Transactivation of cytosolic alanine aminotransferase gene promoter by p300 and c-Myb

Ida G Anemaet, Juan Diego González, María C Salgado, Marina Giralt, Felipe Fernández¹, Isabel V Baanante and Isidoro Metón

Departament de Bioquímica i Biologia Molecular, Facultat de Farmàcia, Universitat de Barcelona, Joan XXIII s/n, 08028 Barcelona, Spain

¹Departament d'Ecologia, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08028 Barcelona, Spain

(Correspondence should be addressed to I Metón; Email: imeton@ub.edu)

Abstract

Alanine aminotransferase (Alt) provides a molecular link between carbohydrate and amino acid metabolism. In the cell context, the predominant Alt isozyme is located in the cytosol. To gain insight into the transcriptional regulation of the cytosolic *alt* gene (*calt*), we cloned and characterized the *calt* promoter from gilthead sea bream (*Sparus aurata*). Transient transfection of sea bass larvae cells with deleted *calt* promoter constructs and electrophoretic mobility shift assays allowed us to identify p300 and c-Myb as new factors in the transcriptional regulation of *calt* expression. Transfection studies carried out with an acetylase-deficient mutant p300 (p300DY) revealed that the acetyltransferase activity of p300 is essential for the p300-mediated transcriptional activation of *S. aurata calt*. We had previously found up-regulation of liver cAlt2, an alternatively spliced isoform of *calt*, under gluconeogenic conditions and in streptozotocin (STZ)-treated *S. aurata*. Quantitative RT-PCR assays showed that increased p300 and c-Myb mRNA levels in the liver of starved *S. aurata* contribute to enhancing the transcription of cAlt2. Consistently, the administration of insulin decreased both p300 and c-Myb expression. The mRNA levels of p300 and c-Myb were also analyzed in the liver of STZ-induced diabetic *S. aurata*. Treatment with STZ increased the expression of p300, whereas it decreased c-Myb. Our findings suggest an involvement of p300 and c-Myb in up-regulation of cAlt2 in the liver of *S. aurata* under starvation. In addition, these results provide evidence for a role of p300 in diabetes.

Journal of Molecular Endocrinology (2010) **45**, 119–132

Introduction

Alanine aminotransferase (Alt, glutamate pyruvate transaminase, EC 2.6.1.2) is a pyridoxal enzyme that catalyzes the reversible transamination between α -L-alanine and α -ketoglutarate to form pyruvate and L-glutamate. By mediating the conversion of these four major intermediate metabolites, Alt provides a molecular link between carbohydrate and amino acid metabolism.

Alt activity in serum is clinically used as a marker of liver integrity or hepatocellular damage; Alt activity is significantly raised in a variety of hepatic injury conditions, including hepatitis, cirrhosis, and drug hepatotoxicity (Dufour *et al.* 2000, Glinghammar *et al.* 2009). Increased Alt activity is observed in rat liver after high protein intake, fasting, and administration of cortisol, and it is also observed in diabetic animals (Rosen *et al.* 1959). Furthermore, β -adrenergic agonists, such as epinephrine, glucagon, and norepinephrine, induce hepatic Alt activity in rats (Begum & Datta 1992).

Recent studies have associated high levels of Alt activity with pathologies such as obesity, muscle diseases, type 1 diabetes, coronary atherosclerotic

disease, and metabolic syndrome (Nathwani *et al.* 2005, Wannamethee *et al.* 2005, West *et al.* 2006, Iacobellis *et al.* 2007, Schindhelm *et al.* 2007). In addition, a rise of serum Alt activity within the reference values in healthy populations is considered a marker for the development of insulin resistance and type 2 diabetes (Vozarova *et al.* 2002, Hanley *et al.* 2004, West *et al.* 2006, Sattar *et al.* 2007).

Two Alt isoforms, ALT1 and ALT2, each encoded by a different gene, have been cloned from humans and mice (Sohocki *et al.* 1997, Yang *et al.* 2002, Jadhao *et al.* 2004). Human *ALT1* gene was cloned based on the amino acid sequence of liver cytosolic ALT (cAlt; Ishiguro *et al.* 1991), and mapped to human chromosome 8q24.3 (Sohocki *et al.* 1997). Yang *et al.* (2002) cloned another human ALT isozyme, ALT2, and mapped this gene to chromosome 16q12.1. Mouse *Alt1* and *Alt2* genes were mapped to chromosomes 15 and 8 respectively (Jadhao *et al.* 2004). Western blotting indicates that human ALT1 is mainly expressed in liver, skeletal muscle, kidney, and, to a lesser extent, in heart, whereas ALT2 is mainly expressed in heart and skeletal muscle. Immunoprecipitation assays demonstrate that ALT1 is the

major isoform expressed in human liver and quantitatively the most important Alt isozyme contributing to basal circulating levels of Alt activity in humans (Lindblom *et al.* 2007).

The hepatic activity of Alt is considered a good indicator of protein utilization in fish (Fynn-Aikins *et al.* 1995, Sanchez-Muros *et al.* 1998, Metón *et al.* 1999). In the liver of gilthead sea bream (*Sparus aurata*), Alt activity decreases following starvation, and is more sensitive than aspartate aminotransferase to changes in diet composition and ration size (Metón *et al.* 1999, Fernández *et al.* 2007). We cloned and reported the subcellular localization of *S. aurata* liver cytosolic (cAlt, homologous to mammalian ALT1) and mitochondrial (mAlt, homologous to mammalian ALT2) Alt isoforms. Expression of mAlt as a C- or N-terminal fusion with the enhanced green fluorescent protein in CHO cells allowed us to demonstrate that the mAlt N-terminus contains a mitochondrial targeting signal that directs the protein into the mitochondria. The primary structure of mAlt was the first amino acid sequence reported for a mitochondrial Alt in animals (Metón *et al.* 2004). A recent study by Yang *et al.* (2009) has indicated that the rat liver ALT2 isoform is likely to contain an N-terminal mitochondrial targeting signal, and has showed by western blotting that ALT2 is present in mitochondrial fractions. In a recent report, we showed that the *S. aurata calt* gene generates two cytosolic transcripts by alternative splicing: cAlt1 and cAlt2. The kinetic characterization and *in vivo* analysis of cAlt1 and cAlt2 expression under various metabolic conditions indicate that the proteins exert distinct roles in the liver of *S. aurata*: cAlt2 expression occurs in liver under gluconeogenic conditions, while cAlt1 is predominant during postprandial utilization of dietary nutrients (Anemaet *et al.* 2008).

Present understanding of control of Alt expression at the transcriptional level is limited. Gray *et al.* (2007) reported a reduction of ALT1 mRNA levels in the liver of Krüppel-like factor 15-deficient mice. Transfection experiments showed that peroxisome proliferator-activated receptor α (PPAR α) agonist and fenofibric acid induce specifically the human *ALT1* promoter in Huh-7 cells, whereas the human *ALT2* promoter is unaffected. ChIP analyses confirmed the binding of PPAR α and PPAR γ to the -574 site in the proximal human *ALT1* promoter (Thulin *et al.* 2008).

Since up-regulation of cAlt2 actually occurs in the liver of streptozotocin (STZ)-induced diabetic *S. aurata* (Anemaet *et al.* 2008), and in order to unravel the basis that governs *calt* transcription, in the present study, we isolated and characterized the *S. aurata calt* promoter. We show that p300 and c-Myb transactivate the *calt* promoter, and provide evidence that p300 may be involved in the pathogenesis of diabetes.

Materials and methods

Experimental animals

Gilthead sea bream (*S. aurata*) fingerlings were obtained from Tinamenor SA (Cantabria, Spain). Fish were maintained in 260-L aquaria supplied with recirculating seawater at 20 °C as described (Fernández *et al.* 2007). Fish were weighed every week to adjust for the quantity of diet supplied for each experiment. Fish were fed daily (1000 h) at 2.5% body weight with a diet containing 46% protein, 9.3% carbohydrates, 22% lipids, 10.6% ash, 12.1% moisture, and 21.1 kJ/g gross energy. The effect of starvation on the expression of p300 and c-Myb was analyzed in the liver of fish deprived of food for 20 days and in fed fish. To study regulation of p300 and c-Myb expression by insulin and STZ, the animals were divided into three groups. Twenty-two hours before sampling, two groups of fish received an i.p. injection with vehicle (0.01 M sodium citrate, pH 4.5), and another group was i.p. injected with STZ (600 mg/kg fish; Sigma-Aldrich). Six hours before sampling, one group of fish previously treated with vehicle received an i.p. dose of bovine insulin (10 units/kg fish; Sigma-Aldrich). Fish were killed by cervical section. Tissue samples were dissected out, immediately frozen in liquid nitrogen, and kept at -80 °C till use. To avoid stress, fish were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester; 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 cALT promoter from *S. aurata* by chromosome walking

To isolate the 5'-flanking region of *calt* gene from *S. aurata*, two rounds of genome walking were performed with the Universal GenomeWalker Kit (Clontech). Four genomic libraries were obtained by blunt-end digestion of *S. aurata* genomic DNA with DraI, EcoRV, PvuII, and StuI (Metón *et al.* 2006). A primary PCR was performed on each library with the gene-specific primer IGAL0502 (Table 1) and the adaptor primer AP-1 provided by the kit. A nested PCR was performed on the primary PCR product, using the gene-specific primer IGAL0501 (Table 1) and the adaptor primer AP-2 provided by the kit. The longer amplification product, a single band of ~600 bp (named s496), was obtained from the StuI library, and purified using the High Pure PCR Purification Kit (Hoffmann-La Roche). According to the DNA sequence obtained by the first round of genome walking, we designed the gene-specific primers IGAL0405 and IGAL0305 (Table 1) to further isolate the 5'-flanking region of *calt* by PCR, following the same amplification conditions. The nested PCR carried out

Table 1 *Sparus aurata* *calt*-specific primers used in the present study

	Sequence 5'–3'	Maps to
Primers		
IGAL0305	CAGCTTTTGAGAAATTAGATATTAACCTGC	–417 to –445
IGAL0405	TTGGCCACATAAAGGATAGTTCAGC	–396 to –420
IGAL0501	GTTGTTTTCCGCCGCTGTCACCTCTGGACTG	172 to 141
IGAL0502	GTTCGCCCGCCTGGTGGGACATGTTG	217 to 193
IGAL0505	CCTTAAAAAATGTAATTACCTGACAGG	–496 to –523
IGAL0605	CCGGTACCATCAAATGTCTTTCCCAAGCCC	–2096 to –2075
IGAL1007	GGAAGCTTGTGTTTTTGTGTTGTTGTTGTTG	196 to 172
IGAL1101	GGGCTACCGAGCGTGGCGTGGCGGTTGGGTGGG	–57 to –33
IGAL1102	GGGGTACCGAGCGTGGCGTGGCGCGCGGGTGGGAATATGAGCAGC- ACGAG	–57 to –16
IGAL1103	GGGAATTCCAGCCGCGCGAGCAGGAGTGC GCGGAAGG	–91 to –55
IGAL12F	CTTTCAGATATCAGTCCAGAGGGTGACAGCGGCGGAAAACAACA- ACAACAACAACAACAAAAACAACA	130 to 195
IGAL13R	AGCTTGTGTTTGTGTTGTTGTTGTTGTTGTTGTTTCCGCCGCTGTC- ACCCCTCTGGACTGATATCTGAAAGGTAC	195 to 130
IGAL3001	GGGAATTCCAGCCAACCGCGAGCAAAAAAGCGCGGAAGGCGTGGC- GTGG	–90 to –45
IGALHND	GGAAGCTTGAAGGAGAGGTTGAGGAGTG	+126 to +107
IGALSAC	GGGAGCTCCTGCCTATCACTTGCATT	–495 to –477
<i>calt</i> –91/–51	GAATTCAGCCAACCGCGAGCAGGAGTGC GCGGAAGGCGTG	–91 to –51
<i>calt</i> –66/–27	GTGCGCGGAAGGCGTGGCGTGGCGGTTGGGTGGGAATATGT	–66 to –27
<i>calt</i> –57/–27	AGGCGTGGCGTGGCGGTTGGGTGGGAATATGG	–57 to –27
<i>calt</i> –41/+6	TTGGGTGGGAATATGAGCAGCACGAGAGGCGCGCGAGAGGCACAAC	–41 to +5
<i>calt</i> –91/–51mutp300	GAATTCAGCCAACCGCGAGCAAAAAAGCGCGGAAGGCGTG	–91 to –51
<i>calt</i> –91/–59mutMyb1	GAATTCAGCCGCGCGAGCAGGAGTGC GCGGG	–91 to –59
<i>calt</i> –57/–27mutMyb2	AGGCGTGGCGTGGCGCGCGGGTGGGAATATGG	–57 to –27

The following primers contain restriction sites (underlined): IGAL0605 (KpnI), IGAL1007 (HindIII), IGAL1101 (KpnI), IGAL1102 (KpnI), IGAL1103 (EcoRI), IGAL3001 (EcoRI), IGALHND (HindIII), and IGALSAC (SacI). IGAL12F and IGAL13R contain protruding ends resulting from restriction with HindIII and KpnI respectively (underlined). Bold and double underlined letters indicate site-directed mutations in primers IGAL1102, IGAL1103, IGAL3001, *calt* –91/–51mutp300, *calt* –91/–59mutMyb1, and *calt* –57/–27mutMyb2. For double-stranded oligonucleotides *calt* –91/–51, *calt* –66/–27, *calt* –57/–27, *calt* –41/+6 and *calt* –91/–51mutp300, *calt* –91/–59mutMyb1, and *calt* –57/–27mutMyb2 used in gel shift experiments, only the forward primers are shown. Mapping positions for IGAL0501, IGAL0502, IGAL1007, IGAL12F, and IGAL13R are calculated from *S. aurata* *calt*2 cDNA. For other primers, mapping positions are calculated from *S. aurata* *calt* gene relative to the transcription start site.

on the EcoRV library gave rise to a band of ~1600 bp (s1603). The DNA fragments s496 and s1603 were ligated into pGEM-T Easy (Promega) to generate pGEM496 and pGEM1603 respectively. 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).

Construction of reporter gene plasmids

The reporter construct pGalt496 was generated by PCR using pGEM496 as a template and oligonucleotides IGALSAC (with a 5'-anchor sequence containing a SacI site; Table 1) and IGALHND (with a 5'-anchor sequence containing a HindIII site; Table 1). The PCR product was digested with SacI/HindIII and ligated into the promoterless luciferase reporter plasmid pGL3-Basic (Promega), previously restricted with the same enzymes. The pGalt2096 construct was originated by ligation of s1603 fragment into pGalt496. To this end, a PCR using pGEM1603 as a template and the

primer pair IGAL0505/IGAL0605 (Table 1) was performed to add a 5'-KpnI anchor to the s1603 fragment. The resulting product was filled-in with Klenow enzyme, digested with KpnI, and ligated into pGalt496, previously digested with SacI, filled-in with Klenow, and restricted with KpnI. The reporter constructs pGalt399, pGalt173, pGalt89, pGalt12, and pGalt2096Δ89–11 were produced by chewing back, fill-in, and self-ligation of pGalt2096 after digestion with MisI/KpnI, Pfl23II/KpnI, EcoRI/KpnI, Paul/KpnI, and EcoRI/Paul respectively. To obtain the pGalt57 reporter construct, a PCR was performed using oligonucleotides IGAL1101/IGALHND and pGalt2096 as a template. The PCR product was digested with KpnI/HindIII and ligated to the main fragment resulting from digestion of pGalt2096 with the same enzymes. The pGalt173mutp300 construct was generated by PCR with the primer pair IGAL3001/GLprimer2 (Promega) using pGalt173 as a template. The amplified product was digested with EcoRI/HindIII and ligated to the longer DNA fragment obtained after restriction of pGalt173 with the

same enzymes. The reporter construct pGalt173mut-Myb1 was obtained by PCR, using the primer pair IGAL1103/IGALHND and pGalt2096 as a template. The PCR product was digested with EcoRI/HindIII and ligated to the main fragment resulting from restriction of pGalt173 with the same enzymes. To generate pGalt57mutMyb2, a PCR was performed with the primer pair IGAL1102/IGALHND and pGalt2096 as a template. The PCR product was restricted with KpnI/HindIII and ligated to the main fragment resulting from digestion of pGalt2096 with the same enzymes. All constructs were verified by cycle sequencing.

Cell transfection and luciferase assay

Sea bass larvae (SBL) cells (Castric 1984) were grown at 20 °C in Modified Eagle's Medium (MEM), supplemented with 10% FCS, 100 IU/ml penicillin, and 100 µg/ml STZ. Transfection experiments on SBL cells grown at 50–60% confluence were performed with FuGENE6 Transfection Reagent (Hoffmann-La Roche) on 6-well plates. Cells were transfected with 0.8 µg of the reporter construct and, when necessary, 100 ng of expression plasmids encoding p300, p300DY, or c-Myb. To correct for variations in transfection efficiency, 200 ng pCMVβ was included in each transfection. To ensure equal DNA amounts, empty plasmids were added in each transfection. The cells were harvested 48 h later, washed in PBS, and incubated for 15 min in 300 µl of cell culture lysis reagent (Promega). After removal of cell debris by centrifugation at 10 000 *g* for 15 s, luciferase activity was measured in 5 µl of the supernatant after the addition of 25 µl of luciferase assay reagent (Promega). Peak light emission was recorded on a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). β-Galactosidase activity in the clear lysate was measured as previously described (Metón *et al.* 2006). Expression plasmids encoding rat p300 (pCMV-p300), a mutant form of p300 lacking endogenous acetyltransferase activity (pCMV-p300DY; Ito *et al.* 2001), and mouse c-Myb (pCMV-cMyb; Oelgeschläger *et al.* 1995) were kindly provided by Dr D Haro (Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona, Barcelona, Spain), Dr Tso-Pang Yao (Department of Pharmacology and Cancer Biology, Duke University, USA), and Dr Bernard Lüscher (Institut für Molekularbiologie, Medizinische Hochschule Hannover, Hannover, Germany) respectively.

Electrophoretic mobility shift assay

Double-stranded oligonucleotides used in gel shift experiments were (only the forward oligonucleotide is shown) p300-cons (5'-CGCACGCGGCAGGGAG-TGAGTGGTAGTGCC-3'), Myb-cons (5'-GGTAACGG-TTCCCAACGGCGGTTGGGGGTAACGGTTGGT-3'),

calt-91/-51, calt-66/-27, calt-57/-27, calt-41/+6, calt-91/-51mutp300, which contains a mutated p300 response element, and calt-91/-59mutMyb1 and calt-57/-27mutMyb2, both containing mutated c-Myb putative sites (Table 1). After 30 min of incubation at 37 °C in the presence of terminal transferase (Hoffmann-La Roche) in a 20 µl reaction, 200 pmol of the double-stranded oligonucleotides were 3' end labeled with digoxigenin-11-ddUTP. The reaction was stopped by adding 2 µl of 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 (DTT), 1% Tween 20, 150 mM KCl, 1 µg of non-specific competitor poly [d(I-C)] (Hoffmann-La Roche), nuclear extracts of SBL cells overexpressing p300 or c-Myb, and the labeled probe. The DNA-protein complexes were electrophoresed at 4 °C on 5% polyacrylamide gel, with 0.5× Tris-borate-EDTA as buffer. DNA was then transferred by contact blotting (2 h at room temperature) to Nytran membranes (Schleicher & Schuell, Keene, NH, USA). DNA was cross-linked to membranes by u.v. irradiation for 3 min. Labeled probes were immunodetected with anti-digoxigenin conjugated with alkaline phosphatase (Hoffmann-La Roche), with CDP-Star (Hoffmann-La Roche) as a chemiluminescent substrate. Membranes were exposed to Hyperfilm ECL (Amersham). For competition experiments, SBL nuclear extracts were pre-incubated for 30 min with a 5- and 20-fold molar excess of unlabeled double-stranded p300-cons or Myb-cons oligonucleotides. For supershift assays, nuclear proteins extracted from p300- or c-Myb-overexpressing SBL cells were incubated in the presence of anti-p300 or anti-c-Myb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at room temperature before the addition of the probe.

Nuclear extracts

Nuclear extracts were prepared from SBL cells. Cells were grown to near confluence, washed, and scraped into 1.5 ml of cold PBS. All the following steps were performed at 4 °C. The cells were pelleted by centrifugation for 10 s at 1000 *g* and resuspended in 400 µl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). Following incubation for 10 min, cells were vortexed for 10 s. Samples were centrifuged for 10 s, and the pellet was resuspended in 20 µl of cold buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) and incubated for 20 min for high salt extraction. Cell debris was removed by centrifugation for 2 min. The supernatant fraction was aliquoted and stored at -80 °C.

Quantitative RT-PCR

Total RNA was extracted from *S. aurata* liver by the Total Quick RNA Cells and Tissues kit (Talent, Trieste, Italy). Five micrograms of total RNA were reverse transcribed by incubation with 5× First Strand Buffer (Invitrogen), 10 mM DTT, 0.5 mM dNTPs, 6.25 ng/μl random hexamers, 40 U/μl rRNasin (Promega), and 200 U/μl M-MLV RT (Invitrogen) for 1 h at 37 °C. The cDNA product was used for subsequent real-time quantitative PCR. The mRNA levels of p300 and c-Myb were determined in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 0.4 μM of each primer, 10 μl of SYBR Green (Applied Biosystems), and 1.6 μl of the diluted cDNA mixture in a final volume of 20 μl. Primer pairs used were IMP303 (5'-GAAC-CAGGTGATGAATGGCTCC-3')/IMP304 (5'-CTTGGT-CATCGCTCCAGGTTGC-3') for p300; and MG0907 (5'-CGTTGGTCCGTGATTGCTAA-3')/MG0908 (5'-CT-CGTAGATGATTCCGGTCTC-3') for c-Myb. Oligonucleotides MC17 (5'-CGTCCACCGCAAATGCTTC-3') and MC18 (5'-GTTGTTGGGCGTTGGTTGG-3') were used to amplify β-actin from all the samples on each plate as a housekeeping gene to normalize expression levels of targets between different samples and to monitor assay reproducibility. Variations in gene expression were calculated by the standard $\Delta\Delta C_t$ method. Oligonucleotides IMP303, IMP304, MG0907, MG0908, MC17, and MC18 were designed from *S. aurata* p300, c-Myb, and β-actin cDNA fragments (GenBank accession numbers GU441458, FJ797059 and X89920).

Statistical analysis

Data were analyzed by one-factor ANOVA using a computer program (StatView, SAS Institute, Cary, NC, USA). Differences were determined by Fisher's protected least significant difference multiple range test.

Results

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

To clone and characterize the promoter region of *calt* gene from *S. aurata*, chromosome walking was performed on genomic DNA isolated from this species. To this end, *calt*-specific oligonucleotides were designed from the previously cloned *S. aurata* *calt2* cDNA (Anemaet *et al.* 2008). A first round of genome walking resulted in the specific amplification of a single fragment of ~600 bp (here named s496) from the *Stu*I library, which corresponds to positions -496/+139 relative to the previously determined transcription start site (Metón *et al.* 2004). The s496 fragment was isolated and cloned into pGEM-T Easy to generate pGEM496. On sequencing, this fragment was completely homologous with the 139 bases at the 5' end of *S. aurata* *calt2* cDNA (Anemaet *et al.* 2008).

A second round of chromosome walking on *S. aurata* genomic DNA was performed, using primers designed from the s496 fragment, to further isolate the 5'-flanking *calt* region. The longer amplification product, an ~1600 bp single fragment (s1603), was obtained from the *Eco*RV library. This fragment was isolated and ligated into pGEM-T Easy to generate pGEM1603. After sequencing, this fragment showed complete homology with the 51 bases at the 5' end of the product obtained by the first round of chromosome walking. Altogether, two consecutive rounds of chromosome walking allowed us to isolate 2235 bp from the 5'-flanking region of *S. aurata* *calt*. This fragment comprises positions -2096 to +139 relative to the transcription start site: its nucleotide sequence was submitted to the DDBJ/EMBL/GenBank Databases under the accession number DQ114991 (Fig. 1).

Figure 1 Sequence analysis of the 5'-flanking region of the *S. aurata* *calt* gene. The sequence upstream from the transcription start site, isolated by chromosome walking, is shown in capital letters. Putative binding sites for transcription factors in s496 fragment are boxed. The transcription start site is indicated by an arrow. The Inr-like element is underlined. The translation start codon for cAlt2 is in bold and double-underlined.

Identification of a functional *S. aurata calt* promoter

To determine whether the isolated genomic DNA-flanking region of *calt* gene contains a functional promoter, nucleotide positions -2096 to $+126$ relative to the transcription start site were subcloned in the promoterless pGL3-Basic plasmid, upstream from the luciferase reporter gene (pGalt2096). The recombinant plasmid pGalt2096 was transiently cotransfected into SBL cells together with a LacZ-containing plasmid (pCMV β) as an internal control for transfection efficiency. The cell lysate was assayed for luciferase and β -galactosidase activities 48 h after transfection. This construct yielded a more than 80-fold increase in luciferase activity over the promoterless vector, pGL3-Basic (Fig. 2). This result indicates that a core functional promoter for basal transcription is contained within the 2096 nucleotides upstream from the transcription start site.

To examine the promoter functional regions involved in modulation of basal *calt* expression in *S. aurata*, sequential 5'-deletion analysis of the promoter fragment was performed. Deletion fragments, with 5' ends ranging from -2096 to -89 and 3' ends at $+126$ (lacking the cAlt2 ATG start codon to prevent any false negative results due to the expression of a bicistronic message), were fused to the luciferase reporter gene and transfected into SBL cells. The longest 5' construct (pGalt2096) gave a more than 80-fold increase in luciferase activity over pGL3-Basic. Sequential 5'-deletion of pGalt2096 revealed reduced promoter activity. Decreased promoter activity was observed using the construct containing the promoter region spanning -89 to $+126$ (pGalt89), which showed induction

40 times greater than the promoterless pGL3-Basic did. No activity was found when the smallest construct (pGalt12; -12 to $+126$) was used. Moreover, when compared to pGalt2096, promoter activity decreased twofold due to deletion of the region encompassing the nucleotide positions -89 to -11 (pGalt2096 Δ 89–11). These results confirmed that the minimal core promoter of the *S. aurata calt* gene is located in the region within 89 bp upstream from the transcriptional start, and suggest that *cis*-acting elements may be located in this area.

Transactivation of *calt* promoter by p300 and c-Myb

In silico analysis of *calt* promoter with TFSEARCH (Heinemeyer *et al.* 1998) revealed several putative transcription factor-binding sites in the proximal promoter region (Fig. 1). Amongst the potential transcription factors were sites for p300 and c-Myb. Cotransfection of SBL cells with the pGalt2096 construct in the presence of a p300 expression plasmid resulted in a more than 23-fold increase in *calt* promoter activity. p300 is a nuclear phosphoprotein that acts as a transcriptional coactivator. p300 promotes gene transcription by bridging between DNA-binding transcription factors and the basal machinery, by providing a scaffold for integrating transcription factors, and by modifying transcription factors and chromatin through acetylation (Kalkhoven 2004, Ghosh & Varga 2007). Reporter constructs containing 5'-deletion of the promoter fragment up to 89 nucleotides upstream from the transcription start site displayed similar p300-mediated enhanced *calt*

Figure 2 Deletion analysis of the 5'-flanking region of *S. aurata calt* in SBL cells. The top left part represents the genomic organization of the 5'-flanking region of *S. aurata calt*. Relevant restriction sites and the translation start codon for cAlt2 are indicated. Nucleotide numbering starts with $+1$ corresponding to the transcription start site, which is indicated by an arrow. Fragments of the *S. aurata calt* promoter with varying 5' ends and an identical 3' end ($+126$) were fused to the luciferase reporter gene in pGL3-Basic vector. SBL cells were transfected with the reporter constructs pGalt2096, pGalt399, pGalt173, pGalt89, pGalt12, pGalt2096 Δ 89–11, or pGL3-Basic, along with pCMV β (lacZ) to normalize for transfection efficiency. Luciferase activity is expressed as a fold increase over promoterless reporter plasmid pGL3-Basic. Results shown are the mean $\pm$ s.d. from at least three independent experiments performed in duplicate.

promoter activity in SBL cells. In contrast, no enhancement of the promoter activity was detected when the shortest construct (pGalt12) was used. Since p300 can interact simultaneously with the basal transcription machinery and with one or more upstream transcription factors (Chan & La Thangue 2001, Kalkhoven 2004), our findings suggested that a *cis* element located within 89 bp upstream from the transcription start site, here named the p300 response element, may be responsible for the p300-mediated transactivation of *calt* promoter. Consistently, no transactivation by p300 was observed when cotransfected with the pGalt2096Δ89–11 reporter construct, which lacks the region within 89 bp upstream from the transcriptional start (Fig. 3A).

Putative c-Myb-binding sites were located within nucleotide positions –85 to –68 and –52 to –35 relative to the transcription start site of *S. aurata calt* and, therefore, close to the p300 response element (–73 to –60). Among other transcription factors, p300 is a well-established coactivator of c-Myb through acetylation of the latter (Tomita *et al.* 2000, Sandberg *et al.* 2005). To address the role of c-Myb in *calt* expression, we performed transfection experiments on SBL cells. Cotransfection of a plasmid encoding c-Myb together with the pGalt2096 construct resulted in a more than threefold increase in *calt* promoter activity (Fig. 3B). Reporter constructs pGalt173 and pGalt89 displayed similar *calt* promoter transactivation after cotransfection with c-Myb. No enhancement of the promoter activity was detected when the shortest construct (pGalt12) was used. Furthermore, no transactivation by c-Myb was observed when cotransfected with pGalt2096Δ89–11 reporter construct, which lacks the region within 89 bp upstream from the transcriptional start. These results suggest that c-Myb may transactivate the *S. aurata calt* promoter by acting via at least one response element located within 89 bp upstream from the transcriptional start. Cotransfection of SBL cells with expression plasmids encoding p300 and c-Myb caused additive effects on the reporter activity of pGalt89 or longer constructs (Fig. 3C).

Although the *S. aurata calt* and human *ALTI* promoter sequences share a low general homology, *in silico* analysis of human *ALTI* with TFSEARCH (Heinemeyer *et al.* 1998) revealed the presence of putative binding sites for p300 and c-Myb close to the transcription start site (data not presented), similarly as for the *S. aurata* gene.

p300 is part of a complex that binds to the *calt* promoter

The shortest reporter construct that showed p300-mediated transactivation (pGalt89) contains a putative p300 response element at positions –73 to –60

Figure 3 Effect of p300 and c-Myb on *S. aurata calt* promoter transcription in SBL cells. The cells were transfected with the promoter constructs pGalt2096, pGalt399, pGalt173, pGalt89, pGalt12, or pGalt2096Δ89–11, with or without expression plasmids encoding p300 (A) or c-Myb (B). (C) SBL cells were transfected with the promoter constructs pGalt2096, pGalt89, or pGalt12, with or without expression plasmids encoding p300 and c-Myb. The promoter activity of the constructs alone was set at 1. The data represent the mean $\pm$ s.d. values of three independent duplicate experiments.

relative to the transcription start site. To analyze whether p300 is part of a protein complex that indeed binds to the putative p300 response element at positions –73 to –60, electrophoretic mobility shift assay (EMSA) experiments were performed. When *calt*–91/–51 (harboring the putative p300 response element) was used as a probe and nuclear extracts were prepared from SBL cells overexpressing p300, one major shifted band was observed. A DNA–protein complex with the same mobility was seen when a consensus p300 probe was used (p300-cons). The shifted band disappeared in competition assays

Figure 4 Effect of mutating the p300 response element on *S. aurata calt* promoter binding and transcription in SBL cells. (A) The left part of the figure shows an electrophoretic mobility shift assay of a competition analysis, using nuclear extracts of SBL cells overexpressing p300 incubated with labeled oligonucleotides p300-cons (lanes 1–4) or calt–91/–51 (lanes 5–8). Lanes 1 and 5 contained no nuclear extract. Lanes 2 and 6 correspond to binding of nuclear extracts to labeled probes without competitor. Lanes 3, 4, 7, and 8 represent competition with 5- and 20-fold molar excess of unlabeled double-stranded competitor (p300-cons). Lanes 9, 10, and 11 show nuclear extracts of p300-overexpressing SBL cells incubated with probes calt–66/–27, calt–41/+6, and calt–91/–51mutp300 respectively. The right part of the figure shows a supershift assay using nuclear extracts of p300-overexpressing SBL cells incubated with labeled oligonucleotide calt–91/–51 in the absence (lanes 12–13) or presence of 2 µg (lanes 14) and 8 µg (lanes 15) of antibody against p300. Lane 12 contained no nuclear extract. DNA–protein complexes are indicated by an arrow. (B) SBL cells were transfected with pGalt173, pGalt173mutp300, or pGalt12, with or without expression plasmids encoding p300 or p300DY. The promoter activity of the constructs alone was set at 1. The data represent the mean $\pm$ s.d. values of three independent duplicate experiments.

performed with 5- and 20-fold molar excess of unlabeled p300-cons (Fig. 4A). No shifted band could be detected when using calt–66/–27 (partial deletion of p300 consensus-like sequence) and calt–41/+6 (absence of p300 consensus-like sequence) probes.

To test whether mutations in the putative p300 site could abolish formation of the DNA–protein complex, we performed bandshift assays using nuclear extracts of SBL cells overexpressing p300 and a labeled probe harboring positions –91 to –52 of the *calt* gene in which the p300 box was mutated (calt–91/–51mutp300). As shown in Fig. 4A, the complex shifted using p300-cons and calt–91/–51 probes disappeared when the calt–91/–51mutp300 probe was used. In addition, a supershift assay was performed

with antibodies against p300. As shown in Fig. 4A, a supershift band was observed when 2 µg of the antibody were used. A higher concentration of antibody (8 µg) blocked the binding of p300 to the probe. Taken together, these data confirm that p300 is part of a complex that binds to the p300 response element at positions –73 to –60 of *calt* gene.

Mutating the p300 response element abolishes transactivation by p300

To generate a reporter construct with a mutated p300 response element (pGalt173mutp300), the same mutations described for the double-stranded oligonucleotide calt–91/–51mutp300 used in bandshift assays were introduced into the promoter construct pGalt173. Cotransfection experiments with pGalt173mutp300 and the p300 expression vector were performed in SBL cells to analyze the level of transactivation due to the wild-type or the mutant reporter construct. p300 did not enhance the transcription of the construct harboring the mutated p300 response element (Fig. 4B). This finding allowed us to hypothesize that the p300 response element at positions –73 to –60, relative to the transcriptional start of *S. aurata calt* promoter, is responsible for transactivation by p300.

Histone acetyltransferase activity of p300 is essential for transcriptional activation of *calt* gene

p300 couples transcription factor recognition to chromatin remodeling through acetylating histones and non-histone proteins, such as a number of transcription factors (Das *et al.* 2009, Marmorstein & Trievel 2009). This brought us to the question of whether p300-mediated acetylation plays a role in p300 transactivation of the *calt* promoter. A point mutant version of p300, p300DY (Ito *et al.* 2001), which lacks histone acetyltransferase (HAT) activity, was cotransfected with *calt* promoter constructs in SBL cells. p300DY did not activate any of the reporter constructs transactivated by wild-type p300, such as pGalt173 (Fig. 4B). These results indicate that the HAT domain of p300 is essential for p300-induced *calt* transactivation.

c-Myb binds to c-Myb consensus elements of *calt* promoter

Two putative c-Myb-binding-like sites are located in the proximal promoter region of *cALT*; one site partially overlaps the p300 response element (–85 to –68) and another element is located downstream (–52 to –35). To investigate whether c-Myb binds to either one or both putative c-Myb response elements, EMSA

experiments were performed. Competition analysis performed in the presence of 5- and 20-fold molar excess of unlabeled consensus c-Myb probe (Myb-cons), using probes *calt*–91/–51 (harboring the upstream response element for c-Myb), *calt*–57/–27 (harboring the downstream response element for c-Myb), and nuclear extracts obtained from SBL cells overexpressing c-Myb, resulted in the disappearance of one major shifted band (Fig. 5A). These data suggest that c-Myb can bind to both c-Myb boxes, located at nucleotides –85 to –68 (upstream site) and –52 to –35 (downstream site) relative to the transcriptional start in the *S. aurata calt* promoter.

Bandshift assays were performed to analyze whether introduction of mutations in the putative upstream and downstream c-Myb sites abolished formation of the

DNA–transcription factor complexes. To this end, nuclear extracts of SBL cells overexpressing c-Myb and labeled probes harboring mutated upstream (*calt*–91/–51mutMyb1) and downstream (*calt*–57/–27mutMub2) c-Myb boxes were used. As shown in Fig. 5A, the shifted complex disappeared when the *calt*–91/–51mutMyb1 and *calt*–57/–27mutMub2 probes were used. Supershift assays carried out with 2 µg-specific antibodies against c-Myb blocked the binding of c-Myb to *calt*–91/–51 and *calt*–57/–27 probes. From these data, we hypothesized that c-Myb might bind to both the upstream and the downstream c-Myb sites located at positions –85 to –68 and –73 to –60 of *calt* gene.

Mutating the downstream c-Myb box abolishes transactivation by c-Myb

To further distinguish whether the upstream and downstream c-Myb boxes contribute equally to the c-Myb-dependent transactivation of the *calt* promoter, we generated the reporter construct pGalt57, which lacks the upstream c-Myb element, but retains the downstream box. Cotransfection of SBL cells with pGalt57 gave similar c-Myb-dependent transactivating rates as reporter constructs harboring both upstream and downstream sites, such as pGalt173 (Fig. 5B). These results suggest that the upstream c-Myb box (–85 to –68) is not functional. As expected, since pGalt57 lacks the p300 response element, p300 had no effect on the reporter activity of pGalt57. The fact that c-Myb was still able to activate the transcription of pGalt57 revealed that this effect was a separate event not related to the p300 response element located at positions –73 to –60.

Two reporter constructs were designed to carry mutated c-Myb upstream and downstream sites: pGalt173mutMyb1, which contains an upstream c-Myb site with the mutations that were introduced in the double-stranded oligonucleotide *calt*–91/–59mutMyb1 used in bandshift assays; and pGalt57mutMyb2, which contains a downstream c-Myb site with the mutations that were introduced in the double-stranded oligonucleotide *calt*–57/–27mutMyb2. SBL cells were cotransfected with the c-Myb expression vector and pGalt173mutMyb1 or pGalt57mutMyb2 to compare the level of transactivation due to the wild-type or the mutant reporter constructs. Similar transactivation values were observed using pGalt173mutMyb1, pGalt173, or pGalt57, which is consistent with the suggestion that the upstream box is not responsible for the transactivating effect of c-Myb. In contrast, c-Myb did not activate the transcription of the construct harboring the mutated downstream c-Myb box (pGalt57mutMyb2, Fig. 5B). This finding led us to conclude that the c-Myb box located at positions –52

Figure 5 Effect of mutating the c-Myb response element on *S. aurata calt* promoter binding and transcription in SBL cells. (A) The left part of the figure shows an electrophoretic mobility shift assay of a competition analysis using nuclear extracts of SBL cells overexpressing c-Myb incubated with labeled oligonucleotides Myb-cons (lanes 1–4), *calt*–91/–51 (lanes 5–8), or *calt*–57/–27 (lanes 9–12). Lanes 1, 5, and 9 contained no nuclear extract. Lanes 2, 6, and 10 show binding of nuclear extracts to labeled probes without competitor. Lanes 3, 4, 7, 8, 11, and 12 correspond to competition with 5- and 20-fold molar excess of unlabeled double-stranded competitor (Myb-cons). The right part of the figure includes a supershift assay using nuclear extracts of c-Myb-overexpressing SBL cells incubated with labeled oligonucleotides *calt*–91/–51 and *calt*–57/–27 in the absence (lanes 13–14 and 17–18) or presence of 2 µg (lanes 15 and 19) of antibody against c-Myb. Lanes 13 and 17 contained no nuclear extract. Lanes 16 and 20 show nuclear extracts of c-Myb-overexpressing SBL cells incubated with probes *calt*–91/–51mutMyb1 and *calt*–57/–27mutMyb2 respectively. DNA–protein complexes are indicated by an arrow. (B) SBL cells were transfected with pGalt173, pGalt173mutMyb1, pGalt57, pGalt57mutMyb2, or pGalt12, with or without expression plasmids encoding c-Myb or p300. The promoter activity of the constructs alone was set at 1. The data represent the mean $\pm$ S.D. values of three independent duplicate experiments.

to -35, relative to the transcriptional start of the *S. aurata calt* promoter, is responsible for the transactivating effect of c-Myb.

Nutritional and hormonal regulation of p300 and c-Myb expression in liver of *S. aurata*

Having established transactivation of the *calt* promoter by p300 and c-Myb, we next asked whether the expression of p300 and c-Myb is controlled by nutritional and hormonal regulation in the liver of *S. aurata*. We showed elsewhere that alternative splicing of *calt* gene in *S. aurata* generates two isoforms: cAlt1 and cAlt2. Specifically, the hepatic expression of *S. aurata* cAlt2 increased under gluconeogenic conditions, such as starvation and STZ-induced diabetes (Anemaet *et al.* 2008).

Quantitative RT-PCR assays showed that starvation similarly affected the expression of p300 and c-Myb. Both p300 and c-Myb mRNA levels significantly increased 1.3- and 1.4-fold respectively in the liver of 20-day-starved *S. aurata* (Fig. 6A). The administration of insulin did not cause significant changes in the expression of p300 6 h after the treatment, although a

tendency to decrease to about 80% of the values in control animals was observed. A significant 55% decrease in c-Myb mRNA levels was found in the liver 6 h after insulin administration (Fig. 6B). STZ is a glucosamine-nitrosourea derivative widely used to generate diabetic animal models through β cell necrosis (Junod *et al.* 1969, Like & Rossini 1976, Wilson & Leiter 1990). As it is the characteristic of the diabetogenic action of STZ, significant hyperglycemia (175.8 ± 53.6 mg/dl, mean $\pm$ s.d., $n=4$ vs 94.3 ± 2.7 mg/dl, mean $\pm$ s.d., $n=4$ in control animals; $P<0.05$) followed 22 h after i.p. administration of 600 mg/kg STZ to *S. aurata*. The administration of STZ significantly up-regulated p300 1.5-fold in the liver of 22-h-treated animals. However, STZ promoted a decrease in c-Myb expression to about 25% of the levels in control fish (Fig. 6B).

Discussion

The liver plays important roles in regulating blood levels of amino acids and synthesizing glucose from excess amino acids. In this tissue, Alt has an essential function in intermediary metabolism to obtain energy and to provide gluconeogenic substrates. To assess the transcriptional regulation of *calt* gene in *S. aurata*, we addressed cloning and characterization of the *calt* promoter. The genomic fragment isolated is the first *calt* gene promoter reported for animals other than humans, and it allowed us to identify two functional *cis* elements involved in transactivation by p300 and c-Myb in the proximal region of the *calt* promoter.

The *S. aurata calt* promoter does not contain a TATA box. However, it includes an initiator (Inr)-like element that overlaps the transcription start site. The functionality of the putative promoter region of fish *calt* was tested by transient transfection of SBL cells with fusion constructs of the isolated genomic fragment to the luciferase reporter gene. Sequential 5'-deletions of the 2235 bp fragment and analysis of promoter activity after transfection of SBL cells with the deleted constructs allowed us to conclude that the promoter region within 89 bp upstream from the transcription start site is essential for transcriptional basal activity of *calt* and constitutes the core functional promoter of this gene.

p300 and CBP have emerged as transcriptional coactivator proteins that interact with and modulate the activity of a wide array of transcription factors bound at upstream activator elements (Shiama 1997). Transient transfection experiments and EMSA analysis demonstrated that p300 confers an activating signal on the *calt* promoter. This is achieved by forming part of a complex that binds to a p300 response element located between -73 and -60 bp upstream from the transcription start site of the *S. aurata* liver *calt* gene

Figure 6 Effect of starvation, insulin, and streptozotocin on p300 and c-Myb mRNA levels in liver of *S. aurata*. (A) Quantitative RT-PCR assays of p300 and c-Myb mRNA levels performed on total RNA isolated from liver of fed and 20-day-starved fish. The mRNA levels of p300 and c-Myb relative to β -actin mRNA are expressed as mean $\pm$ s.d. of four fish. Statistical significance related to fed animals is indicated as follows: * $P<0.05$; ** $P<0.01$. (B) Quantitative RT-PCR assays carried out on total RNA isolated from liver of fish after treatment with vehicle (control; 0.01 M sodium citrate, pH 4.5), insulin (INS; 10 U/kg fish; 6 h), or streptozotocin (STZ; 600 mg/kg fish; 22 h). The mRNA levels of p300 and c-Myb relative to β -actin mRNA are expressed as mean $\pm$ s.d. of four animals. Statistical significance related to control animals is indicated as follows: * $P<0.05$.

promoter. Recruitment of proteins with HAT activity, such as p300, to the Inr element of the *calt* promoter may provide an open DNA configuration precisely at the site of transcription initiation. Histone acetylation in the proximal *calt* promoter region probably makes chromatin more accessible to other transcription factors and the basal transcriptional machinery. Targeting of p300 to the Inr element might enable the recruitment of TFIID to the *calt* promoter, as suggested for other TATA-less Inr-containing promoters (Abraham *et al.* 1993, Swope *et al.* 1996).

Apart from acetylating histones, p300 has been shown to acetylate several transcription factors in a regulated manner and is recruited to the various promoters in response to several stimuli. Many of the p300 mutations identified in tumors actually result in the loss of acetyltransferase activity (Gayther *et al.* 2000), suggesting that the ability of p300 to acetylate one or more cell proteins may be critical for their functions in growth control. Since an acetyltransferase-deficient point mutant of p300 (p300DY) failed to induce the acetylation of the tumor suppressor p53, Ito *et al.* (2001) concluded that binding between p53 and p300 is necessary for p300-mediated p53 acetylation. Transfection experiments in SBL cells using p300DY show that an acetylase-deficient mutant p300 was unable to induce *calt* promoter activity, indicating that the acetyltransferase activity of p300 is essential for the p300-mediated transcriptional activation of *S. aurata calt*.

Our findings suggest that p300 may induce *calt* promoter activity by acetylating histones, to promote a transcriptionally favorable structure of the nucleosome at the transcriptional start, and transcription factors such as c-Myb. The c-Myb gene encodes a proto-oncogene that leads to viral myeloblastosis in avian species (Beug *et al.* 1982). The role of c-Myb as a transcription factor has been extensively studied in hematopoietic stem cells. However, the expression of c-Myb seems to be required in a number of other tissues (Ramsay 2005). The transcriptional activity of c-Myb and other transcription factors is regulated through binding the KIX domain of p300 (Kasper *et al.* 2002, Kauppi *et al.* 2008). Two putative c-Myb boxes were found in the *S. aurata calt* promoter at positions –85 to –68 and –52 to –35 relative to the transcriptional start. Although EMSA assays suggested that c-Myb can bind both sites, site-directed mutagenesis revealed that the downstream element (positions –52 to –35) was the only functional one and responsible for c-Myb-dependent transactivation of the *calt* promoter. Since c-Myb was still able to activate the transcription of *calt* promoter reporter constructs lacking the p300 response element, we can conclude that the c-Myb-dependent transactivation of *calt* occurred irrespective of the p300 response element located at

positions –73 to –60. However, it is well known that the DNA-binding activity of c-Myb is increased by p300-mediated acetylation (Tomita *et al.* 2000). Furthermore, replacement of c-Myb-acetylated residues (lysines) by arginine dramatically decreases the transactivating capacity of c-Myb (Sano & Ishii 2001). Thus, p300-mediated acetylation of c-Myb could enhance the binding of c-Myb to the *S. aurata calt* promoter. Conceivably, p300-mediated acetylation of c-Myb at lysine residues neutralizes the positive charge and results in a conformational change, which, in turn, may increase the DNA-binding activity of c-Myb and thus the transcriptional activity of *calt*.

Previously, we showed that the hepatic mRNA levels of *S. aurata* cAlt1 and cAlt2 depend largely on nutritional and hormonal status. Long-term starvation increases cAlt2 mRNA levels in the liver of *S. aurata*, whereas short-term refeeding and administration of insulin down-regulate cAlt2 expression. These conditions lead to opposite effects on the mRNA levels of cAlt1. Consistent with stimulation of cAlt2 in conditions associated with increased gluconeogenesis, STZ-treated fish present a marked increase in the hepatic expression of cAlt2. These findings led us to hypothesize that increased cAlt2 expression can be responsible for the serum elevation of Alt activity levels in animals under conditions related to insulin resistance and development of type 2 diabetes (Anemaet *et al.* 2008). To understand the molecular mechanism that governs *calt* gene transcription, we analyzed the expression of p300 and c-Myb in the liver of starved and insulin- and STZ-treated *S. aurata*. In agreement with cAlt2 expression, the mRNA levels of p300 and c-Myb increased in starved animals and decreased in fish liver after the administration of insulin. It remains uncertain whether the suppressing effect of insulin on cAlt2 expression results from a direct or indirect pathway. As it has been described in mammals, we cannot rule out an indirect action of insulin on *calt* expression through inhibition of gluconeogenic substrates release from muscle and adipose tissue, rather than direct hepatic insulin effects. Hence, hyperglycemia has been shown to potentiate the indirect actions of insulin (Fisher & Kahn 2003). This may well account for down-regulation of cAlt2 expression in the liver of *S. aurata* following the administration of glucose or insulin (Anemaet *et al.* 2008).

Based on the data given, both p300 and c-Myb are likely to contribute to increased cAlt2 expression in starved fish. Interestingly, and in a similar way as for cAlt2, up-regulation of p300 expression was also observed in STZ-induced diabetic *S. aurata*. In this regard, p300 is recognized as an important element in the pathogenesis of various diseases (Kalkhoven 2004), and there is increasing evidence that links p300 with diabetes (Chen *et al.* 2010). Consistent with our

findings, increased p300 mRNA levels have been found in the heart of STZ-induced diabetic mice (Kaur *et al.* 2006), leading the authors to conclude that p300 may play an important role in glucose- and diabetes-induced fibronectin synthesis. Moreover, curcumin decreases diabetes-induced oxidative stress in the kidney through reversion of p300 up-regulation in STZ-treated rats (Chiu *et al.* 2009). Indeed, it has been suggested that abnormal interactions between mutant transcription factors and p300 contribute to the pathogenesis of diabetes (Eeckhoutte *et al.* 2001, Fajans *et al.* 2001, Ban *et al.* 2002, Stanojevic *et al.* 2004, Fernandez-Zapico *et al.* 2009). The transcriptional regulation of *calt* expression in *S. aurata* does not necessarily apply to human *ALTL*. However, since a p300 putative response element is present in the human *ALTL* promoter, further research is needed to explore the relationship between the expression of p300, ALT, and diabetes in humans.

In conclusion, besides characterization for the first time of an *alt* promoter in animals other than humans, the results of the present study suggest a contribution of p300 and c-Myb to up-regulation of cAlt2 expression in the liver of *S. aurata* subjected to starvation. Our findings also argue for a role of p300 in up-regulation of cAlt2 in STZ-induced diabetic fish. Upcoming efforts will be addressed to unravel the specific mechanisms that govern alternative splicing of *calt* gene and cAlt1 transcription.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the MCYT (Spain) grants BIO2006-01857 and BIO2009-07589. I G Anemaet and J D González were recipients of FI-IQUC (Generalitat de Catalunya, Spain) and FPU (Ministerio de Educación y Ciencia, Spain) fellowships.

Author contribution statement

I G Anemaet isolated the *calt* promoter, obtained reporter constructs, and performed transfection and EMSA studies. J D González, M C Salgado, and M Giralte developed the quantitative RT-PCR analysis. F Fernández, I V Baanante, I Metón, I G Anemaet, J D González, and M C Salgado performed fish treatments and obtained the samples. I Metón, F Fernández, and I V Baanante designed the experiments. I G Anemaet, I Metón, and I V Baanante analyzed the data and wrote the manuscript.

Acknowledgements

The authors thank Dr D Haro (Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona, Barcelona, Spain), Dr Tso-Pang Yao (Department of Pharmacology and Cancer Biology, Duke

University, USA), and Dr Bernard Lüscher (Institut für Molekularbiologie, Medizinische Hochschule Hannover, Hannover, Germany) for providing the p300, p300DY, and c-Myb expression vectors respectively.

References

- Abraham SE, Lobo S, Yaciuk P, Wang HG & Moran E 1993 p300, and p300-associated proteins, are components of TATA-binding protein (TBP) complexes. *Oncogene* **8** 1639–1647.
- Anemaet IG, Metón I, Salgado MC, Fernández F & Baanante IV 2008 A novel alternatively spliced transcript of cytosolic alanine aminotransferase gene associated with enhanced gluconeogenesis in liver of *Sparus aurata*. *International Journal of Biochemistry & Cell Biology* **40** 2833–2844.
- Ban N, Yamada Y, Someya Y, Miyawaki K, Ihara Y, Hosokawa M, Toyokuni S, Tsuda K & Seino Y 2002 Hepatocyte nuclear factor-1alpha recruits the transcriptional co-activator p300 on the GLUT2 gene promoter. *Diabetes* **51** 1409–1418.
- Begum NA & Datta AG 1992 Effect of adrenergic agonists and antagonists on alanine amino transferase, fructose-1:6-bisphosphatase and glucose production in hepatocytes. *Molecular and Cellular Biochemistry* **113** 93–103.
- Beug H, Hayman MJ & Graf T 1982 Myeloblasts transformed by the avian acute leukemia virus E26 are hormone-dependent for growth and for the expression of a putative myb-containing protein, p135 E26. *EMBO Journal* **1** 1069–1073.
- Castric J 1984 Obtention et étude de quelques caractéristiques d'une lignée cellulaire du bar, *Morone labrax* (Linné). Mémoire. Faculté des Sciences de Brest, Université de Bretagne Occidentale, France.
- Chan HM & La Thangue NB 2001 p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *Journal of Cell Science* **114** 2363–2373.
- Chen S, Feng B, George B, Chakrabarti R, Chen M & Chakrabarti S 2010 Transcriptional co-activator p300 regulates glucose induced gene expression in the endothelial cells. *American Journal of Physiology. Endocrinology and Metabolism* **298** E127–E137.
- Chiu J, Khan ZA, Farhangkhoei H & Chakrabarti S 2009 Curcumin prevents diabetes-associated abnormalities in the kidneys by inhibiting p300 and nuclear factor-kappaB. *Nutrition* **25** 964–972.
- Das C, Lucia MS, Hansen KC & Tyler JK 2009 CBP/p300-mediated acetylation of histone H3 on lysine 56. *Nature* **459** 113–117.
- Dufour DR, Lott JA, Nolte FS, Gretch DR, Koff RS & Seeff LB 2000 Diagnosis and monitoring of hepatic injury. II. Recommendations for use of laboratory tests in screening, diagnosis, and monitoring. *Clinical Chemistry* **46** 2050–2068.
- Eeckhoutte J, Formstecher P & Laine B 2001 Maturity-onset diabetes of the young type 1 (MODY1)-associated mutations R154X and E276Q in hepatocyte nuclear factor 4alpha (HNF4alpha) gene impair recruitment of p300, a key transcriptional co-activator. *Molecular Endocrinology* **15** 1200–1210.
- Fajans SS, Bell GI & Polonsky KS 2001 Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. *New England Journal of Medicine* **345** 971–980.
- Fernández F, Miquel AG, Córdoba M, Varas M, Metón I, Caseras A & Baanante IV 2007 Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (*Sparus aurata*, L.) fingerlings. *Journal of Experimental Marine Biology and Ecology* **343** 1–10.
- Fernandez-Zapico ME, van Velkinburgh JC, Gutierrez-Aguilar R, Neve B, Froguel P, Urrutia R & Stein R 2009 MODY7 gene, KLF11, is a novel p300-dependent regulator of Pdx-1 (MODY4) transcription in pancreatic islet beta cells. *Journal of Biological Chemistry* **284** 36482–36490.

- Fisher SJ & Kahn CR 2003 Insulin signaling is required for insulin's direct and indirect action on hepatic glucose production. *Journal of Clinical Investigation* **111** 463–468.
- Fynn-Aikins K, Hughes SG & Vandenberg GW 1995 Protein retention and liver aminotransferase activities in Atlantic salmon fed diets containing different energy sources. *Comparative Biochemistry and Physiology, Part A, Physiology* **111** 163–170.
- Gayther SA, Batley SJ, Linger L, Bannister A, Thorpe K, Chin SF, Daigo Y, Russell P, Wilson A, Sowter HM *et al.* 2000 Mutations truncating the EP300 acetylase in human cancers. *Nature Genetics* **24** 300–303.
- Ghosh AK & Varga J 2007 The transcriptional coactivator and acetyltransferase p300 in fibroblast biology and fibrosis. *Journal of Cellular Physiology* **213** 663–671.
- Glinghammar B, Rafter I, Lindstrom AK, Hedberg JJ, Andersson HB, Lindblom P, Berg AL & Cotgreave I 2009 Detection of the mitochondrial and catalytically active alanine aminotransferase in human tissues and plasma. *International Journal of Molecular Medicine* **23** 621–631.
- Gray S, Wang B, Orihuela Y, Hong EG, Fisch S, Halder S, Cline GW, Kim JK, Peroni OD, Kahn BB *et al.* 2007 Regulation of gluconeogenesis by Kruppel-like factor 15. *Cell Metabolism* **5** 305–312.
- Hanley AJ, Williams K, Festa A, Wagenknecht LE, D'Agostino RB Jr, Kempf J, Zinman B & Haffner SM 2004 Elevations in markers of liver injury and risk of type 2 diabetes: the insulin resistance atherosclerosis study. *Diabetes* **53** 2623–2632.
- Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Ananko EA, Podkolodnaya OA, Kolpakov FA *et al.* 1998 Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. *Nucleic Acids Research* **26** 362–367.
- Iacobellis G, Moschetta A, Buzzetti R, Ribaudo MC, Baroni MG & Leonetti F 2007 Aminotransferase activity in morbid and uncomplicated obesity: predictive role of fasting insulin. *Nutrition, Metabolism, and Cardiovascular Diseases* **17** 442–447.
- Ishiguro M, Takio K, Suzuki M, Oyama R, Matsuzawa T & Titani K 1991 Complete amino acid sequence of human liver cytosolic alanine aminotransferase (GPT) determined by a combination of conventional and mass spectral methods. *Biochemistry* **30** 10451–10457.
- Ito A, Lai CH, Zhao X, Saito S, Hamilton MH, Appella E & Yao TP 2001 p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. *EMBO Journal* **20** 1331–1340.
- Jadhao SB, Yang RZ, Lin Q, Hu H, Anania FA, Shuldiner AR & Gong DW 2004 Murine alanine aminotransferase: cDNA cloning, functional expression, and differential gene regulation in mouse fatty liver. *Hepatology* **39** 1297–1302.
- Junod A, Lambert AE, Stauffacher W & Renold AE 1969 Diabetogenic action of streptozotocin: relationship of dose to metabolic response. *Journal of Clinical Investigation* **48** 2129–2139.
- Kalkhoven E 2004 CBP and p300: HATs for different occasions. *Biochemical Pharmacology* **68** 1145–1155.
- Kasper LH, Boussouar F, Ney PA, Jackson CW, Reh J, van Deursen JM & Brindle PK 2002 A transcription-factor-binding surface of coactivator p300 is required for haematopoiesis. *Nature* **419** 738–743.
- Kauppi M, Murphy JM, de Graaf CA, Hyland CD, Greig KT, Metcalf D, Hilton AA, Nicola NA, Kile BT, Hilton DJ *et al.* 2008 Point mutation in the gene encoding p300 suppresses thrombocytopenia in *Mpl*^{−/−} mice. *Blood* **112** 3148–3153.
- Kaur H, Chen S, Xin X, Chiu J, Khan ZA & Chakrabarti S 2006 Diabetes-induced extracellular matrix protein expression is mediated by transcription coactivator p300. *Diabetes* **55** 3104–3111.
- Like AA & Rossini AA 1976 Streptozotocin-induced pancreatic insulinitis: new model of diabetes mellitus. *Science* **193** 415–417.
- Lindblom P, Rafter I, Copley C, Andersson U, Hedberg JJ, Berg AL, Samuelsson A, Hellmold H, Cotgreave I & Glinghammar B 2007 Isoforms of alanine aminotransferases in human tissues and serum – differential tissue expression using novel antibodies. *Archives of Biochemistry and Biophysics* **466** 66–77.
- Marmorstein R & Trievel RC 2009 Histone modifying enzymes: structures, mechanisms, and specificities. *Biochimica et Biophysica Acta* **1789** 58–68.
- Metón I, Mediavilla D, Caseras A, Cantó E, Fernández F & Baanante IV 1999 Effect of diet composition and ration size on key enzyme activities of glycolysis–gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (*Sparus aurata*). *British Journal of Nutrition* **82** 223–232.
- Metón I, Egea M, Fernández F, Eraso MC & Baanante IV 2004 The N-terminal sequence directs import of mitochondrial alanine aminotransferase into mitochondria. *FEBS Letters* **566** 251–254.
- 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.
- Nathwani RA, Pais S, Reynolds TB & Kaplowitz N 2005 Serum alanine aminotransferase in skeletal muscle diseases. *Hepatology* **41** 380–382.
- Oelgeschläger M, Krieg J, Luscher-Firzlaff JM & Luscher B 1995 Casein kinase II phosphorylation site mutations in c-Myb affect DNA binding and transcriptional cooperativity with NF-M. *Molecular and Cellular Biology* **15** 5966–5974.
- Ramsay RG 2005 c-Myb a stem-progenitor cell regulator in multiple tissue compartments. *Growth Factors* **23** 253–261.
- Rosen F, Roberts NR & Nichol CA 1959 Glucocorticosteroids and transaminase activity. I. Increased activity of glutamicpyruvic transaminase in four conditions associated with gluconeogenesis. *Journal of Biological Chemistry* **234** 476–480.
- Sanchez-Muros MJ, Garcia-Rejon L, Garcia-Salguero L, de la Higuera M & Lupianez JA 1998 Long-term nutritional effects on the primary liver and kidney metabolism in rainbow trout. Adaptive response to starvation and a high-protein, carbohydrate-free diet on glutamate dehydrogenase and alanine aminotransferase kinetics. *International Journal of Biochemistry & Cell Biology* **30** 55–63.
- Sandberg ML, Sutton SE, Pletcher MT, Wiltshire T, Tarantino LM, Hogenesch JB & Cooke MP 2005 c-Myb and p300 regulate hematopoietic stem cell proliferation and differentiation. *Developmental Cell* **8** 153–166.
- Sano Y & Ishii S 2001 Increased affinity of c-Myb for CREB-binding protein (CBP) after CBP-induced acetylation. *Journal of Biological Chemistry* **276** 3674–3682.
- Sattar N, McConnachie A, Ford I, Gaw A, Cleland SJ, Forouhi NG, McFarlane P, Shepherd J, Cobbe S & Packard C 2007 Serial metabolic measurements and conversion to type 2 diabetes in the west of Scotland coronary prevention study: specific elevations in alanine aminotransferase and triglycerides suggest hepatic fat accumulation as a potential contributing factor. *Diabetes* **56** 984–991.
- Schindhelm RK, Dekker JM, Nijpels G, Bouter LM, Stehouwer CD, Heine RJ & Diamant M 2007 Alanine aminotransferase predicts coronary heart disease events: a 10-year follow-up of the Hoorn Study. *Atherosclerosis* **191** 391–396.
- Shiama N 1997 The p300/CBP family: integrating signals with transcription factors and chromatin. *Trends in Cell Biology* **7** 230–236.
- Sohocki MM, Sullivan LS, Harrison WR, Sodergren EJ, Elder FF, Weinstock G, Tanase S & Daiger SP 1997 Human glutamate pyruvate transaminase (GPT): localization to 8q24.3, cDNA and genomic sequences, and polymorphic sites. *Genomics* **40** 247–252.
- Stanojevic V, Habener JF & Thomas MK 2004 Pancreas duodenum homeobox-1 transcriptional activation requires interactions with p300. *Endocrinology* **145** 2918–2928.

- Swope DL, Mueller CL & Chrivia JC 1996 CREB-binding protein activates transcription through multiple domains. *Journal of Biological Chemistry* **271** 28138–28145.
- Thulin P, Rafter I, Stockling K, Tomkiewicz C, Norjavaara E, Aggerbeck M, Hellmold H, Ehrenborg E, Andersson U, Cotgreave I *et al.* 2008 PPARalpha regulates the hepatotoxic biomarker alanine aminotransferase (ALT1) gene expression in human hepatocytes. *Toxicology and Applied Pharmacology* **231** 1–9.
- Tomita A, Towatari M, Tsuzuki S, Hayakawa F, Kosugi H, Tamai K, Miyazaki T, Kinoshita T & Saito H 2000 c-Myb acetylation at the carboxyl-terminal conserved domain by transcriptional co-activator p300. *Oncogene* **19** 444–451.
- Vozarova B, Stefan N, Lindsay RS, Saremi A, Pratley RE, Bogardus C & Tataranni PA 2002 High alanine aminotransferase is associated with decreased hepatic insulin sensitivity and predicts the development of type 2 diabetes. *Diabetes* **51** 1889–1895.
- Wannamethee SG, Shaper AG, Lennon L & Whincup PH 2005 Hepatic enzymes, the metabolic syndrome, and the risk of type 2 diabetes in older men. *Diabetes Care* **28** 2913–2918.
- West J, Brousil J, Gazis A, Jackson L, Mansell P, Bennett A & Aithal GP 2006 Elevated serum alanine transaminase in patients with type 1 or type 2 diabetes mellitus. *QJM* **99** 871–876.
- Wilson GL & Leiter EH 1990 Streptozotocin interactions with pancreatic beta cells and the induction of insulin-dependent diabetes. *Current Topics in Microbiology and Immunology* **156** 27–54.
- Yang RZ, Blaileanu G, Hansen BC, Shuldiner AR & Gong DW 2002 cDNA cloning, genomic structure, chromosomal mapping, and functional expression of a novel human alanine aminotransferase. *Genomics* **79** 445–450.
- Yang RZ, Park S, Reagan WJ, Goldstein R, Zhong S, Lawton M, Rajamohan F, Qian K, Liu L & Gong DW 2009 Alanine aminotransferase isoenzymes: molecular cloning and quantitative analysis of tissue expression in rats and serum elevation in liver toxicity. *Hepatology* **49** 598–607.

Received in final form 16 June 2010

Accepted 22 June 2010

Made available online as an Accepted Preprint 23 June 2010