Sp1 and Sp3 regulate glucokinase gene transcription in the liver of gilthead sea bream (*Sparus aurata*)

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Abstract

To better understand the transcriptional machinery that governs glucokinase (GCK) expression, we have cloned and characterized the proximal promoter region of *GCK* from gilthead sea bream (*Sparus aurata*). The 5'-flanking region of *GCK* was isolated by chromosome walking. SMART RACE-PCR allowed us to locate the transcription start site 98 bp (bp) upstream from the translational start. Transfection analysis in HepG2 cells revealed the presence of a functional promoter in the 1397 bp 5'-flanking isolated fragment (positions -1321 to +76 relative to the transcription start site). Sequential 5'-deletion analysis indicated a core functional promoter for basal transcription within the 288 bp upstream from the transcription start site. Transient transfection experiments performed in HepG2 cells and electrophoretic mobility shift assays denoted that Sp1 binds and transactivates *GCK* promoter, whereas Sp3 repressed Sp1-mediated activation of *GCK* by competing for the same binding site. Mutations in the Sp binding site completely abolished the enhancing effect of Sp1. Treatment with insulin stimulated *GCK* expression, and increased Sp1 levels in *S. aurata* liver. We propose a new mechanism that involves Sp1 and Sp3 to mediate insulin activation of *GCK* transcription.

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Introduction

Glucokinase (GCK, ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1), or hexokinase IV, catalyzes the phosphorylation of glucose to glucose-6-phosphate (Glu-6-P). In mammals, GCK is mainly expressed in liver and pancreatic β -cells. The hepatic enzyme contributes to the control of glycemia through glucose metabolism and its storage as glycogen, whereas the pancreatic isoform acts as a glucose sensor that controls insulin secretion in β -cells. In mammals, starvation and diabetes down-regulate *GCK* expression in the liver, whereas refeeding on high carbohydrate diets and treatment with insulin stimulate *GCK* expression. Glucocorticoids potentiate the action of insulin, whereas 3,5,3'-triiodo-L-thyronine and biotin also stimulate *GCK* transcription, but to a lesser extent than insulin (Iynedjian *et al.* 1989, Magnuson *et al.* 1989, Magnuson 1990, Narkewicz *et al.* 1990, Chauhan & Dakshinamurti 1991, Iynedjian 1993). The stimulatory effect of insulin on hepatic *GCK* expression is reversed by cAMP (Iynedjian *et al.* 1989). The mammalian *GCK* gene has a dual promoter transcription unit. The upstream promoter is active in pancreatic islets β -cells, duodenum, pituitary, and brain, whereas the downstream promoter is active in hepatocytes (Magnuson & Shelton 1989, Iynedjian *et al.* 1996). The mechanism involved in liver-specific expression of *GCK* is not well understood. Hypoxia-inducible factor-1 (Roth *et al.* 2004), sterol-regulatory element-binding protein-1 (SREBP-1; Foretz

et al. 1999), signal transducer and activator of transcription 5B (STAT 5B; Sawka-Verhelle *et al.* 2000), upstream stimulatory factors (USFs; Iynedjian 1998), hepatocyte nuclear factor (HNF)-4 (Roth *et al.* 2002), and the transcriptional coactivator p300 (Roth *et al.* 2004) have been reported as insulin-dependent *GCK* gene activators. A recent report challenges involvement of STAT 5B in *GCK* transcription (Ribaux *et al.* 2002). Whether SREBP-1 mediates insulin action on *GCK* expression is also controversial (Gregori *et al.* 2006, Hansmann *et al.* 2006). Other HNFs, FoxO1, and peroxisome proliferator-activated receptor- γ have also been proposed to play a role in *GCK* transcription in the liver (Cha *et al.* 2001, Lannoy *et al.* 2002, Kim *et al.* 2004, Zhang *et al.* 2006).

In fish, glucose intolerance and limited use of dietary carbohydrates have been reported. Compared with mammals, these animals show prolonged hyperglycemia after a glucose load and when feeding on high carbohydrate diets (Christiansen & Klungsøyr 1987, Cowey & Walton 1989, Wilson 1994, Moon 2001, Metón *et al.* 2003). The relative inability of carnivorous fish to utilize carbohydrates has been reported as being due, in part, to absence of GCK activity in the liver (Wilson 1994, Moon 2001). Recently, the cDNA coding for GCK has been isolated from the piscine liver and the functionality of the fish GCK cDNA was shown by *in vitro* transcription/translation and after transient transfection in COS-7 cells (Caseras *et al.* 2000). These findings indicate that GCK is functional in fish liver,

although control of *GCK* gene expression remains poorly understood.

To gain insight into the molecular mechanism regulating *GCK* expression, we have cloned and characterized the proximal promoter region of *GCK* from gilthead sea bream (*Sparus aurata*). We conclude that Sp1 transactivation of *GCK* promoter and repression of Sp1-mediated activation by Sp3 may be involved in insulin action on *GCK* expression.

Materials and methods

Animal treatments

Gilthead sea bream (*S. aurata*) obtained from Tinamenor (Cantabria, Spain) were maintained as described previously (Salgado *et al.* 2004). Fish were fed daily (1000 h) at 1% body weight. To study the regulation of *GCK*, Sp1, and Sp3 expression by insulin *in vivo*, 24 h after the last meal (1000 h), two different groups received an i.p. injection of bovine insulin (10 U/kg fish; Sigma) or vehicle (saline). Fish were killed by cervical section 6 h after treatment. Tissue samples were dissected out, immediately frozen in liquid N₂ and kept at −80 °C until use. To avoid stress, fish were anesthetized with MS-222 (1:12 500) before handling. The experimental procedures met the guidelines of the animal use committee of the Universitat de Barcelona.

Cloning of the 5'-flanking region of *GCK* gene from *S. aurata* by chromosome walking

The 5'-flanking region of *GCK* was isolated by PCR using 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 library was ligated to the GenomeWalker adaptor provided in the kit. A primary PCR was performed on each library with the gene-specific primer ME0001 (5'-CCGCAGGTTT GACTAAATGAGCC-3') and the AP-1 primer provided in the kit. A nested PCR was performed on the primary PCR product using the gene-specific primer ME0004 (5'-GCCTCGGTATGTGTCTCTAGGTGTGTGG-3') and the AP2 primer from the kit. The longer amplification product, a single 1.4 kb band, was obtained from the StuI library and ligated into pGEM T easy plasmid (Promega) to generate pGEM-GK1400. Two independent clones were fully sequenced on both strands following the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit instructions (Applied Biosystems, Foster City, CA, USA).

Characterization of the transcription start site

The 5' end of the hepatic *S. aurata* *GCK* cDNA was determined using the SMART RACE cDNA

Amplification kit (Clontech). One microgram poly A⁺ RNA obtained from liver of *S. aurata* was converted into cDNA using PowerScript reverse transcriptase (RT) at 42 °C for 1.5 h in the presence of 5'-CDS primer, for first-strand synthesis, and SMART II A oligonucleotide, which serves as an extended template for RT after annealing an oligo-dC previously added to the end of the mRNA template by the terminal transferase activity of PowerScript RT. Primary and nested PCR were conducted with primer pairs ME0001/Universal Primer Mix A (from the kit) and ME0004/Nested Universal Primer (from the kit) respectively. The single 106 bp band generated was purified and ligated into pGEM T easy plasmid (Promega). Identical nucleotide sequence corresponding to the 5' end of *GCK* cDNA was obtained by sequence analysis of two independent clones.

RT-PCR analysis of *GCK* mRNA tissue specificity

cDNA templates for PCR amplification were synthesized from 5 µg total RNA obtained from *S. aurata* frozen tissues using Moloney Murine Leukemia Virus (M-MLV) RT (BRL Life Technologies). The RT reaction products were subjected to PCR amplification using MEGK01 (5'-CCGTGTGATGCTGGTGAAGG-3') and MEGK02 (5'-GGAGAAGGTGAAACCGAGAGG-3') as primers to amplify a 202 bp fragment of *S. aurata* *GCK* cDNA. As a control of RNA integrity, a 711 bp fragment of *S. aurata* β-actin was amplified in parallel with primers BA0199 (5'-GACAACGGATCCGGTATGTGC-3') and BA0299 (5'-GACCTGTCCGTCGGGCAGCTC-3'). Aliquots of each reaction were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

Construction of reporter gene plasmids

The *S. aurata* *GCK* promoter sequence located between positions −606 to +76 relative to the transcription start site was generated by PCR, using pGEM-GK1400 as template and oligonucleotides ME0107 (5'-CTCCTAAATTGACACCCAC-3') and ME0004, and subcloned into the SmaI site of the promoterless luciferase reporter plasmid pGL3-Basic (Promega) to generate pGK606. The pGK1321 construct was made by ligation of the NcoI/NdeI *GCK* promoter fragment, obtained from pGEM-GK1400, to the MluI/NdeI site of pGK606. To this end, NcoI and MluI ends were made blunt before NdeI digestion and subsequent ligation. The reporter constructs designated as pGK288 and pGP21 were produced by self-ligation of filled-in ends of pGK606 after digestion with EcoRV/NheI and PvuII/NheI respectively. The pGK288mutSp construct was made by ligation of the MluI/PvuII fragment of a PCR product, obtained

using pGK288 as template and oligonucleotides ME0506 (5'-GAGAACTTCAGCTGTTTTATGCTCA GGTGAAGT**TTATTTTATACACCGCACGTAAAGGC** CCACCCC-3'; the mutated Sp box is bold and double-underlined) and RVprimer3 (positions +4760 to +4779 in pGL3-Basic vector), into pGK288 previously digested with MluI/PvuII. The pGK72 and pGK72mutSp constructs were generated by PCR amplification using pGK606 as template, and primer pairs ME0202 (5'-CC**CCCGGGCCTTTACGTGCGG**-3'; the SmaI site included is bold and underlined)/GL primer2 (positions +111 to +89 in pGL3-Basic vector) and ME0310 (5'-CC**CCCGGGCCTTTACGTGCGGTG TATAAAATAAACTTCACCTGAGCATAAAAAACAG**-3'; the SmaI site included is bold and underlined; the mutated Sp box is bold and double-underlined)/GLprimer2 respectively. The PCR products were subcloned into the SmaI/HindIII site of pGL3-Basic. Self-ligation of pGK606 previously digested with EcoRV/PvuII was performed to generate pGK606Δ288-21. The reporter construct harboring the rat liver GCK promoter (prGK1418) was constructed by ligation of a PCR product, obtained by amplification using oligonucleotides ME0503 (5'-GCTCCCTCCTCTCCGGAGGC-3') and ME0504 (5'-GGGGTCGTTGGGAAGTAGAG-3') and genomic DNA from rat as template, into the SmaI site of pGL3-Basic. All constructs were verified by sequencing using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

Cell transfection and luciferase assay

HepG2 (ATCC HB 8065) were 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 calcium phosphate co-precipitation 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 with 400 ng expression vectors encoding Sp1 or Sp3. To correct for variations in transfection efficiency, 500 ng CMV-β (lacZ) was included in each transfection. To ensure equal DNA amounts, empty plasmids were added in each transfection. Four hours after addition of the precipitate, cells were shocked in 10% dimethylsulfoxide in serum-free medium for 2 min. The cells were harvested 16 h later, washed in PBS and incubated for 15 min in 300 µl Cell Culture Lysis Reagent (Promega). After the removal of cell debris by centrifugation at 10 000 *g* for 15 s, luciferase activity was measured in the supernatant after the addition of Luciferase Assay Reagent (Promega) in 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 β-mercaptoethanol, 0.9 mM 2-nitrophenyl-β-D-galactopyranoside (ONPG), and 0.1 M sodium phosphate pH 7.5. After incubation at 37 °C, the reaction was stopped by addition of 0.5 ml of 0.5 M Na₂CO₃, and the intensity of the yellow color was determined by its optical density at 420 nm. Culture medium for Chinese hamster ovary (CHO) cells was Ham's F-12 nutrient mixture supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. Sea bass larvae (SBL) cells were cultured at 20 °C in MEM, complemented with Hank's salts, 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. CHO and SBL cells were transiently transfected with reporter and expression constructs using FuGene 6 (Roche), according to the manufacturer's instructions. As an internal control, CMV-β was included in each transfection. Cells were harvested 40 h following transfection, and luciferase, and β-galactosidase activity were assayed as for HepG2 cells. The SBL cell line was a generous gift of Dr J Castric (Technopôle Brest-Iroise, Brest, France). Expression plasmids encoding rat Sp1 (pRC-CMVSp1) and rat Sp3 (pRC-CMVSp3) were kindly provided by Dr G Suske (Institute of Molecular Biology and Tumor Research, Marburg, Germany; Hagen *et al.* 1994).

Electrophoretic mobility shift assay

Double-stranded oligonucleotides used in gel shift experiments were (only the forward oligonucleotides are shown): Sp1-cons (5'-AAGAGGGTGGGCGGGAG CAACCA-3'), GK-56/-37 (5'-TGTGGCCAGCCCACTTC ACC-3'), and GK-56/-37mutSp (5'-TGT**TATAAAATAAAC** TTCACC-3'). Double-stranded oligonucleotides, 100 pmols, were 3'-end labeled with digoxigenin-11-ddUTP using terminal transferase (Roche) in a 20 µl reaction for 30 min at 37 °C. The reaction was stopped by adding 2 µl 0.2 M EDTA. Binding reactions were carried out in a total volume of 20 µl containing 100 mM Hepes, pH 7.6, 5 mM EDTA, 50 mM (NH₄)₂SO₄, 5 mM dithiothreitol, 1% Tween 20, 150 mM KCl, 1 µg non-specific competitor poly [d(I-C)] (Roche), HepG2 extracts prepared from Sp1 or Sp3 overexpressing cells or *in vitro* translated Sp1 and Sp3 proteins, and the labeled probe. *In vitro* transcription/translation of Sp1 and Sp3 proteins were made with TNT T7 quick coupled transcription/translation system (Promega) in a reaction mixture incubated for 1 h at 4 °C. The DNA-protein complexes were electrophoresed at 4 °C on 5% polyacrylamide gel and using 0.5× tris-borate-EDTA (TBE) as buffer. DNA was thereafter transferred by contact blotting (2 h at room temperature) to Nytran membranes (Schleicher & Schuell, Keene, NH, USA).

DNA was cross-linked to membranes by UV irradiation for 3 min. Labeled probes were immunodetected with anti-digoxigenin conjugated to alkaline phosphatase (Roche) and using CDP-Star (Roche) as chemiluminescent substrate. Membranes were finally exposed to Hyperfilm ECL (Amersham). For competition experiments, HepG2 extracts were preincubated for 30 min with a 10- to 200-fold molar excess of unlabeled double-stranded Sp1-cons oligonucleotide. For super-shift assays, 2 µg polyclonal anti-human Sp1 and/or Sp3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were incubated with *in vitro* translated Sp1 and Sp3 proteins for 30 min at room temperature before addition of the probe.

Northern blotting analysis

Total RNA was isolated from liver of *S. aurata* with the Total Quick RNA Cells & Tissues Kit (Talent, Trieste, Italy), electrophoresed on a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and crosslinked to the membrane by UV irradiation. A GCK homologous probe was labeled by incorporation of digoxigenin-11-dUTP during PCR with primer pair MEGK01/MEGK02. Prehybridizations of the membranes were performed at 50 °C for 2 h in 7% SDS (wt/vol), 50% formamide, 5× SSC, 2% blocking reagent (Roche), 0.1% laurylsarcosine (wt/vol), 50 mM sodium phosphate (pH 7.0). The probe was added and hybridization was carried out overnight at 50 °C. High stringency washes were performed as described (Salgado *et al.* 2004). The 18S rRNA was used to correct for loading irregularities. Labeled probes were immunodetected with anti-digoxigenin conjugated to alkaline phosphatase and CDP-Star as described above.

Western blotting analysis

Proteins in liver crude extracts from *S. aurata* were separated on 10% SDS-PAGE gels, transferred to nylon membranes and immunoblotted with polyclonal anti human Sp1 and Sp3 (Santa Cruz Biotechnology) following the ECL Western blotting (Amersham) procedure. An anti-rabbit peroxidase-conjugated secondary antibody was used for chemiluminescent detection.

Results

Cloning of the 5'-flanking region of the *S. aurata* GCK gene

A 1397 bp fragment upstream from the translation start codon of GCK was isolated by chromosome walking on *S. aurata* genomic DNA using oligonucleotides designed from the piscine hepatic GCK cDNA (Fig. 1). GCK expression was assessed by RT-PCR in various tissues of *S. aurata*. In piscine tissues, expression of GCK mRNA was essentially found in the liver. The putative transcription initiation site of GCK mRNA was determined using the SMART RACE PCR approach (Fig. 2A). A single fragment was obtained and cloned into pGEM T easy. Upon sequencing, this fragment exhibited complete homology to the 47 bases in the 5' untranslated region of the previously cloned *S. aurata* GCK cDNA (Caseras *et al.* 2000). Analysis of two independent clones indicated that *S. aurata* GCK mRNA initiates 98 nucleotides upstream of the translation start codon. Sequence analysis of the 1397 bp 5'-flanking region using MOTIF-TRANSFAC 6.0 (Heinemeyer *et al.* 1999) revealed the presence of a TATA box at positions -33 to -19 relative to the

Figure 1 Sequence analysis of the 5'-flanking region of *S. aurata* GCK. The upstream sequence isolated by chromosome walking is shown in capitals. An arrow indicates the transcription start site. The translation start codon is in bold and underlined. Putative binding sites for transcription factors are boxed.

Figure 2 Mapping of the transcription initiation site of *S. aurata* GCK mRNA and functional analysis of the 5'-flanking region of GCK in HepG2 cells. (A) Resolution of SMART RACE PCR products on a 4% agarose gel is shown. The assay, performed with 1 µg hepatic Poly A⁺ RNA (lane 1), produced a 106 bp band that was purified, ligated into pGEM T easy and sequenced. M, DNA size marker (ϕX174 DNA digested with HinfI). (B) The top left part represents the genomic organization of the 5'-flanking region of *S. aurata* GCK. Relevant restriction sites and exon 1, depicted as a white box, are indicated. Nucleotide numbering starts with +1 corresponding to the transcriptional start. Reporter constructs having varying 5' ends and an identical 3' end (+76) were transfected in HepG2 cells. Luciferase activity is expressed as a fold increase over promoterless reporter plasmid pGL3-Basic. Results presented are the mean ± s.d. from at least three independent duplicate experiments.

transcription start site, and several putative transcription factor binding sites. Amongst the potential transcription factors were sites for Sp, USF, SREBP-1, and STAT proteins (Fig. 1). The nucleotide sequence reported in this paper was submitted to the DDBJ/EMBL/GenBank databases under the accession number AY206500.

Functionality of *S. aurata* GCK promoter

To determine whether the genomic DNA flanking exon 1 contains a functional promoter, the DNA fragment isolated by chromosome walking was subcloned in the promoterless plasmid pGL3-Basic, upstream of the luciferase reporter gene. The recombinant plasmid pGK1321 (−1321/+76) was transiently co-transfected into HepG2 cells together with a *lacZ*-containing plasmid, as internal control for transfection efficiency. This construct exhibited more than a fivefold increase in luciferase activity relative to the promoterless vector, pGL3-Basic (Fig. 2B). This result indicated that the region comprised within 1321 nucleotides upstream of the transcription start site of *S. aurata* GCK contains a functional promoter.

To examine the promoter functional regions involved in the modulation of basal GCK expression in *S. aurata*, sequential 5'-deletion analysis of the promoter fragment was carried out. Deletion fragments, with 5' ends ranging from −1321 to −21 and 3' ends at +76, were fused to the luciferase reporter gene and transfected into HepG2 cells. The longest 5' construct (pGK1321, −1321 to +76) yielded a fivefold increase in luciferase activity relative to pGL3-Basic (Fig. 2B). Similar results were obtained using pGK606 (−606 to +76) and pGK288 (−288 to +76). The

reporter construct containing the region −72 to +76 (pGK72) exhibited ~50% of promoter activity compared with longer constructs. No activity was found using the smallest construct (pGK21; −21 to +76) or a deleted construct harboring promoter sequences located between −606 and +76 bp relative to the transcription start site, but lacking the region comprised between positions −288 to −21 (pGK606Δ288-21). These results clearly indicate that the promoter region between −288 and −21 bp is important for the basal transcription of the *S. aurata* GCK gene, suggesting that *cis*-acting elements may be located in this region. The high GC and GT content of this region led us to investigate a possible role of Sp proteins in the transcriptional activation of GCK.

Transactivation of GCK promoter by Sp1 and repression of Sp1-mediated transcription by Sp3

To examine the role of the putative Sp binding sites in the transcriptional activity of the GCK gene, co-transfection experiments in HepG2 cells were performed. Reporter constructs containing 5'-deletion analysis of the promoter fragment were introduced into HepG2 cells together with expression plasmids encoding the Sp family members Sp1 or Sp3. Co-transfection of Sp1 with reporter constructs equal or longer than pGK72 resulted in a three- to fivefold induction of the promoter activity relative to the basal activity of the corresponding promoter constructs. No enhancement of the promoter activity could be detected when the shortest construct (pGK21) was used. These results suggest that a functional Sp binding site may be located within 72 bp upstream of the transcription start site. Consistent with these results, no transactivation by Sp1

was observed when co-transfected with pGK606 Δ 288-21 reporter construct, which lacks the region between positions -288 and -21 (Fig. 3A). Sp3 alone did not affect the activity of any of the promoter fragments tested. However, co-transfection of a 1:1 mixture of expression vectors for Sp1 and Sp3 resulted in inhibition of the Sp1-dependent transactivation using pGK72 or longer reporter constructs. Since matches for Sp1 are found in the promoter sequences of mammalian *GCK* (Magnuson *et al.* 1989, Postic *et al.* 1995, Samson & Wong 2002), we also analyzed the effect of Sp1 and Sp3 on the rat *GCK* promoter. To this end, we co-transfected HepG2 cells with expression vectors for Sp1 and Sp3, and a reporter construct harboring a promoter sequence of rat *GCK* (pGK1418; positions -1418 bp to $+109$ bp relative to the transcription start site) that includes a putative Sp binding site (positions -441 to -433 ; Magnuson *et al.* 1989). As in *S. aurata*, Sp1 transactivated rat *GCK* promoter and Sp3 repressed Sp1-mediated transcription (Fig. 3A).

As Sp1 and Sp3 behaved functionally different and can compete for the same binding site, we investigated the potential function of Sp3 as a repressor of *S. aurata*

GCK promoter. Various amounts (0.8 – 3.2 μ g) of the expression vectors encoding Sp1 and Sp3 were co-transfected together with the pGK72 reporter construct into HepG2 cells. As shown in Fig. 3B, the promoter activity decreased in a dose-dependent manner when the cells were co-transfected with 0.8 μ g pRC-CMVSp1 and increasing amounts of pRC-CMVSp3. However, co-transfection with 0.8 μ g of pRC-CMVSp3 and increasing amounts of pRC-CMVSp1 resulted in a dose-dependent increase of promoter transcriptional activity. These results suggest that Sp1 and Sp3 compete for the same binding site in the *GCK* promoter.

Sp1 and Sp3 bind to the putative Sp binding site of *GCK* promoter

The shorter reporter construct that exhibited Sp1-mediated transactivation (pGK72) contains a GC-rich region at positions -53 to -44 relative to the transcription start site of *S. aurata* *GCK*. To show that Sp1 and Sp3 can indeed bind to the putative Sp binding site at position -53 to -44 , bandshift experiments were performed. Using probe GK-56/-37 (harboring

Figure 3 Effect of Sp1 and Sp3 on *S. aurata* *GCK* promoter transcription in HepG2 cells. (A) The cells were transfected with pGL3-Basic, the *S. aurata* *GCK* promoter constructs pGK1321, pGK606, pGK288, pGK72, pGK21, or pGK606 Δ 288-21, or the rat *GCK* promoter construct prGK1418, with or without expression plasmids encoding Sp1 or Sp3. (B) The cells were co-transfected with pGK72 and different amounts of pRC-CMV Sp1 and/or pRC-CMV Sp3. The promoter activity of the constructs alone was set at 1. The data represent the mean $\pm$ s.d. values of two independent duplicate experiments.

the putative *Sp* binding site) and HepG2 extracts prepared from cells overexpressing *Sp1*, one major shifted band could be observed. A DNA–protein complex with the same mobility was observed when a consensus *Sp1* probe was used (*Sp1-cons*). The shifted band was eliminated by competition with 200-fold molar excess of unlabeled *Sp1-cons* (Fig. 4A). Similar results were obtained using HepG2 extracts prepared from *Sp3* overexpressing cells (Fig. 4B). Specificity of *Sp1* and *Sp3* binding to the *Sp* box at positions –53 to –44 of *S. aurata GCK* was validated through supershift experiments using the labeled GK-56/-37 probe and *in vitro* translated *Sp1* and *Sp3*. *Sp1* and *Sp3* gave rise to complexes of similar mobility. In the presence of *Sp1* antibody, the complex observed with *Sp1* protein was partially supershifted. Similar results were obtained using a *Sp3* antibody in the presence of *Sp3* protein. A supershifted band of less mobility was observed when both anti-*Sp1* and anti-*Sp3* antibodies were included in a single reaction with *Sp1* and *Sp3* proteins (Fig. 4C). Altogether, these data confirm that both *Sp1* and *Sp3* bind the *Sp* box at positions –53 to –44 of *GCK*.

To test whether we could abolish formation of the DNA–protein complex by mutating the putative *Sp* binding site, we performed bandshift assays using HepG2 extracts of *Sp1* overexpressing cells and a labeled probe harboring positions –56 to –37 of the *GCK* gene in which the GC box was mutated (GK-56/-37mut*Sp*). As shown in Fig. 4D, the complex shifted using *Sp1-cons* and GK-56/-37 probes totally disappeared when the GK-56/-37mut*Sp* probe was used.

Mutating the *Sp* binding site abolishes transactivation by *Sp1*

The same mutations as described for the double-stranded oligonucleotide GK-56/-37mut*Sp* used in bandshift assays were introduced into the promoter constructs pGK288 and pGK72 to generate reporter constructs with a mutated *Sp* site (pGK288mut*Sp* and pGK72mut*Sp* respectively). Co-transfection experiments in HepG2 cells with the promoter constructs and expression vectors for *Sp1* and *Sp3* were performed to compare transactivation of the wild type and mutated reporter constructs. *Sp1* was not able to enhance the transcription of the constructs harboring the mutated *Sp* site (Fig. 5A). From these studies, we concluded that the *Sp* binding site at positions –53 to –44, relative to the major transcription start site of the *S. aurata GCK* promoter, is responsible for transactivation by *Sp1*. In addition, *Sp3* is a repressor of *Sp1*-mediated activation by competing for the same binding site.

The activating effect of *Sp1* on *GCK* transcription was also tested in nonliver-derived cells. Transient transfection of CHO and SBL cells with an expression plasmid encoding *Sp1* and the *S. aurata GCK* reporter constructs

pGK72 and pGK72mut*Sp* showed that the effect of *Sp1* was dependent on the cell line. Similarly, as in HepG2 cells, *Sp1* activated *GCK* transcription in CHO cells and did not transactivate a *GCK* reporter construct harboring a mutated *Sp* site at positions –53 to –44. In contrast to HepG2 and CHO cells, no effect of *Sp1* was observed in SBL cells (Fig. 5B).

Regulation of *Sp1* protein levels and *GCK* expression by insulin in liver of *S. aurata*

Although *Sp1* binding sites are common to many insulin-responsive genes and matches for *Sp1* are found in the promoter sequences of rat and mouse *GCK* (Magnuson *et al.* 1989, Postic *et al.* 1995, Samson & Wong 2002), the putative involvement of *Sp* proteins in transactivation of *GCK* has not been studied. To study the action of insulin and the role of *Sp1* and *Sp3* on *GCK* expression, we analyzed *GCK* mRNA abundance and protein levels of *Sp1* and *Sp3* in the liver of insulin-treated *S. aurata*. Analysis by northern blot revealed that *GCK* mRNA increased after insulin treatment in the piscine liver (Fig. 6A). Western blot analysis showed that insulin increased *Sp1* protein levels, whereas no effect on *Sp3* was observed (Fig. 6B).

Discussion

It is well known that insulin stimulates *GCK* expression in the liver. However, knowledge of the transcription factors implicated in control of *GCK* transcription has received limited attention and the molecular mechanism responsible for this effect remains unclear. In this study, we have isolated and characterized the promoter region of *GCK* from *S. aurata*. The genomic fragment isolated allowed us to identify a *cis*-element in the proximal region of the *GCK* promoter implicated in transactivation by *Sp1*. Additionally, we present evidence that supports a role for *Sp* proteins in insulin-dependent activation of *GCK* by insulin.

The 5'-flanking region of *S. aurata GCK* was cloned by chromosome walking. Mapping of the transcriptional start indicated the presence of a start site located 98 bp upstream relative to the translation start codon. Our localization of the transcription start site predicts a 2058 bp mRNA, which is consistent with the 2.1 kb transcript previously identified by northern blot (Caseras *et al.* 2000). The functionality of the putative promoter region of fish *GCK* was tested by transient transfection of HepG2 cells with fusion constructs of the 1397 bp isolated by chromosome walking and sequential 5' deletions of this fragment to the luciferase gene. We found that the promoter region within 288 bp upstream to the transcription start site is essential for transcriptional basal activity of *GCK* and thus

Figure 5 Effect of Sp1 and Sp3 on the promoter activity of *GCK* containing a mutated Sp box. (A) pGK288, pGK288mutSp, pGK72, and pGK72mutSp were co-transfected in HepG2 cells and with expression vectors encoding Sp1 and/or Sp3. (B) CHO and SBL cells were transfected with pGK72 and pGK72mutSp, and with or without an expression plasmid encoding Sp1. The luciferase activity of the reporter constructs alone was set at 1. The data represent the mean $\pm$ s.d. values of two independent duplicate experiments.

constitutes the core functional promoter of this gene. The high GC and GT content of this region led us to investigate a possible role of Sp proteins in the transcriptional activation of *GCK*.

GC and GT boxes are DNA-elements frequently present in many promoters and enhancers of genes that are under cell cycle regulation, hormonal activation, and developmental patterning. The Sp family of proteins comprises a large number of homologous transcription factors that can control transcriptional activity of target genes through binding to GC/GT boxes in their regulatory regions (Suske 1999, Bouwman & Philipsen 2002, Kaczynski *et al.* 2003). Sp proteins have been found in species ranging from invertebrates to humans, including fish (Baudler *et al.* 1997, Zhao *et al.* 2003). Sp family members contain three zinc fingers and glutamine- and serine/

threonine-rich *trans*-activation domains. Despite the high degree of structural homology, Sp proteins behave in a functionally different manner. Regulation of transcription via GC/GT boxes by Sp proteins is a tightly controlled complex process. Sp1 and Sp3 recognize and bind to the GC/GT boxes with identical affinity (Hagen *et al.* 1994). Sp1 is implicated in the activation of a large number of regulated and constitutively expressed genes, whereas Sp3 comprises an additional inhibitory domain and acts as an activator or as a repressor of Sp1-mediated activation depending on the promoter and cellular context (Suske 1999, Bouwman & Philipsen 2002, Kaczynski *et al.* 2003). Since Sp1 and Sp3 are present in the same cell types and compete for the same binding sites *in vivo*, the relative abundance of both transcription factors allows regulation of target genes. In the present study, we show

Figure 4 Electrophoretic mobility shift assay. Competition analysis using (A) Sp1 or (B) Sp3 overexpressing HepG2 extracts incubated with labeled oligonucleotides Sp1-cons (lanes 1–4) or GK-56/-37 (lanes 5–8). Lanes 1 and 5 contained no extract. Lanes 2 and 6 show binding of HepG2 extracts to labeled probes without competitor. Lanes 3, 7 and 4, 8 show competition with 10- and 200-fold molar excess respectively of unlabeled double-stranded competitor (Sp1-cons). (C) Gel shift with labeled probe GK-56/-37 using *in vitro* translated Sp1 (Sp1; lanes 1, 2, 5–7) and/or Sp3 (Sp3; lanes 3–7), and supershift performed with antibodies against Sp1 (Sp1 Ab; lanes 2, 5 and 7) and/or Sp3 (Sp3 Ab; lanes 4, 6, and 7). (D) Shift assays performed using HepG2 extracts incubated with labeled oligonucleotides Sp1-cons, GK-56/-37 or GK-56/-37mutSp (mutated Sp1 binding sequence). DNA–protein complexes and supershifted bands are indicated by arrows and open arrows respectively. FP, free probes.

Figure 6 Effect of insulin on *GCK* expression and immunodetectable Sp1 and Sp3 in the liver of *S. aurata*. (A) Representative northern blot of *GCK* mRNA levels in liver of fish after 6 h treatment with saline (control) or insulin (10 U/kg fish). The integrity and relative amounts of RNA loaded were checked and corrected by 18 S rRNA ethidium bromide staining on the same gel. (B) Western blotting analysis of Sp1 and Sp3 protein levels in liver of fish after 6 h treatment with saline (control) or insulin (10 U/kg fish).

that both Sp1 and Sp3 are capable of interacting specifically with the GC box located between -53 bp and -44 bp upstream of the transcription start site of *GCK*.

To determine the functional role of Sp1 and Sp3 on the *GCK* promoter, transient transfection studies were carried out in HepG2 cells. We demonstrate that Sp1 confers an activating signal through binding to the *GCK* promoter. Mutations in the GC box abolish binding of Sp1 to the mutated site. As a result Sp1 was no longer able to enhance the transcription of *GCK* in transient transfection experiments. These results clearly indicate that Sp1 is implicated in the transcriptional activation of *GCK* promoter. On the other hand, Sp3 did not drive reporter gene expression, and furthermore it behaved as a repressor of Sp1-mediated transactivation by competing for the same binding site in the *GCK* promoter. Interestingly, transfection experiments performed with a promoter reporter construct of rat *GCK* harboring a putative Sp binding site gave rise to similar results as with *S. aurata GCK*, suggesting that regulation of *GCK* transcription by Sp proteins may not be a specific effect for fish species. On the other hand, an elevated Sp1 to Sp3 ratio resulted in stimulation of the *S. aurata GCK* promoter activity, whereas the gene expression was inhibited when the Sp3 to Sp1 ratio was high. In support of this model, it has been shown that a mutant of Sp3 lacking the DNA-binding domain is not able to suppress Sp1-mediated transactivation of the artificial promoter BCAT-2 (Hagen *et al.* 1994). In the present study, most of the transfection experiments were performed in a liver-derived cell line (HepG2). However, we also analyzed whether the activating effect of Sp1 on *S. aurata GCK* is cell type-dependent. As in HepG2 cells, Sp1 transactivated *GCK* transcription in CHO cells, whereas it did not cause any effect on SBL cells. Dependence of the cell lines to Sp1 action may result from cell-type dependent features such as post-translational regulation of Sp1 and the Sp1/Sp3 ratio, which in turn may confer specificity of *GCK* tissue expression.

In addition to the steady-state levels of Sp1, hormones can alter its binding and transcriptional activity through post-translational modifications. Insulin stimulates synthesis, *O*-glycosylation and phosphorylation of Sp1 (Bouwman & Philipsen 2002, Samson & Wong 2002, Chu & Ferro 2005, Majumdar *et al.* 2006). The fact that Sp1 binding sites are found in the insulin-responsive regions of numerous genes suggests that Sp1 is involved in mediating insulin action. In particular, Sp1 plays an important role in the transcription of several glycolytic enzymes, such as pyruvate kinase and aldolase (Hermisse *et al.* 1996, Schafer *et al.* 1996, Netzker *et al.* 1997). Here, we found that insulin increased *GCK* mRNA levels in the liver of *S. aurata*. These results are consistent with previous studies in *S. aurata* that indicate decreased *GCK* mRNA in starved fish, postprandial rise of *GCK* expression, and increased *GCK* mRNA abundance in liver of fish fed high carbohydrate diets (Caseras *et al.* 2000, 2002). Indeed, we recently showed that insulin stimulates the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and down-regulates the gluconeogenic glucose-6-phosphatase catalytic subunit in the liver of this species (Salgado *et al.* 2004, Metón *et al.* 2006). In addition to stimulation of hepatic *GCK* expression, we found that insulin increased Sp1 protein levels in *S. aurata*, whereas Sp3 remained unaffected. These results suggest that increased Sp1/Sp3 ratio in insulin-treated animals may enhance *GCK* transcription.

In conclusion, the present study has shown that Sp1 binds to and transactivates *GCK* promoter, and points to involvement of Sp proteins in insulin regulation of *GCK* expression.

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References

- Baudler M, Duschl J, Winkler C, Scharlt M & Altschmied J 1997 Activation of transcription of the melanoma inducing Xmrk oncogene by a GC box element. *Journal of Biological Chemistry* **272** 131–137.
- Bouwman P & Philipsen S 2002 Regulation of the activity of Sp1-related transcription factors. *Molecular and Cellular Endocrinology* **195** 27–38.
- Caseras A, Metón I, Fernández F & Baanante IV 2000 Glucokinase gene expression is nutritionally regulated in liver of gilthead sea bream (*Sparus aurata*). *BBA – Gene Structure and Expression* **1493** 135–141.
- Caseras A, Metón I, Vives C, Egea M, Fernández F & Baanante IV 2002 Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (*Sparus aurata*). *British Journal of Nutrition* **88** 607–614.
- Cha JY, Kim HI, Im SS, Li TZ & Ahn YH 2001 HNF1 and/or HNF3 may contribute to the tissue specific expression of glucokinase gene. *Experimental and Molecular Medicine* **33** 59–63.
- Chauhan J & Dakshinamurti K 1991 Transcriptional regulation of the glucokinase gene by biotin in starved rats. *Journal of Biological Chemistry* **266** 10035–10038.
- Christiansen DC & Klungsoyr L 1987 Metabolic utilization of nutrients and the effects of insulin in fish. *Comparative Biochemistry and Physiology* **88B** 701–711.
- Chu S & Ferro TJ 2005 Sp1: regulation of gene expression by phosphorylation. *Gene* **348** 1–11.
- Cowey CB & Walton MJ 1989 Intermediary metabolism. In *Fish Nutrition*, pp 260–321. Ed JE Halver. San Diego: Academic Press.
- Foretz M, Guichard C, Ferre P & Foufelle F 1999 Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. *PNAS* **96** 12737–12742.
- Graham FL & Van der Eb AJ 1973 A new technique for assay of infectivity of human adenovirus 5 DNA. *Virology* **52** 456–467.
- Gregori C, Guillet-Deniau I, Girard J, Decaux JF & Pichard AL 2006 Insulin regulation of glucokinase gene expression: evidence against a role for sterol regulatory element binding protein 1 in primary hepatocytes. *FEBS Letters* **580** 410–414.
- Hagen G, Müller S, Beato M & Suske G 1994 Sp1-mediated transcriptional activation is repressed by Sp3. *EMBO Journal* **13** 3843–3851.
- Hansmann F, Mordier S & Iynedjian PB 2006 Insulin induction of glucokinase and fatty acid synthase in hepatocytes: analysis of the roles of sterol-regulatory-element-binding protein-1c and liver X receptor. *Biochemical Journal* **399** 275–283.
- Heinemeyer T, Chen X, Karas H, Kel AE, Kel OV, Liebich I, Meinhardt T, Reuter I, Schacherer F & Wingender E 1999 Expanding the TRANSFAC database towards an expert system of regulatory molecular mechanisms. *Nucleic Acids Research* **27** 318–322.
- Hermisse U, Schafer D, Netzer R & Brand K 1996 The aldolase A promoter in proliferating rat thymocytes is regulated by a cluster of SP1 sites and a distal modulator. *Biochemical and Biophysical Research Communications* **225** 997–1005.
- Iynedjian PB 1993 Mammalian glucokinase and its gene. *Biochemical Journal* **293** 1–13.
- Iynedjian PB 1998 Identification of upstream stimulatory factor as transcriptional activator of the liver promoter of the glucokinase gene. *Biochemical Journal* **333** 705–712.
- Iynedjian PB, Jotterand D, Nouspikel T, Asfari M & Pilot PR 1989 Transcriptional induction of glucokinase gene by insulin in cultured liver cells and its repression by the glucagon-cAMP system. *Journal of Biological Chemistry* **264** 21824–21829.
- Iynedjian PB, Marie S, Wang H, Gjinovci A & Nazaryan K 1996 Liver-specific enhancer of the glucokinase gene. *Journal of Biological Chemistry* **271** 29113–29120.
- Kaczynski J, Cook T & Urrutia R 2003 Sp1- and Kruppel-like transcription factors. *Genome Biology* **4** 206.
- Kim SY, Kim HI, Park SK, Im SS, Li T, Cheon HG & Ahn YH 2004 Liver glucokinase can be activated by peroxisome proliferator-activated receptor-gamma. *Diabetes* **53** S66–S70.
- Lannoy VJ, Decaux JF, Pierreux CE, Lemaigre FP & Rousseau GG 2002 Liver glucokinase gene expression is controlled by the onecut transcription factor hepatocyte nuclear factor-6. *Diabetologia* **45** 1136–1141.
- Magnuson MA 1990 Glucokinase gene structure. *Diabetes* **39** 523–527.
- Magnuson MA & Shelton KD 1989 An alternate promoter in the glucokinase gene is active in the pancreatic beta cell. *Journal of Biological Chemistry* **264** 15936–15942.
- Magnuson MA, Andreone TL, Printz RL, Koch S & Granner DK 1989 Rat glucokinase gene: structure and regulation by insulin. *PNAS* **86** 4838–4842.
- Majumdar G, Harrington A, Hungerford J, Martinez-Hernandez A, Gerling IC, Raghow R & Solomon S 2006 Insulin dynamically regulates calmodulin gene expression by sequential O-glycosylation and phosphorylation of Sp1 and its subcellular compartmentalization in liver cells. *Journal of Biological Chemistry* **281** 3642–3650.
- Metón I, Egea M & Baanante IV 2003 New insights into the regulation of hepatic glucose metabolism in fish. In *Recent Research Developments in Biochemistry*, vol 4, pp 125–149. Ed SG Pandalai. Kerala, India: Research Signpost.
- Metón I, Egea M, Anemaet IG, Fernández F & Baanante IV 2006 Sterol regulatory element binding protein-1a transactivates 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene promoter. *Endocrinology* **147** 3446–3456.
- Moon TW 2001 Glucose intolerance in teleost fish: fact or fiction? *Comparative Biochemistry and Physiology* **29B** 243–249.
- Narkiewicz MR, Iynedjian PB, Ferre P & Girard J 1990 Insulin and triiodothyronine induce glucokinase mRNA in primary cultures of neonatal rat hepatocytes. *Biochemical Journal* **271** 585–589.
- Netzer R, Weigert C & Brand K 1997 Role of the stimulatory proteins Sp1 and Sp3 in the regulation of transcription of the rat pyruvate kinase M gene. *European Journal of Biochemistry* **245** 174–181.
- Postic C, Niswender KD, Decaux JF, Parsa R, Shelton KD, Gouhot B, Pettepher CC, Granner DK, Girard J & Magnuson MA 1995 Cloning and characterization of the mouse glucokinase gene locus and identification of distal liver-specific DNase I hypersensitive sites. *Genomics* **29** 740–750.
- Ribaux P, Gjinovci A, Sadowski HB & Iynedjian PB 2002 Discrimination between signaling pathways in regulation of specific gene expression by insulin and growth hormone in hepatocytes. *Endocrinology* **143** 3766–3772.
- Roth U, Jungermann K & Kietzmann T 2002 Activation of glucokinase gene expression by hepatic nuclear factor 4alpha in primary hepatocytes. *Biochemical Journal* **365** 223–228.
- Roth U, Curth K, Unterman TG & Kietzmann T 2004 The transcription factors HIF-1 and HNF-4 and the coactivator p300 are involved in insulin-regulated glucokinase gene expression via the phosphatidylinositol 3-kinase/protein kinase B pathway. *Journal of Biological Chemistry* **279** 2623–2631.
- Salgado MC, Meton I, Egea M & Baanante IV 2004 Transcriptional regulation of glucose-6-phosphatase catalytic subunit promoter by insulin and glucose in the carnivorous fish, *Sparus aurata*. *Journal of Molecular Endocrinology* **33** 783–795.

- Samson SL & Wong NC 2002 Role of Sp1 in insulin regulation of gene expression. *Journal of Molecular Endocrinology* **29** 265–279.
- Sawka-Verhelle D, Tartare-Deckert S, Decaux JF, Girard J & Van Obberghen E 2000 Stat 5B, activated by insulin in a Jak-independent fashion, plays a role in glucokinase gene transcription. *Endocrinology* **141** 1977–1988.
- Schafer D, Hamm-Kunzelmann B, Hermfisse U & Brand K 1996 Differences in DNA-binding efficiency of Sp1 to aldolase and pyruvate kinase promoter correlate with altered redox states in resting and proliferating rat thymocytes. *FEBS Letters* **391** 35–38.
- Suske G 1999 The Sp-family of transcription factors. *Gene* **238** 291–300.
- Wilson RP 1994 Utilization of dietary carbohydrate by fish. *Aquaculture* **124** 67–80.
- Zhang W, Patil S, Chauhan B, Guo S, Powell DR, Le J, Klotsas A, Matika R, Xiao X, Franks R *et al.* 2006 FoxO1 regulates multiple metabolic pathways in the liver: effects on gluconeogenic, glycolytic, and lipogenic gene expression. *Journal of Biological Chemistry* **281** 10105–10117.
- Zhao J, Cao Y, Zhao C, Postlethwait J & Meng A 2003 An SP1-like transcription factor Spr2 acts downstream of Fgf signaling to mediate mesoderm induction. *EMBO Journal* **22** 6078–6088.

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