Transcriptional regulation of glucose-6-phosphatase catalytic subunit promoter by insulin and glucose in the carnivorous fish, *Sparus aurata*

M C Salgado, I Metón, M Egea and I V Baanante
Departament de Bioquímica i Biologia Molecular, Facultat de Farmàcia, Universitat de Barcelona, Diagonal 643, 08028 Barcelona, Spain

(Requests for offprints should be addressed to I V Baanante; Email: baanantevazquez@ub.edu)

Abstract

Increase in glucose-6-phosphatase catalytic subunit (G6Pase, G6pc) transcription enhances hepatic glucose production in non-insulin-dependent diabetes mellitus (NIDDM). The fact that carnivorous fish is an alternative model to study NIDDM led us to clone and characterise the first G6pc promoter region reported for fish and non-mammalian animals. The 5'-flanking region of G6pc from gilthead sea bream (*Sparus aurata*) was isolated by chromosome walking. With SMART RACE-PCR, the transcription start site was located 106 base pairs (bp) upstream of the translational start. Transfection analysis in HepG2 cells located a functional promoter in the 850 bp 5'-flanking isolated fragment (positions –770 to +80 relative to the transcription start). Sequential 5'-deletion analysis of the promoter fragment revealed that a core functional promoter for basal transcription is comprised within the 190 bp upstream of the transcription start site. *In vivo*, glucose and insulin reduced G6Pase mRNA levels in the fish liver. Transfection experiments in HepG2 cells showed that insulin repressed *S. aurata* G6pc under high-glucose conditions. Synergistic activation of piscine G6pc promoter was induced by cotransfection with expression plasmids for hepatocyte nuclear factor-4α (HNF-4α) and peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1α). No direct relationship was found between PGC-1α coactivation of HNF-4α transactivation and the repressive effect of insulin. Interestingly, insulin hardly affected G6pc promoter activity in the absence of glucose, suggesting that a reduced capacity of insulin-dependent repression of piscine G6pc may lead to insulin resistance in carnivorous fish.

*Journal of Molecular Endocrinology* (2004) **33**, 783–795

Introduction

In mammals, liver glucose-6-phosphatase (EC 3·1·3·9, G6Pase, G6pc) plays a key role in blood glucose homeostasis by catalysing the dephosphorylation of glucose-6-phosphate (G-6P) to glucose, the terminal reaction of gluconeogenesis and glycogenolysis. According to the substrate-transport model, G6Pase is a multicomponent complex located in the endoplasmic reticulum. Hydrolysis of G-6P involves the coupled functions of different membrane-spanning translocases that mediate penetration of G-6P and efflux of inorganic phosphate (Pi) and glucose (Van de Werve et al. 2000, Clottes et al. 2002, Foster & Nordlie 2002, Van Schaftingen & Gerin 2002). Expression of the catalytic subunit of G6Pase is restricted to liver, kidney, small intestine and brain (Gautier-Stein et al. 2003, Guionie et al. 2003). Hepatic G6pc gene expression is hormonally and nutritionally regulated. G6pc expression is stimulated by glucose, glucocorticoids, cAMP, fatty acids, leptin and β2-adrenergic receptor agonists, whereas it is inhibited by tumour necrosis factor α, interleukin-6 and insulin (Streeper et al. 2000). Studies *in vivo* and *in vitro* indicate that G6pc expression in rats is stimulated by glucose and xylitol, irrespective of insulin, through different signalling pathways (Massillon et al. 1996, 1998, Massillon 2001, Pagliassotti et al. 2003). Insulin inhibits gluconeogenesis by suppressing the expression of key enzymes, such as G6Pase. In HepG2 cells, repression of G6pc transcription by insulin requires two regions in the proximal promoter, designated...
A and B. Hepatocyte nuclear factor (HNF)-1 binds to region A, and enhances the effect of insulin (Streeper et al. 1998). Recently, insulin was postulated to downregulate G6pc expression by inhibiting the binding of a transcriptional activator, the forhead transcription factor Foxo1 (also known as FKHR), to insulin response sequence (IRS) motifs located in region B (Vander Kooy et al. 2003). The dual-specificity protein kinase DYRK1A synergistically potentiates transactivation of G6pc by Foxo1 (Von Groote-Billinggaier et al. 2003). Short-chain fatty acids induce G6pc transcription via recruitment of HNF-4α to the promoter (Massillon et al. 2003). Recent studies have shown that transcriptional activation of the G6pc promoter by HNF-4α and Foxo1 involves the coactivator protein peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1α) (Herzig et al. 2001, Yoon et al. 2001, Bousted et al. 2003, Puigserver et al. 2003, Rhee et al. 2003). CRE-binding protein (CREB) mediates activation of G6pc transcription through binding to a cAMP response element (CRE), which contributes to the regulation of the gene transcription by both cAMP and glucocorticoids (Schmoll et al. 1999). Additionally, binding of HNF-3γ and Sp family proteins is essential for the basal activity of G6pc promoter (Lin et al. 1997, Wasner et al. 2001).

In carnivorous fish, glucose intolerance and limited use of dietary carbohydrates have been reported. Compared with mammals, these animals show prolonged hyperglycaemia after a glucose load and when fed high-carbohydrate diets (Christiansen & Klungsøyr 1987, Cowey & Walton 1989, Baanante et al. 1991, Wilson 1994, Moon 2001, Metón et al. 2003). This metabolic profile mimics non-insulin-dependent diabetes mellitus (NIDDM) in humans, and carnivorous fish are thus considered as an alternative model in which to study NIDDM (Wilson 1994, Moon 2001). In patients with NIDDM, hepatic gluconeogenesis is increased; the inability of insulin to control enzymes such as G6Pase contributes to hyperglycaemia (Streeper et al. 1997, Clore et al. 2000). Consistent with a model whereby a reduction of G6Pase activity affects glucose homeostasis, silencing of hepatic G6pc reduces postprandial serum glucose levels in mice (Huang et al. 2004). In the present study, to understand regulation of G6pc transcription in the carnivorous fish glucose-intolerant model, we have cloned the first G6pc catalytic subunit promoter reported for fish and non-mammalian animals. In addition, we characterised regulation of the proximal promoter region activity of piscine G6pc by glucose and insulin, and transactivation by HNF-4α and the coactivator PGC-1α.

Materials and methods

Animal treatments

Gilthead sea bream (Sparus aurata) obtained from Tinamenor (Cantabria, Spain) were maintained, at 20 °C, in 260-l aquaria supplied with running seawater in a closed system with an active pump filter and UV lamps. The photoperiod was a 12 h/12 h dark/light cycle. Fish were fed daily (1000 h) at 1% body weight with a diet containing 46% protein, 9·3% carbohydrates, 22% lipids, 10·6% ash and 12·1% moisture, and 21·1 kJ/g gross energy. To study regulation of G6pc expression by insulin and glucose in vivo, we divided the animals into four groups of six fish each. At 24 h after the last meal (at 10 h), three different groups received an intraperitoneal injection of glucose (2 g/kg fish), bovine insulin (10 units/kg fish) (Sigma) or glucose (2 g/kg fish) plus insulin (10 units/kg fish). The other group was injected with vehicle (saline). At 6 h after treatment, fish were killed by cervical section. Blood was collected, and liver samples were dissected out, immediately frozen in liquid N₂ and kept at −80 °C until use.

To avoid stress, fish were anaesthetised with MS-222 (1:12 500) before handling. The experimental procedures met the guidelines of the animal use committee of the University of Barcelona.

Cloning of the 5′-flanking region of G6pc from S. aurata by chromosome walking

The 5′-flanking region of G6pc was isolated by PCR with the Universal GenomeWalker Kit (Clontech). Briefly, four libraries were obtained by blunt-end digestion of S. aurata genomic DNA with DraI, EcoRV, PvuII and StuI. Each batch of digested genomic DNA was ligated to the GenomeWalker adaptor provided in the kit. Primary PCR was performed on each library with the gene-specific primer MCGP01 (5′-TCGACCCCCCAGCTGTGCAGAAGATC-3′) and the AP-1 primer provided in the kit. Seven initial cycles were carried out, with 25-s denaturation at 94 °C, and 3-min
annealing and DNA synthesis at 72 °C, followed by 32 cycles in which the annealing and DNA synthesis temperature was 67 °C, and a final extension step of 7 min at 67 °C. Nested PCR was performed on the primary PCR product with the gene-specific primer MCGP02 (5'-GATGGCAGCTCTTTCTGCGTA 3') and the AP2 primer from the kit. The PCR reaction mixture was incubated for 2 min at 94 °C and subjected to five cycles of denaturation at 94 °C for 25 s, annealing at 68 °C for 30 s and DNA synthesis at 72 °C for 90 s, followed by 20 cycles in which the annealing temperature was 65 °C, and a final extension step of 5 min at 72 °C. The longer amplification product, a single 850 bp band, was obtained from the DraI library and ligated into pGEM T Easy plasmid (Promega) to generate pGEM-P850. Two independent clones were fully sequenced on both strands, according to the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit instructions (Applied Biosystems, Foster City, CA, USA).

Characterisation of the transcription start site

The 5' end of the hepatic S. aurata G6Pase cDNA was determined by the SMART RACE cDNA Amplification Kit (Clontech). This generates full-length cDNAs in reverse transcription reactions (Zhu et al. 2001). In brief, 1 µg poly A + RNA obtained from liver of S. aurata was converted into cDNA with PowerScript RT (Clontech, Palo Alto, CA, USA) at 42 °C for 1·5 h. 5'-RACE ready cDNA was obtained with the 5'-CDS primer, for first-strand synthesis, and the SMART II A oligonucleotides from the kit. After the end of the mRNA template is reached, the terminal transferase activity of PowerScript RT adds several dC residues that allow annealing of the SMART II A oligonucleotide, which serves as an extended template for RT. A touchdown PCR was conducted with the Universal Primer Mix A from the kit and MCGP01 oligonucleotides. Five initial cycles were carried out, with 30-s denaturation at 94 °C and 1-min annealing and extension at 72 °C, followed by five cycles with 30-s denaturation at 94 °C, 30-s annealing at 70 °C and 1-min extension at 72 °C, and 20 cycles with 30-s denaturation at 94 °C, 30-s annealing at 68 °C and 1-min extension at 72 °C. The single 187 bp band generated was purified and ligated into pGEM T Easy plasmid (Promega). Identical nucleotide sequence corresponding to the 5' end of G6Pase cDNA was obtained by sequence analysis of two independent clones.

Construction of reporter gene plasmids

The S. aurata G6pc–luciferase reporter fusion construct harbouring promoter sequences located between positions −770 and +80 relative to the transcription start site (pGP770) was generated by PCR. To this end, a forward MCGP05 primer (5'-CCGCTAGCCAGCCGGCCGCTGGCTAACA 3'), extending from positions −770 to −751, and containing a 5'-anchor sequence with an NheI site (underlined), a reverse MCGP06 oligonucleotide (5'-TGGATCCCGGCGTACCTGGCCTGCTGCGTG 3') between positions +87 and +107 with a 5'-anchor sequence containing a HindIII site (underlined), and pGEM-P850 as a template were used. The PCR product was cloned into the NheI/HindIII digested pGL3-Basic promoterless luciferase reporter plasmid (Promega). The reporter constructs designated as pGP629, pGP190 and pGP51 were produced by self-ligation of filled-in ends of pGP770 after digestion with EcoRV/HindIII, SmaI/HindIII and XhoI/HindIII respectively. All constructs were verified by sequencing with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA).

Cell transfection and luciferase assay

The human hepatoma derived cell line HepG2 (ATCC HB 8065) was cultured in Dulbecco's modified Eagle's medium (D-MEM), supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. The cells were grown at 37 °C in 5% CO₂. The calcium phosphate coprecipitation method was used for transient transfection of HepG2 at 45–50% confluence in six-well plates (Graham & Van der Eb 1973). Cells were transfected with 4 µg reporter construct, and when necessary, with 800 ng expression vector encoding human insulin receptor B, or 400 ng expression vectors encoding HNF-4α or PGC-1α. To correct for variations in transfection efficiency, 500 ng CMV-β (lacZ) were included in each transfection. To ensure equal DNA amounts, empty plasmids were added in each transfection. At 4 h after addition of the precipitate, cells were shocked in 10% dimethylsulphoxide in
serum-free medium for 2 min. Fresh medium or serum-free medium (supplemented with glucose or recombinant human insulin (Sigma)) was added, and the cells were harvested 16 h later, washed in PBS and incubated for 15 min in 300 µl culture lysis reagent (Promega). After removal of cellular debris by centrifugation at 10 000 g for 15 s, luciferase activity was measured in 5 µl supernatant after addition of 30 µl luciferase assay reagent (Promega). Peak light emission was recorded on a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA).

β-Galactosidase activity of 30–100 µl clear lysate was measured in a 1-ml reaction containing 0·3 mM MgCl₂, 13·5 mM 30–100 µl clear lysate was measured in a 1-ml reaction containing 0·3 mM MgCl₂, 13·5 mM β-mercaptoethanol, 0·9 mM ONPG (2-nitrophenyl-β-d-galactopyranoside) and 0·1 M sodium phosphate, pH 7·5. After addition of 0·5 ml 0·5 M Na₂CO₃, and the reaction was stopped by irradiation for 3 min. A G6Pase homologous probe was cross-linked to the membranes by UV irradiation for 3 min. Autoradiograms of Northern blots were quantified by scanning densitometry.

**Hepatocyte isolation and semiquantitative RT-PCR**

*S. aurata* hepatocytes were isolated by collagenase digestion. Minced tissue was incubated in L15 medium containing 0·5 mg/ml collagenase (Sigma) for 30 min at 20 °C. The cell suspension was centrifuged at 100 g for 3 min, and the supernatant was discarded. Cells were then washed three times with PBS and suspended in DMEM medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin for 30 min at 20 °C. For analysis of the effect of glucose and insulin on G6pase expression, the cells were incubated for 4 h at 20 °C in serum-free DMEM medium supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin, and with or without 5 mM glucose or 10 nM recombinant human insulin (Sigma). Cell viability, estimated by Trypan blue exclusion, was >95%. Total RNA from cells was obtained with the Total Quick RNA Cells & Tissues kit (Talent). cDNA templates for PCR amplification were synthesised from 1 µg total RNA with MMLV-RT (BRL Life Technologies, Carlsbad, CA, USA) for 60 min at 37 °C in the presence of random hexamer primers. The RT reaction products were subjected to PCR amplification. F6 (5’-CTCCTTGAGACCTCCTGCGC-3’) and F8R (5’-CG GCTGCTGACATCAGAGTG-3’) were used as primers to amplify a 673 bp fragment of G6Pase (Metón et al. 2004). *S. aurata* β-actin was used as an internal control for quality and quantity of RNA. Each PCR reaction contained cDNA template, PCR buffer, 0·2 mM

**Northern blotting analysis**

Total RNA was isolated from liver samples of *S. aurata* with the Total Quick RNA Cells & Tissues kit (Talent). An amount of 20 µg of total RNA was denatured and then loaded onto a 1% agarose gel containing 4-75% formaldehyde. Electrophoresis was performed in denaturing conditions for 5 h at 35 V; RNA was then transferred overnight to Nytran membranes (Schleicher & Schuell) in 5 × SSC (1 × SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7·5). RNA was cross-linked to the membranes by UV irradiation for 3 min. A G6Pase homologous probe was labelled by incorporation of digoxigenin-11-dUTP during PCR with primer pairs F1 (5’-GGTCTGGAGAGCTGGC TCAAC-3’) / F2R (5’-CG CACTTCTGCGCTTC TCCA-3’) which correspond to nucleotides 223–243 and 759–740 respectively in the *S. aurata* G6Pase cDNA (Metón et al. 2004). Prehybridisations of the membranes proceeded for 2 h at 50 °C in 7% SDS (w/v), 50% formamide, 5 × SSC, 2% blocking reagent (Roche), 0·1% N-laurylsarcosine (w/v) and 50 mM sodium phosphate, pH 7·0. The probes were added and hybridisation was performed overnight at 50 °C. Membranes were then washed twice for 15 min at room temperature in 2 × SSC, 0·1% SDS and twice for 15 min at 68 °C in 0·2 × SSC and 0·1% SDS. For normalisation, hybridisation of membranes with β-actin was carried out. Labelled probes were immunodetected with antidigoxigenin conjugated to alkaline phosphatase (Roche) and CDP-Star (Roche) as chemiluminescent substrate. Membranes were finally exposed to Hyperfilm ECL (Amersham).
dNTP, 250 µM of each primer and 1 U Taq DNA polymerase (Biotools). After initial denaturation at 94 °C for 2 min, a number of cycles (20–30) at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min were performed to verify that the PCR products amplify linearly and to determine the optimal number of cycles allowing their detection without saturation of the signal. Aliquots of each reaction were electrophoresed on a 2% agarose gel, and band intensities were compared by imaging of ethidium bromide staining.

Statistics

Data were analysed by one-factor analysis of variance by a computer program (StatView, SAS Institute, Cary, NC, USA). Differences were determined by Fisher’s PLSD multiple range test, with significance level at $P < 0.01$.

Results

Cloning of the 5’-flanking region of the S. aurata G6pc gene

An 850 bp fragment upstream of the translation start codon of G6pc was isolated by chromosome walking on S. aurata genomic DNA, using oligonucleotides designed from the hepatic G6Pase catalytic subunit cDNA previously cloned from this species (Metón et al. 2004) (Fig. 1A). The putative transcription initiation site of G6Pase mRNA was determined by the SMART RACE PCR approach (Zhu et al. 2001). A single fragment was obtained and cloned into pGEM T Easy. Upon sequencing, this fragment exhibited complete homology to the 39 bases in the 5′ untranslated region (UTR) of the previously cloned S. aurata G6Pase cDNA. Analysis of two independent clones indicated that S. aurata G6Pase mRNA initiates 106 nucleotides upstream of the translation start codon (Fig. 1B). Sequence analysis of the 850 bp 5′-flanking region with MOTIF-TRANSFAC 6.0 (Heinemeyer et al. 1999) revealed lack of basal elements such as a TATA box or a CAAT box, and presence of several putative transcription factor binding sites in the proximal region of the promoter. Among the potential transcription factors were sites for CREB, HNF-1, HNF-3β and HNF-4α, all implicated in the transcriptional activation of G6pc promoter in mammals (Fig. 1A). The nucleotide sequence reported in this paper was submitted to the DDBJ/EMBL/GeneBank databases under accession no. AY344583.

Identification of a functional S. aurata G6pc promoter

To determine whether the genomic DNA flanking exon 1 contains a functional promoter, the 850 bp
fragment isolated by chromosome walking was subcloned in the promoterless plasmid pGL3-Basic, upstream of the luciferase reporter gene. The recombinant plasmid pGP770 (−770/+80) was transiently cotransfected into HepG2 cells together with a lacZ-containing plasmid, as internal control for transfection efficiency. The cell lysate was assayed for luciferase and β-galactosidase activity 20 h after transfection. This construct exhibited more than 25-fold increase in luciferase activity relative to the promoterless vector, pGL3-Basic (Fig. 2). This result indicated that the region comprised within 770 nucleotides upstream of the transcription start site of S. aurata G6pc contains a functional promoter.

Transcriptional activity of G6pc promoter deletions

To examine the functional regions of the promoter involved in modulation of basal G6pc expression in S. aurata, sequential 5′-deletion analysis of the promoter fragment was carried out. To this end, deletion fragments, with 5′ ends ranging from −770 to −51 and 3′ ends at +80, were fused to the luciferase reporter gene and transfected into HepG2 cells. After 20 h, luciferase activity was measured in crude cell lysate. The longest 5′ construct (pGP770; −770 to +80) yielded a 25-fold increase in luciferase activity relative to pGL3-Basic (Fig. 2). Similar results were obtained with pGP629 (−629 to +80). Upon further deletion of 580 bases (pGP190; −190 to +80), a noticeable but non-significant increase with respect to pGP770 and pGP629 was observed. Thus, the pGP190 construct exhibited the highest increase in luciferase activity, 35-fold. These results indicate that motifs located between −770 and −190 do not play an essential role in the G6pc promoter basal activity in HepG2 cells. The lower promoter activity was observed with the construct containing the promoter region spanning −51 to +80 (pGP51),
which exhibited a 13-fold increase compared with the promoterless pGL3-Basic and a 64% reduction in luciferase activity compared with pGP190. Therefore, positive cis-elements may be found between nucleotide positions –190 and –51, as well as in the region comprised within 51 nucleotides upstream of the transcription start site.

Regulation of hepatic glucose-6-phosphatase expression by glucose and insulin in vivo and in isolated hepatocytes

To investigate in vivo effects of glucose and insulin on G6pc expression in the liver of S. aurata, four groups of fish were injected with glucose, insulin, glucose plus insulin, or saline (vehicle). Hepatic G6Pase mRNA levels were assessed 6 h after the treatment, by Northern blotting analysis. Administration of glucose, insulin or glucose plus insulin reduced G6Pase mRNA to 50%, 40% and 28% respectively, of control (saline) (Fig. 3A). Similar results were observed in G6pc expression in isolated hepatocytes by semiquantitative RT-PCR after treatment for 4 h with insulin and glucose plus insulin. No reduction of G6Pase mRNA levels was observed in hepatocytes incubated in the presence of glucose alone (Fig. 3B). The effect of insulin and glucose administration on serum glucose levels was also determined. Compared with control fish (146±51 ± 6.08 mg/dl, mean ± s.d., n = 5), insulin treatment significantly reduced glycaemia, to 54% (79±44 ± 14±85 mg/dl, n = 5), whereas both glucose and glucose plus insulin caused a 12–15-fold increase (1682±30 ± 684±04 mg/dl, n = 6; and 2276±87 ± 585±39 mg/dl, n = 6, respectively).

Regulation of G6pc promoter activity by glucose and insulin in HepG2 cells

In view of the effect of glucose and insulin on hepatic G6Pase mRNA levels in vivo and on isolated hepatocytes, and to study further the molecular mechanism by which glucose and insulin regulate G6pc expression in S. aurata, HepG2 cells transiently transfected with G6pc promoter fragments fused to the luciferase reporter gene were incubated in the presence or absence of glucose and insulin. Addition of up to 25 mM glucose to the culture medium caused a marked, dose-dependent increase in G6pc promoter activity (Fig. 4A). Addition of insulin in the absence of glucose did not affect luciferase activity with the longer 5′ construct (pGP770) or the shorter 5′ construct (pGP51). However, in the presence of 5 mM glucose, insulin repressed promoter activity of both pGP770 and pGP51 constructs (Fig. 4B). Thus, insulin overrode the effect of glucose on G6pc promoter activity in S. aurata, and the putative cis-element for insulin action may be located downstream of position –51 relative to the transcription start site.

Synergistic activation of G6pc promoter by HNF-4α and PGC-1α

Efficient promoter activation often requires the synergistic effect of transcription factors and coactivator proteins. At present, the mechanism by which insulin downregulates the expression of gluconeogenic genes remains unclear. Recently, Puigserver et al. (2003) suggested that insulin suppresses gluconeogenesis stimulated by PGC-1α through disruption of PGC-1α-FoxO1 interaction. We tested whether simultaneous overexpression of PGC-1α and HNF-4α resulted in G6pc promoter activation to levels higher than with one transcription factor alone. The pGP770 construct was activated about fivefold by PGC-1α alone, whereas HNF-4α caused fourfold activation. The combination of HNF-4α and PGC-1α resulted in 20-fold activation of the G6pc promoter (Fig. 5). In order to identify the region responsible for the synergistic effect of HNF-4α and PGC-1α, the same experiment was carried out in the 5′ deletion constructs pGP629, pGP190 and pGP51. HepG2 cells cotransfected with pGP629, pGP190 or pGP51 and expression plasmids for HNF-4α and PGC-1α lacked the synergistic effect (Fig. 5), suggesting that the region located between positions –770 and –629 relative to the transcription start site is required for this effect.

Discussion

Disturbed regulation of G6pc expression by insulin contributes to increased hepatic glucose production and to NIDDM in mammals. The advent of carnivorous fish as a glucose-intolerant system for the study of non-insulin-dependent diabetes (Wilson 1994, Caseras et al. 2000, Moon 2001) led us to isolate and characterise modulation of the promoter region of G6pc from S. aurata by glucose
Figure 3 Effect of glucose and insulin on hepatic G6Pase mRNA in vivo and in isolated hepatocytes from S. aurata. (A) Northern blot and densitometric analysis of G6Pase mRNA levels in liver of fish 6 h after treatment with saline (control), glucose (2 g/kg fish), insulin (10 units/kg fish) or glucose (2 g/kg fish) plus insulin (10 units/kg fish). An amount of 20 µg total RNA was loaded in each lane. G6Pase mRNA levels are expressed as mean±S.D. of four or five fish. (B) Semiquantitative RT-PCR and densitometric analysis of G6Pase mRNA levels in isolated hepatocytes 4 h after no treatment (control) or treatment with glucose (5 mM), insulin (10 nM) or glucose (5 mM) plus insulin (10 nM). G6Pase mRNA levels are expressed as mean±S.D. of two independent duplicate experiments. Different letters denote significant differences among treatments ($P<0.01$).
Understanding the transcriptional regulation of G6pc in carnivorous fish may explain why these animals are glucose intolerant. We previously reported that long-term starvation and energy restriction increase hepatic G6pc expression at mRNA level in S. aurata (Caseras et al. 2002). Consistent with strong dependence upon G6pc expression of hepatic glucose cycling in mammals (Nordlie et al. 1999, Pagliassotti et al. 2003), postprandial short-term modulation of G6Pase mRNA in the liver of regularly fed S. aurata occurs, with minimal mRNA levels 4–15 h after food intake (Caseras et al. 2002). In the present study, we have isolated the 5′/p9′-flanking region of the S. aurata G6pc and analysed its promoter activity in HepG2 cells by transient transfection. This genomic fragment allowed us to assess transcriptional regulation of the piscine G6pc promoter by glucose and insulin, and to identify trans-regulatory factors.

The functionality of the putative promoter region of fish G6pc was tested by transient transfection of HepG2 cells with fusion constructs of the 850 bp isolated by chromosome walking and sequential 5′ deletions of this fragment to the luciferase gene. The longest construct (pGP770) exhibited 25-fold higher luciferase activity than the promoterless pGL3-Basic. Variations in transfection efficiencies were corrected for using CMV-β (lacZ) as an internal control. The data represent the mean±S.D. values of two independent duplicate experiments.

**Figure 4** Effect of glucose and insulin on the activity of S. aurata G6pc promoter in HepG2 cells. (A) HepG2 cells transiently transfected with the pGP770 promoter construct were cultured in the presence (2, 5 and 25 mM) or absence of glucose in the medium, and luciferase activity was measured 16 h later. The promoter activity of pGP770 in the absence of glucose was set at 1. Different letters indicate significant differences (P<0·01). (B) HepG2 cells were transiently transfected with pGL3-Basic or the promoter constructs pGP770 or pGP51, and 800 ng expression vector encoding human insulin receptor B. After transfection, the cells were cultured in the presence (5 mM) or absence of glucose, and with 10 or 100 nM insulin or without human insulin in serum-free medium. The promoter activity of pGL3-Basic in the absence of glucose and insulin was set at 1. Variations in transfection efficiencies were corrected with CMV-β (lacZ) as an internal control. The data represent the mean±S.D. values of three independent duplicate experiments.

**Figure 5** Effect of HNF-4α and PGC-1α on the activity of S. aurata G6pc promoter in HepG2 cells. The cells were transiently transfected with pGL3-Basic or the promoter constructs pGP770, pGP629, pGP190 or pGP51, either with or without 400 ng expression plasmids encoding HNF-4α or PGC-1α. The promoter activity of reporter constructs alone was set at 1. Variations in transfection efficiencies were corrected for using CMV-β (lacZ) as an internal control. The data represent the mean±S.D. values of two independent duplicate experiments.
between –770 and –190 did not significantly affect the promoter activity as compared with the pGP770 construct. Therefore, it can be inferred that this region is not essential for promoter basal activity. Deletion between –190 and –51 resulted in a significant decrease in the promoter activity. The shorter construct –51 to +80 (pGP51) conferred moderate promoter activity. These data suggest the presence of positive regulatory element(s) within 190 bp upstream of the transcription start site, and indicate that this region is a core functional promoter for basal transcriptional activation of G6pc.

In rats, starvation and hormones that increase cAMP stimulate G6pc expression, whereas refeeding and insulin administration decrease it (Argaud et al. 1996, Minassian et al. 1999). Insulin inhibits basal G6pc expression as well as G6pc stimulation by glucose, glucocorticoids, cAMP and fatty acids. Studies in vivo and in vitro have shown that glucose stimulates G6pc expression in rats. However, the induction of G6pc by glucose is paradoxical and has been linked to a balancing effect with that of insulin to avoid complete depletion of the enzyme during meal absorption, to control the hepatic glycogen storage and to prepare the transition to fasting periods (Argaud et al. 1997, Chatelain et al. 1998, Massillon 2001).

In the present study, repression of G6pc occurred in insulin-treated fish and isolated hepatocytes. In contrast to glucose-dependent activation of G6pc expression in rats, G6Pase mRNA levels decreased in S. aurata after glucose administration. Interestingly, glucose did not modulate G6pc expression in isolated hepatocytes, suggesting that glucose-dependent repression in vivo may result from a prevailing effect of insulin in a physiological state that leads to increased insulin levels. Treatment with glucose plus insulin resulted in a marked reduction of G6Pase mRNA both in vivo and in isolated hepatocytes. These findings argue for an in vivo repression of G6pc in the liver of S. aurata caused by a hyperglycaemic state following food intake. This agrees with suppression of hepatic glucose production by hyperinsulinaemia and hyperglycaemia through inhibition of G6Pase in rats (Guignot & Mithieux 1999), and with the postprandial rise in blood glucose levels followed by a decrease of hepatic G6pc expression previously observed in regularly fed fish (Caseras et al. 2002). The lack of stimulatory effect of glucose on fish G6pc in vivo is consistent with previous results indicating that increasing carbohydrate dietary content does not affect hepatic G6Pase mRNA levels in S. aurata (Caseras et al. 2002). Likewise, Hornbuckle et al. (2001) did not observe a stimulatory effect of glucose on G6Pase mRNA in conscious dogs, whereas insulin had an inhibitory effect. Thus, control of G6pc expression in carnivorous animals such as S. aurata and dogs followed a similar mechanism, which in turn differs from that in rats (omnivore). In HepG2 cells transfected with 770 bp of the S. aurata G6pc promoter region, absence of insulin in the culture medium allowed a positive regulatory effect of glucose on the transcriptional activity of the fish G6pc promoter. Since glucose administration leads to increased insulin release in vivo, the observation that glucose decreased G6Pase mRNA levels in the liver of S. aurata may result from a prevailing inhibitory effect of insulin over the effect of glucose on G6pc promoter. The possible contribution of glucose-derived metabolites and other hormones to suppression of G6pc transcription in fish cannot be excluded.

In contrast to the strong inhibitory effect of insulin on rat G6pc promoter activity in H4 IIE hepatoma cells in the absence of glucose (Chen et al. 2000), insulin hardly affected the transcriptional activity of S. aurata G6pc promoter in HepG2 cells under a no-glucose condition. However, a repressing effect of insulin was observed in the presence of glucose, a situation that is probably closer to the postprandial state in the piscine liver. Hence, as in mammals, insulin overrode glucose-mediated activation of S. aurata G6pc promoter. Since the ability of insulin to repress hepatic glucose production is reduced in NIDDM, the fact that insulin had no significant effect on the activity of S. aurata G6pc promoter under low-glucose conditions suggests that glucose availability determines insulin-dependent repression of piscine G6pc, and thus may contribute to insulin resistance in carnivorous fish.

Recent studies illustrate the role of the coactivator PGC-1α in the transcriptional activation of mammalian gluconeogenic genes, such as G6pc and phosphoenolpyruvate carboxykinase 1 (Pck1). PGC-1α has been shown to mediate induction of mouse G6pc by HNF-4α (Boustead et al. 2003, Rhee et al. 2003). Puigserver et al. (2003) showed that coactivation by PGC-1α is critical for Foxo1-dependent transcriptional activation of the G6pc
and PekI promoters in mice, leading the authors to propose a model in which gluconeogenic gene expression is inhibited by insulin via a PKB-dependent disruption of the complex formed with the coactivator protein PGC-1α and Foxo1. Indeed, Pgc1 promoter activity is induced by coexpression of Foxo1 via the IRS within the Pgc1 promoter in HepG2 cells, and coexpression of PKB was found to mimic the inhibitory effect of insulin on Pgc1 promoter activity (Daitoku et al. 2003). In addition, glucagon may stimulate G6pc expression via PGC-1α. The expression of Pgc1 is induced by glucagon via cAMP, leading to increased G6pc expression, probably by the interaction of PGC-1α with HNF-4α and the glucocorticoid receptor. In liver, fasting markedly induces PGC-1α expression (Lin et al. 2002), and overexpression of PGC-1α in primary hepatocytes increases glucose production and the expression of G6pc and Pek1 (Yoon et al. 2001). Furthermore, in several models of NIDDM that are associated with increased gluconeogenesis, Pgc1 gene expression is also elevated (Hara et al. 2002, Yoon et al. 2003). On the other hand, heterozygous mutations in the HNF4α gene give rise to maturity onset diabetes of the young type 1 (Ryffel 2001). We investigated the effect of overexpression of HNF-4α and PGC-1α on G6pc promoter activity in HepG2 cells cotransfected with plasmids ranging from –770 to +80 relative to the transcription start site in the S. aurata G6pc promoter and sequential 5′ deletions of this fragment. A synergistic effect was observed through cotransfection with both HNF-4α and PGC-1α and the longest promoter construct, suggesting that coactivation of HNF-4α occurs in S. aurata. Loss of synergism was observed with the shorter constructs, indicating that the sequence between –770 and –629 in the S. aurata G6pc promoter region is essential for this effect, presumably by binding of HNF-4α to this region. These findings suggest that the major role attributed to PGC-1α in turning on the gluconeogenic programme in mammals may also occur in fish. Since the pGP51 construct was modestly activated by overexpression of PGC-1α, a second cis-element involved in PGC-1α stimulation within the 51 bp upstream of the transcription start of the S. aurata G6pc promoter cannot be ruled out. However, this putative site would be neither necessary nor sufficient for PGC-1α coactivation of HNF-4α. The fact that insulin repressed the promoter activity of pGP51 construct excludes a model involving suppression of PGC-1α coactivation of HNF-4α in the piscine G6pc promoter to explain insulin repression of S. aurata G6pc under high-glucose conditions, as occurs with insulin-dependent disruption of the PGC-1α-Foxo1 complex in mice (Puigserver et al. 2003). Further studies are needed to identify cis-elements involved in insulin repression of fish G6pc.

Acknowledgements

The authors wish to thank Drs M D Walker, B M Spiegelman and J Whittaker for providing the HNF-4α, PGC-1α and insulin receptor B expression vectors respectively. This study was supported by grants from MCYT (Spain), BMC2000–0761 and BIOC2003–01098. M Egea is the recipient of a fellowship from the University of Barcelona. We thank the Language Advisory Service of the University of Barcelona for correcting the English manuscript.

References


Clore JN, Stillman J & Sugerman H 2000 Glucose-6-phosphatase flux *in vitro* is increased in NIDDM. *Diabetes* 49 969–974.


Lin B, Morris DW & Chou JY 1997 The role of HNF1α, HNF3γ, and cyclic AMP in glucose-6-phosphatase gene activation. *Biochimica et Biophysica Acta* 138B 145–153.


Pagliassotti MJ, Wei Y & Bizeau ME 2003 Glucose-6-phosphatase activity is not suppressed but the mRNA level is increased by a sucrose-enriched meal in rats. *Journal of Nutrition* 133 32–37.


Ryffel GU 2001 Mutations in the human genes encoding the transcription factors of the hepatocyte nuclear factor (HNF)1 and HNF4 families: functional and pathological consequences. *Journal of Molecular Endocrinology* 27 11–29.


Received 19 July 2004
Accepted 9 August 2004
Made available online as an Accepted Preprint
15 September 2004