter [37]. Consistent with this, we found that IGF-II P3 is by far more active than IGF-II P1 and P2 in INS-1 cells when tested in transient transfection assays, indicating that the predominant 3.6 kb mRNA is transcribed from P3. The fold increase of IGF-II mRNA from low to high glucose concentrations in this cell line, was almost identical to the fold increase of IGF-II P3 expression (luciferase activities) in the transfected INS-1 cells cultured at the two glucose concentrations (approximately threefold). This observation, along with the high stability of IGF-II mRNA at high and low glucose concentrations, suggests that the glucose-induced IGF-II gene up-regulation in this cell line, at least in part, is transcriptionally controlled. The IGF-II gene, therefore, falls into the group of genes that are regulated by glucose, such as insulin and the enzymes that are involved in glucose and fatty acid metabolism i.e., acetyl CoA carboxylase and fatty acid synthase. The effects of glucose on acetyl CoA carboxylase (ACC) [46] and pyruvate kinase [47] in INS-1 cells, and ACC and fatty acid synthase in adipocyte tissue [48] have been shown to be mimicked by glucose 6-phosphate, the first product in the glucose metabolism pathway. The effect of glucose on these genes was quite rapid and a net increase in mRNA level could be detected at 4 h following incubation at high glucose. A glucose-response element in the promoter of L-type pyruvate kinase gene [49], and similar motifs in the promoter 2 of the ACC gene have been demonstrated [46]. The glucose-induced IGF-II mRNA increase differs from those of ACC and pyruvate kinase in two respects. Firstly, the induction has a long lag period (24 h), and secondly, no sequence corresponding to this "glucose-response element" could be identified in the rat IGF-II P3 [50]. These findings suggest the presence of an intermediary glucose-responsive molecule(s) involved in the IGF-II gene up-regulation by glucose, yet to be determined. Recently, it has been reported that part of the glucose-induced insulin gene expression is through the increase in nuclear transcription factors that bind the Far element, present in the rat insulin I

gene promoter [51]. No such element was identified in the rat IGF-II P3.

Glucose-induced beta-cell activities, such as insulin secretion, are believed to be mediated by a rise in cyclic AMP, and/or activation of protein kinase C [43, 44, 52]. To assess whether the same mechanism is involved in glucose-induced IGF-II gene expression, the levels of IGF-II mRNA in INS-1 cells in the presence of forskolin, a cyclic AMP increasing agent, or PMA, a protein kinase C activator were determined. We found that the glucose-induced IGF-II gene expression in this cell line is not mediated by the two transducing systems. These observations differ from the regulation of IGF-II gene expression in other systems. Cyclic AMP increasing agents in human fetal pancreas islet-like cell clusters [23], human fetal adrenal [53, 54], and rat thyroid follicular cells [55] increase the IGF-II mRNA levels, and PMA causes the reduction of basal levels of IGF-II mRNA in human fetal adrenal cells [54]. These findings suggest that IGF-II gene regulation could vary depending on the developmental stage and specificity of the tissues.

Insulin and IGF-II in rats are tandemly located on the rat chromosome 1 [27]. This, along with the finding that IGF-II could be detected only in the beta cells of adult rat, human, and dog pancreatic islets [19], has raised the assumption that the two genes are co-regulated. Indeed, recently, a parallel regulation of the two tandemly located genes, IGF-II and H19 (H19 expresses an mRNA to which no protein has been assigned), was reported in cultured human fetal adrenal cells [53]. The comparison of insulin and IGF-II gene expression under different culture conditions in this beta-cell model argues against this hypothesis. Firstly, the glucose concentration for optimal gene induction is different for the two genes. During long-term treatment, at 5 mmol/l glucose the insulin gene was highly expressed, while IGF-II was expressed at basal levels. Conversely, at 15 mmol/l of glucose where the IGF-II gene was maximally expressed, the insulin gene was markedly down-regulated. Secondly, forskolin exerts opposing effects on insulin and IGF-II gene expression. Cyclic AMP increased insulin mRNA levels in INS-1 cells, both at low and high glucose concentrations, consistent with the previous findings in normal islets [56] and beta-cell lines [57, 58]. However, it strongly inhibited the glucose-induced IGF-II mRNA increase. Thirdly, although PMA increased the insulin mRNA level at high glucose concentrations (as has been previously demonstrated in a clonal hamster beta-cell line, HIT-T15 [57]) it had no detectable effects on the IGF-II gene expression in this cell line.

Glucose, at high concentrations, exhibited negative effects on insulin mRNA accumulation and on the IGF-II P3-luciferase expression in the transient transfection assay during the long-term (48-h and

longer) treatment. The threshold of the inhibitory glucose concentration for the two genes, however, varied greatly (10 and 25 mmol/l for insulin and IGF-II, respectively). This phenomenon, reminiscent of "glucose toxicity" [59, 60] was observed for other genes in this cell line (data not shown). The negative effects of high glucose concentration on insulin gene expression during long-term exposure have been demonstrated previously in the HIT-T15 cell line [61]. The mechanism of this "glucose toxicity" was determined to be partly due to a change in the ability of a glucose-sensitive transcription factor to interact with the insulin gene promoter. Whether the same mechanism is effective in this broad range of "gene-silencing" activities of high glucose concentrations in INS-1 cells remains to be determined.

IGF-II is defined as an autocrine growth factor, and recently its role as a tumour progression factor of beta cells [62] and other cell types [63], when expressed at abnormally high levels, has been reported. Although the direct involvement of the endogenous IGF-II in INS-1 cell proliferation remains to be determined, the effects of glucose on IGF-II mRNA up-regulation and DNA synthesis (induction of thymidine uptake) are superimposable both in time and concentration. This is also the case when the effects of PMA and forskolin on IGF-II mRNA and DNA synthesis are compared. Whether this is a cause-and-effect relationship is under investigation; however, it clearly indicates that IGF-II gene activation and DNA synthesis in this cell line are regulated in parallel.

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