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Characterization of a far upstream located promoter expressing the acetyl-CoA carboxylase-alpha in the brain of cattle

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ABSTRACT

The expression of the bovine acetyl-CoA carboxylase-alpha-encoding gene (*ACACA*) was known to be controlled by three promoters. Here, we characterized a fourth promoter (PI) located 41 kb upstream of the adjacent nutritionally-regulated promoter PIA on bovine chromosome 19. Our results showed that PI is an intergenic promoter driving expression of *ACACA* and conceivably, by analogy with the homologous genomic arrangement in sheep a component of the chromatin-modifying complex gene (*TADA2L*). 5′-RACE experiments defined the 3′ boundary of the promoter and a novel exon 1 comprising 263 bp. It features at position +226 an ORF encoding an N-terminally extended ACC-α enzyme. The PI sequence is GC-rich, has no TATA box and CAAT box, similar to the homologous promoters in sheep, mouse and human. Expression profiles showed that PI is the promoter driving expression of the dominant *ACACA*-transcript in brain. Reporter gene assays in HC-11 cells indicated that deletion of extended promoter segments harboring putative cAMP response elements (CRE) clustered in the distal promoter region and specificity protein 1 (Sp1) attachment sites lowered PI activity.

1. Introduction

The rate-limiting step in the biosynthesis of long-chain fatty acid in the cytosol is the carboxylation of acetyl-CoA to form malonyl-CoA by the enzyme acetyl-CoA carboxylase α (ACC, EC 6.4.1.2) also known as ACC1 or ACACA (Kim, 1997; Wakil and Abu-Elheiga, 2009; Wakil et al., 1983). Three promoters (PIA, PII, PIII) have so far been found to control the transcription initiation of the *ACACA* in cattle (Mao and Seyfert, 2002; Mao et al., 2001, 2002; Molenaar et al., 2003; Shi et al., 2010). PIA (previously known as PI) is the nutritionally controlled promoter mainly active in liver, adipose tissue and mammary gland (Mao et al., 2001; Shi et al., 2010, 2012). PII is the housekeeping promoter (Mao and Seyfert, 2002), while PIII is induced during lactation in the mammary gland and drives the expression of the most abundant *ACACA* transcript in this organ (Mao et al., 2002).

In sheep, mouse and human the most 5′ located promoter (PI) of the *ACACA* was identified as a GC-rich bidirectional promoter, expressing *ACACA* together with the oppositely oriented *TADA2L* gene, encoding a component of chromatin-modifying complexes (Travers et al., 2005). PI was originally identified in human adipose tissue and found to be highly expressed in brain (Ha et al., 1994; Travers et al., 2005). The PI of mouse and rat is located 43 kb upstream of the nutritionally regulated promoter PIA. To date, there was no report of a bovine promoter PI of *ACACA*, due to a gap in the sequence information of the genome contig in this region. We hypothesized for two reasons that the bovine *ACACA* might also harbor this conserved PI. First, the currently known most distal promoter from cattle (PIA) shares a high identity with the second, more downstream located promoter of sheep and rodents. Second, the most 5′-located promoter PI of human *ACACA* shows high identity with ovine PI (Barber et al., 2005), but has low identity with bovine PIA. It therefore appears that the already known bovine promoter PIA is distinct from the upstream most promoter of human and ovine *ACACA*. Hence, the bovine PIA might physically not be the most upstream located promoter at 5′ terminus of bovine *ACCA*. This implies the uncertainty that potentially an upstream located promoter might control or influence the activity of the nutritionally regulated promoter PIA in cattle.

In the current study, we have therefore searched for such a promoter. We isolated and sequenced the most 5′-located promoter (PI) of the bovine *ACACA*. We show that PI is a GC-rich promoter being particularly expressed in brain. Reporter gene assays indicate that binding sites for the cAMP response element binding protein (CREB) and specificity protein 1 (Sp1) might be involved in controlling the PI promoter activity. The investigations of this study may provide insights for better targeted analyses of the regulation of fatty acid metabolism in the central nervous system.

Abbreviations: *ACACA*, acetyl-CoA carboxylase-alpha; CRE, cAMP response elements; cDNA, DNA complementary to RNA; ORF, open reading frame; 5′ or 3′-RACE, 5′ or 3′-rapid amplification of cDNA ends; RT-qPCR, Reverse transcription and quantitative real-time PCR; Sp1, Specificity protein 1; TADA2L, transcriptional adaptor 2-like; tsp, transcriptional start point; 5′-UTR, 5′ untranslated region.

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2. Materials and methods

2.1. Reverse transcription (RT-) and quantitative real-time RT-qPCR

Tissue cubes were snap frozen in liquid nitrogen immediately after slaughter and stored at −80°°C. TRIzol (Invitrogen) was used to prepare total RNA hereof after a storage time of no longer than 3–4 weeks. Quality of the RNA was assessed by running out 1 μg on denaturing formaldehyde containing gels and approved if ethidium bromide staining showed very clearly that the 28s RNA band was stronger than the 18s RNA band. The purified RNA was stored frozen (−80 °C). Primer Ac990r attaching at around ~990 nt (encoded by exon 9) downstream of the so far known transcriptional start site was annealed to initiate cDNA synthesis using the Superscript II Reverse Transcriptase (Invitrogen), as described (Mao et al., 2001). The product was purified with the High Pure purification kit (Roche). This template served to retrieve the partial exon 1 fragment in RT-PCR using the forward primer (PI_f) binding to exon 1 and reverse primer (bAcEx6rn) attaching to exon 6. The PCR products were resolved on 2% agarose gel. The specific bands were retrieved and cloned into the pGEM T-easy vector (Promega, Madison, WI) for sequencing. Abundances of *ACACA* transcripts derived from the respective promoters were measured with the FastStart Syber-Green I reaction kit and the LightCycler instrument (both from Roche, Basel, Switzerland) essentially as described (Molenaar et al., 2003). Briefly, we applied per measurement cDNA equivalent to an input of 75 ng of total RNA and a mixture of all amplification primers (25 pg from each). All primers are listed in Supplementary Tab. 1. The PCR cycle program started with an initial denaturation (95 °C, 5 min) and then featured 40 cycles of annealing (10 s, 60 °C), elongation (20 s, 72 °C), fluorescence acquisition (5 s, 83 °C) and melting (15 s, 95 °C). Authenticity of the amplicons was validated by their homogenous melting profile, but also by determining the size of the products on ethidium bromide stained agarose gels. Assays for all different transcripts had initially been validated by sub-cloning and sequencing of the respective amplicons retrieved from Light Cycler runs. Relative mRNA copy numbers derived from PI, PIA, PII and PIII or total *ACACA* were titrated against external standards consisting of serial dilutions (10^6—10 copies) of plasmid clones harboring the respective amplicons of the diagnostic exons.

2.2. Genomic walking

The genomic region harboring partial PI of the bovine *ACACA* was isolated from BAC478 (Mao et al., 2001). Genomic walker libraries were established from this BAC clone using the Genome Walker™ Universal Kit (BD Biosciences, San Jose, CA, USA). Separate aliquots of BAC478 DNA were digested with *Sma*I, *Eco*RV, *Pvu*II, *Dra*I, *Stu*I, respectively. Each individual library was amplified with forward adaptor-primer 1 provided by the kit and the gene-specific reverse primer (Ex1_r1, Tab. 1) using the Expand high-Fidelity PCR system Kit (Roche, Basel, Switzerland). PCR products were diluted 50-fold and 1 μl was used as template in the secondary PCR with the forward adapter-primer 2 of the kit and the nested gene-specific reverse primer (Ex1_r2, Tab. 1). Identified fragments were cloned into the pGEM T-easy vector for sequencing.

2.3. GC-rich PCR

GC-rich PCR was performed using GC-RICH PCR System (Roche) supplemented with 10% DMSO. The forward primer (PI_f2, Tab. 1) was derived *in silico* from the *TADA2L* gene (Sequence number NW_001493653), and the initial reverse primer (Ex1_r1, Tab. 1) was derived *in silico* from the highly homologous ovine *ACACA* sequence using BAC478 as template. The PCR product (1.2 kb) was cloned into pGEM-T easy vector for sequencing of both strands.

2.4. Rapid amplification of cDNA ends (5′-RACE)

5′-RACE was performed using the GeneRacer™ kit (Invitrogen) as prescribed by the manufacturer. RACE-amplification was used to identify the 5′-terminus of the PI-expressed mRNA. The cDNA template was generated from 5 μg of total RNA from brain, primed in reverse with the oligonucleotide Ac990r. *ACACA* specific reverse primers bACex6rn, nested by primer Ex1_r1 (Tab. 1) binding on the reverse strands of exon 6 and exon 1 respectively were used as PCR amplification primers in combination with the nested forward primers of the 5′-RACE adapter. The final amplicon (~300 bp) was separated on 2% agarose gel and cloned into pGEM T-easy vector for sequencing.

2.5. Reporter gene constructs8B

The PI promoter with a partial segment from exon 1 was cloned from position -952 to position +255 (clone -952) into the *Sma*I site of the promoter-less firefly luciferase expression plasmid pGL3basic (Promega). In order to examine the fine regulation of PI, two deletions were constructed. Firstly, an internal segment of 577 bp was deleted, between the *Stu*I sites at position -790 and -214 (clone ΔStu). Secondly, PI was truncated down to the proximal 214 bp (clone -214) by deleting the distal 738 bp in the construct PI-luc with *Kpn*I and *Stu*I. The resulting fragment was blunted (Klenow fill-in reaction) and relegated. All subclones were confirmed by restriction analysis and sequencing.

2.6. Cell culture, transient transfection and reporter gene assays

The established murine mammary epithelial cell line (HC-11) was cultured as described (Murani et al., 2009) in RPMI medium, supplemented with 10% fetal calf serum (FCS), 5 μg/ml insulin, 1 ng/ml epidermal growth factor (EGF), and 50 μg/ml gentamicin. One and a half micrograms of the reporter construct was co-transfected into the HC-11 cells with 15 ng of phRL-TK *renilla* reporter (Promega, Madison, WI) using Lipofectamine™ 2000 reagent (Invitrogen). Twenty-four hours later, cells were split into 24-well-plate. Luciferase activity was assessed 48 h later (Dual-luciferase® reporter Assay system, Promega) as described (Mao et al., 2001). Firefly luciferase activity of the reporter plasmid was normalized against the activity of renilla luciferase expressed by the phRL-TK control plasmid. The normalized data were expressed as multiples of the activity of the promoter-less vector pGL3basic (Promega). All transfections were conducted and assayed in triplicate.

2.7. Statistical analysis

The statistical significance of differing mean values was assessed with a one way ANOVA analysis including a Tukey–Kramer correction for multiple testing using the SAS 9.3 soft ware package (SAS Institute, Cary, NC, USA). A p value <0.05 was considered to indicate a significant difference.

3. Results and discussion

3.1. Identification and isolation of PI and Ex1

Previously our lab had identified a BAC clone (BAC478) harboring a 5′-segment of the bovine *ACACA*, including PIA (Mao et al., 2001). Sequencing the end of BAC478 with Sp6 indicated now that the far upstream region contains segments from the *TADA2L* gene. This suggested, by analogy with the situation in sheep (Travers et al., 2005) that BAC478 might contain the complete most 5′-located promoter (PI) of the bovine *ACACA* (Fig. 1A). BLAST analysis with that sequence identified a bovine genomic sequence contig (sequence number NW_001493653) supporting the correctness of our assumption. Hence, *ACACA* and *TADA2L* are arranged in the genome of cattle