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

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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-4 α (HNF-4 α) and peroxisome proliferator-activated receptor- γ coactivator-1 (PGC-1 α). No direct relationship was found between PGC-1 α coactivation of HNF-4 α 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.

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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 β_3 -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 forkhead transcription factor Foxo1 (also known as FKHR), to insulin response sequence (IRS) motifs located in region B (Vander Kooi *et al.* 2003). The dual-specificity protein kinase DYRK1A synergistically potentiates transactivation of *G6pc* by Foxo1 (Von Groote-Bidlingmaier *et al.* 2003). Short-chain fatty acids induce *G6pc* transcription via recruitment of HNF-4 α to the promoter (Massillon *et al.* 2003). Recent studies have shown that transcriptional activation of the *G6pc* promoter by HNF-4 α and Foxo1 involves the coactivator protein peroxisome proliferator-activated receptor- γ coactivator-1 (PGC-1 α) (Herzig *et al.* 2001, Yoon *et al.* 2001, Boustead *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-3 γ 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 & Klungsoyr 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-4 α and the coactivator PGC-1 α .

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'-TCGACCCCCCAGCTGT GCAGAAGATC-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'-GATGGCTCAGCCTCTGCCTGAGGATG-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'-GCGCTAGCGACGGCCCGGGCTGGTAAA-3'), extending from positions -770 to -751, and containing a 5'-anchor sequence with an NheI site (underlined), a reverse MCGP06 oligonucleotide (5'-GCAAGCTTGATGGCTCAGCCTCTGCCTG-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 cell 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 MgCl_2 , 13.5 mM β -mercaptoethanol, 0.9 mM ONPG (2-nitrophenyl- β -D-galactopyranoside) and 0.1 M sodium phosphate, pH 7.5. After incubation at 37 °C (usually 20 min to 1 h), the reaction was stopped by addition of 0.5 ml 0.5 M Na_2CO_3 , and the intensity of the yellow colour was determined by its optical density at 420 nm. The expression plasmids encoding rat HNF-4 α (pcDNA3.HNF4 α ; Bartoov-Shifman *et al.* 2002), mouse PGC-1 α (pSV2-PGC1; Puigserver *et al.* 1998) and human insulin receptor B (InsRB; Yoshimata *et al.* 1988) were kindly provided by Drs M.D. Walker (Weizmann Institute of Science, Israel), B.M. Spiegelman (Harvard Medical School, USA), and J. Whittaker (Case Western Reserve University, USA) respectively.

Northern blotting analysis

Total RNA was isolated from liver samples of *S. aurata* with the Total Quick RNA Cells & Tissues kit (Talent, Trieste, Italy). 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 $5 \times \text{SSC}$ ($1 \times \text{SSC} = 150 \text{ mM NaCl}$ 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'-GTCCTGGGAGACTGGC TCAAC-3')/F2R (5'-CCACTTCTGGGCTTTC 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, $5 \times \text{SSC}$, 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 $2 \times \text{SSC}$, 0.1% SDS and twice for 15 min at 68 °C in $0.2 \times \text{SSC}$ 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). 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 *G6pc* 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'-CTC TCCTTGACCTCCCTGGC-3') and F8R (5'-CG GCTGCTGACATCAGAGT GG-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

Figure 1 Sequence analysis of the 5'-flanking region of the *S. aurata* *G6pc* and mapping of the transcription initiation site of G6Pase mRNA. (A) The upstream sequences isolated by chromosome walking are shown in capitals. An arrow indicates the transcription start site. The translation start codon ATG is in boldface and underlined. Putative binding sites for transcription factors and IRS motifs are boxed. (B) Resolution of SMART RACE PCR products on a 2% agarose gel is shown. The assay, performed with 1 µg hepatic poly A⁺ RNA, produced a 187 bp band that was purified, ligated into pGEM T Easy and sequenced.

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

Figure 2 Deletion analysis and promoter activity of the 5'-flanking region of *S. aurata G6pc* in HepG2 cells. The top left part represents the genomic organisation of the 5'-flanking region of *S. aurata G6pc*. Relevant restriction sites and exon 1, depicted as a white box, are indicated. Nucleotide numbering starts with +1, corresponding to the transcription initiation site. The *S. aurata G6pc* promoter having varying 5' ends and an identical 3' end (+80) was fused to the luciferase reporter gene in pGL3-Basic vector. The constructs were transfected in HepG2 cells along with CMV- β (*lacZ*) to normalise for transfection efficiency. Luciferase activity is expressed as a fold increase over promoterless reporter plasmid pGL3-Basic. Results presented are the mean $\pm$ S.D. from at least three independent experiments performed in duplicate.

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 $\pm$ 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-4 α and PGC-1 α

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-1 α through disruption of PGC-1 α -Foxo1 interaction. We tested whether simultaneous overexpression of PGC-1 α and HNF-4 α resulted in *G6pc* promoter activation to levels higher than with one transcription factor alone. The pGP770 construct was activated about fivefold by PGC-1 α alone, whereas HNF-4 α caused fourfold activation. The combination of HNF-4 α and PGC-1 α resulted in 20-fold activation of the *G6pc* promoter (Fig. 5). In order to identify the region responsible for the synergistic effect of HNF-4 α and PGC-1 α , 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-4 α and PGC-1 α 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 ($P < 0.01$).

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 $\pm$ s.d. values of three independent duplicate experiments.

and insulin. 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'-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 promoter region of *S. aurata* *G6pc* was cloned by chromosome walking. Mapping of the transcriptional start indicated the presence of a start site located 106 bp upstream of the translation start codon. Our localisation of the transcription start site predicts an 1815 bp mRNA, which is consistent with the 1.8 kb transcript identified by Northern blotting (Metón *et al.* 2004).

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. Deletions of the promoter region

Figure 5 Effect of HNF-4 α and PGC-1 α 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-4 α or PGC-1 α . 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 $\pm$ 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-1 α in the transcriptional activation of mammalian gluconeogenic genes, such as *G6pc* and phosphoenolpyruvate carboxykinase 1 (*Pck1*). PGC-1 α has been shown to mediate induction of mouse *G6pc* by HNF-4 α (Boustead *et al.* 2003, Rhee *et al.* 2003). Puigserver *et al.* (2003) showed that coactivation by PGC-1 α is critical for Foxo1-dependent transcriptional activation of the *G6pc*

and *Pck1* 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-1 α 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-1 α . The expression of *Pgc1* is induced by glucagon via cAMP, leading to increased *G6pc* expression, probably by the interaction of PGC-1 α with HNF-4 α and the glucocorticoid receptor. In liver, fasting markedly induces PGC-1 α expression (Lin *et al.* 2002), and overexpression of PGC-1 α in primary hepatocytes increases glucose production and the expression of *G6pc* and *Pck1* (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 *HNF4a* gene give rise to maturity onset diabetes of the young type 1 (Ryffel 2001). We investigated the effect of overexpression of HNF-4 α and PGC-1 α 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-4 α and PGC-1 α and the longest promoter construct, suggesting that coactivation of HNF-4 α 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-4 α to this region. These findings suggest that the major role attributed to PGC-1 α in turning on the gluconeogenic programme in mammals may also occur in fish. Since the pGP51 construct was modestly activated by overexpression of PGC-1 α , a second *cis*-element involved in PGC-1 α 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-1 α coactivation of HNF-4 α . The fact that insulin repressed the promoter activity of pGP51 construct excludes a

model involving suppression of PGC-1 α coactivation of HNF-4 α 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-1 α -Foxo1 complex in mice (Puigserver *et al.* 2003). Further studies are needed to identify *cis*-elements involved in insulin repression of fish *G6pc*.

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