

Tissue expression analysis, cloning and characterization of the 5'-regulatory region of the bovine *FABP3* gene

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Abstract *Fatty acid binding protein 3 (FABP3)* is a member of the *FABP* family which bind fatty acids and have an important role in fatty acid metabolism. A large number of studies have shown that the genetic polymorphisms of *FABP3* are positively correlated with intramuscular fat (IMF) content in domestic animals, however, the function and transcriptional characteristics of *FABP3* in cattle remain unclear. Real-time PCR analysis revealed that bovine *FABP3* was highly expressed in cardiac tissue. The 5'-regulatory region of bovine *FABP3* was cloned and its transcription initiation sites were identified. Sequence

analysis showed that many transcriptional factor binding sites including TATA-box and CCAAT-box were present on the 5'-flanking region of bovine *FABP3*, and four CpG islands were found on nucleotides from -891 to +118. Seven serial deletion constructs of the 5'-regulatory region evaluated in dual-luciferase reporter assay indicated that its core promoter was 384 base pairs upstream from the transcription initiation site. The transcriptional factor binding sites RXR α , KLF15, CREB and Sp1 were conserved in the core promoter of cattle, sheep, pigs and dogs. These results provide further understanding of the function and regulation mechanism of bovine *FABP3*.

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Introduction

Fatty acid binding proteins (FABPs) belong to a small molecular weight (14–15 kDa) and highly conserved cytoplasmic protein family that bind fatty acids with high affinity [1]. They have a crucial role in intracellular fatty acid transport and metabolism [2]. In mammals, there are at least nine members in the *FABP* family which consists of *FABP1* to *FABP9* [3, 4].

Fatty acid binding protein 3 (FABP3), also known as *H-FABP*, mainly expresses in mature cardiomyocytes and skeletal muscle, but also exists in other tissues [5]. *FABP3* plays a role in fatty acid transport and utilization in cardiac or skeletal muscle [6]. In addition, *FABP3* is essential for cold tolerance and efficient fatty acid oxidation in the brown adipose tissue of mice [7]. Over-expression of the *FABP3* gene can promote adipogenesis in 3T3-L1 pre-adipocytes [8]. Recently, *FABP3* has been considered as a

candidate gene associated with intramuscular fat (IMF) content in domestic animals.

A large number of studies have shown that the genetic polymorphism of *FABP3* is positively correlated with IMF content in pigs [9–16]. In goats, the expression level of *FABP3* is significantly correlated with IMF content [17]. Furthermore, the expression level of *FABP3* is significantly different between low- and high-marbled groups in Korean cattle [18]. It is therefore important to explore the transcriptional regulation mechanism of *FABP3*, which helps to improve IMF content in domestic animals.

To date, there are many reports about the expression regulation of *FABP3* [19–22]. However, the information about transcriptional regulation of *FABP3* in cattle is limited. To better understand the function and the transcriptional characteristics of bovine *FABP3*, we analyzed the spatial expression pattern and identified the transcription initiation sites of the bovine *FABP3*. In addition, the 5'-regulatory region of bovine *FABP3* was cloned and investigated in skeletal muscle cells and adipocytes.

Materials and methods

Ethics statement

All animal procedures were performed according to the guidelines of the China Council on Animal Care and the protocols were approved by the Experimental Animal Management Committee (EAMC) of Northwest A&F University.

Molecular cloning and sequence analysis

Genomic DNA was extracted from Qinchuan cattle blood, as described previously [23]. To obtain the 5'-regulatory region, the *FABP3* gene was searched in the UCSC genome database (<http://genome.ucsc.edu/cgi-bin/hgGateway>). The primers (*FABP3*-PF/R, Supplemental Table 1) were designed based on the bovine genomic sequence (bos-Tau8_refGene_NM_174313 range = chr2:122719225-122783830) to amplify approximately 2 kb of 5'-regulatory sequence. The PCR product was purified from an agarose gel and subsequently cloned into the pGEM-T-Easy vector (Promega Corp.) prior to sequencing. The 5'-regulatory region was analyzed for potential transcription factor binding sites using the TESS (<http://www.cbil.upenn.edu/cgi-bin/tess>), Genomatix suite (<http://www.genomatix.de/>) and TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>). CpG islands were predicted using MethPrimer (<http://www.urogene.org/methprimer/>).

The 4 kb 5'-regulatory region of *FABP3* from eight species (Supplemental Table 2) were obtained from the

genome database at the National Center for Biotechnology Information (NCBI). Multi-alignments of 5'-regulatory region among the eight species were performed using ClustalX 2.0 [24].

5'-Rapid amplification of cDNA ends (5'-RACE)

To identify the transcription initiation sites, 5'-RACE from total RNA of *longissimus dorsi* muscle of adult Qinchuan cattle was performed according to the manufacturer's protocol using the SMARTTM RACE Kit (Clontech Inc, Palo Alto, CA, USA). The PCR primers (*FABP3*-GSP1 and *FABP3*-GSP2, Supplemental Table 1) were adopted to acquire the 5'-end of the *FABP3* gene. Reaction conditions were performed in the first PCR as described previously [25, 26]. The second PCR template was a 20-fold dilution of the first PCR products.

Real-time PCR analysis of spatial expression pattern

Sixteen tissues (*longissimus dorsi* muscle, kidney, heart, *biceps femoris* muscle, liver, subcutaneous fat, spleen, lung, cecum, rumen, reticulum, omasum, abomasum, testis fat, large intestine and small intestine) were obtained from three adult Qinchuan cattle. Total RNA was extracted from the samples using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase I (MBI Fermentas, St. Leon-Rot, Germany). RNA quality was assessed with agarose gel electrophoresis and a ND-1000 Spectrophotometer (NanoDrop Technologies, USA). If the 260/280 ratio of the RNA was outside the range 1.8–2.1, the sample was discarded. The RNA was then reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega Corp., Madison, WI, USA). *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was selected as the endogenous reference. The primers of *FABP3*-RT-F/R and *GAPDH*-F/R (Supplemental Table 1) [27] were used for this assay. The cycling conditions were a single cycle of 95 °C for 10 s, 50 cycles of 95 °C for 3 s, 60 °C for 25 s, then a single cycle of 95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s. Other steps were followed with SYBR Premix Ex TaqTMII (TaKaRa, Biotechnology Co. Ltd, Dalian, P.R.C.) using the ABI 7500 RT-PCR system (Applied Biosystems, Foster City, Calif, USA). The results were analyzed using the $2^{-\Delta\Delta C_t}$ method.

Cell culture, transfection and dual-luciferase reporter assay

Mouse myoblasts of the C2C12 and 3T3-L1 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) (Gibco-Invitrogen, Carlsbad, CA, USA) under humidified air

containing 5 % CO₂ at 37 °C. Seven serial deletion sub-clones were amplified with 5'-nested PCR primers (*FABP3*-F1 ~ F7, Supplemental Table 1) and a common 3' primer (*FABP3*-R, Supplemental Table 1), which were cloned into the *SacI*-*XhoI* sites of pGL3-basic vector (Promega Corp., Madison, WI, USA). After sequencing verification, the plasmids were extracted with an Endo-free Plasmid Mini Kit (Omega Biotek, Norcross, GA, USA). The plasmids (0.8 µg) and the lipofectamine 2000 (2 µl) were co-transfected into C2C12 or 3T3-L1 cells grown in 24-well plates. To normalize the transfection efficiency, the cells were co-transfected with 10 ng of Renilla luciferase reporter plasmids (pRL-TK vector, Promega Corp.). The pGL3-basic vector was used as the external control. At 5 h after transfection, the media was replaced by DMEM with 2 % horse serum (HS) (Gibco-Invitrogen, Carlsbad, CA, USA) to induce differentiation of C2C12 myoblasts to myotubes for 40 h. At 45 h after transfection, the cells were lysed and analysed with a dual-luciferase reporter assay according to the manufacturer's instructions [25, 26].

Statistical analysis

All values are expressed as the mean ± standard deviation (SD). Differences between groups (gene expression levels of real-time PCR between tissues, relative luciferase activities between different constructs) were tested with the Student's T test (two-tailed). The result was considered significant when the p-value was less than 0.05. The result was considered highly significant when the p-value was less than 0.01.

Results and discussion

Spatial expression pattern of bovine *FABP3* gene

The results of real-time PCR analysis showed the highest expression of bovine *FABP3* in heart, followed by *biceps femoris* muscle, *longissimus dorsi* muscle, large intestine, small intestine, subcutaneous fat, cecum, rumen, lung, reticulum, omasum, liver and abomasum (Fig. 1). Fluorescence signals were not detected in spleen, kidney or testis fat. The expression level of *FABP3* in heart was highly significantly different from others. The results were generally consistent with that of *FABP3* in rats [28].

Transcription initiation sites of the bovine *FABP3* gene

To date, there are no published reports about transcription initiation sites in the bovine *FABP3* gene. In the present study, 524 and 402 bp fragments were amplified in the first

and second PCR, respectively (Fig. 2). Three different 5' ends were discovered in the first exon based on sequencing results of 13 positive clones. There were four, three and six positive clones extended to the 62, 60 and 59 bp respectively upstream from the translational start site (Fig. 4). The guanine residue (G) in the 5'-flanking sequence, 62 bp upstream from the translational start site, was designated as +1. After comparison with the *Bos Taurus* Assembly (BTA) reference AC_000159.1 (range from 122723225 to 122783830) and NC_007300.5 (range from 127602177 to 127609785), the identified transcription initiation site (+1) extended 16 bp to 5'-end (5'- GCTGGTCCCAGAGTCC-3').

Characterization of the bovine *FABP3* gene 5'-regulatory region

A 1998 bp fragment of the bovine *FABP3* gene 5'-regulatory region spanning nucleotides from -1822 to +176 was cloned and submitted to GenBank database (GenBank No. KJ649748). Several transcription factor binding sites were recognized by sequence analysis of 5'-flanking sequences (Fig. 3). *FABP3* mRNA was expressed most highly in heart, consistent with the hypothesis that promoters of tissue-specific expression genes usually contain a TATA-box [29]. In addition, four CpG islands were predicted in the bovine *FABP3* gene promoter using Meth-Primer (Fig. 4), which indicated that the transcription activity might be dependent on the CpG islands methylation level. In human, the methylation status of *FABP3* is associated with phenotypes of cardiovascular disease [30].

Compared with the 5'-regulatory sequence (GenBank No. KJ649748) of bovine *FABP3*, the 5'-flanking sequences of cattle, sheep, pigs and dogs shared 99.95, 91.73, 77.11 and 29.58 % sequence similarity. However, the 5'-flanking sequences of human, rats, mice and chickens did not show significant sequence similarity with that of bovine *FABP3*. The result indicates that the function and transcription mechanism of bovine *FABP3* might be similar to that of sheep.

Transcriptional activity of the bovine *FABP3* gene promoter

To identify the transcriptional activity of the bovine *FABP3* gene 5'-regulatory region, seven serial deletion constructs in pGL3-basic containing -1822/+176, -1584/+176, -1284/+176, -984/+176, -684/+176, -384/+176 and -84/+176 were generated. The transcriptional activities of the different plasmids constructs were determined by transfecting them into C2C12 cells. The results of dual-luciferase reporter assay indicated that transcriptional activity of the construct -384/+176 was

Fig. 1 Tissue distribution analysis of bovine *Fatty acid binding protein 3 (FABP3)* mRNA. The values of *FABP3* mRNA were normalized to housekeeping *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* mRNA. Error bars indicate the SD (n = 3). The significance levels of T tests in comparison to the expression level of heart are indicated with **P < 0.01

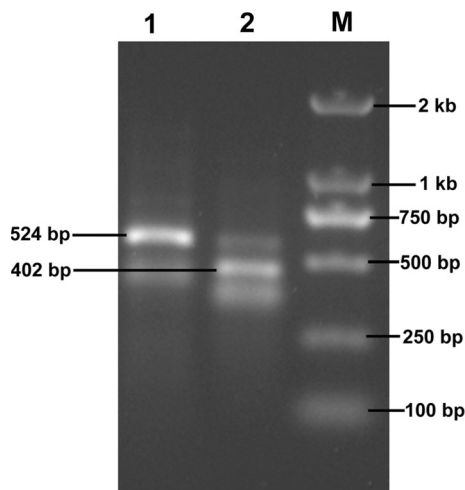
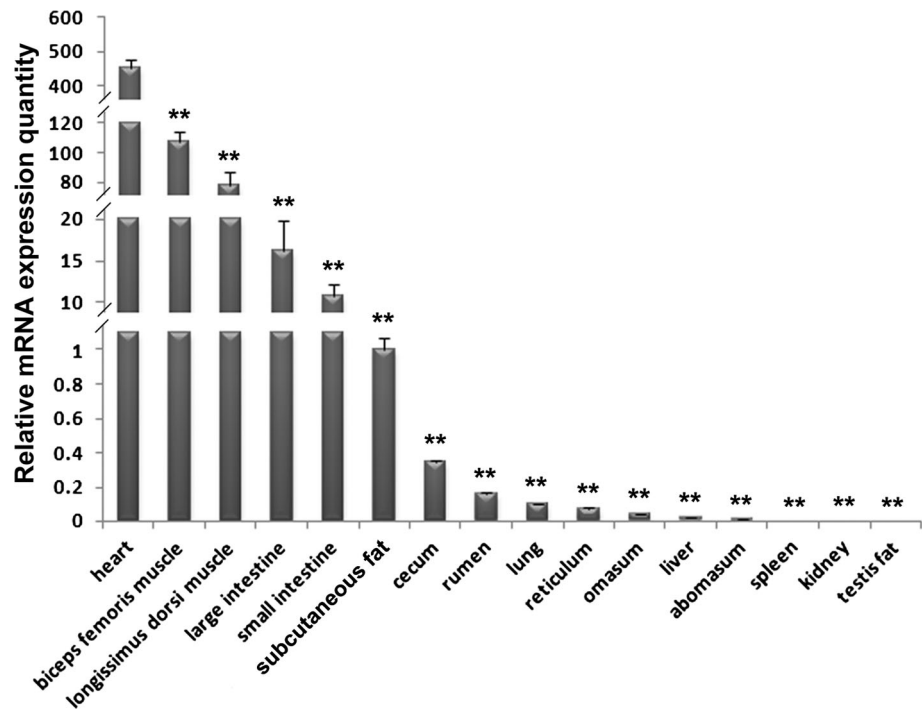


Fig. 2 The results of 5'-Rapid amplification of cDNA ends (5'-RACE) analysis. Lane 1 and 2 represent the first and second PCR products, respectively. Lane M represents the marker of DL2000

approximately ten-fold greater than pGL3-basic without promoter (Fig. 5a). The activities of the other constructs were lower than that of the construct $-384/+176$ and higher than that of pGL3-basic. When the C2C12 cells were induced by HS after transfection, the transcriptional activity of the construct $-384/+176$ was significantly increased and highest in the seven constructs (Fig. 5a). While the construct plasmids were transfected into 3T3-L1 cells, the transcriptional activity of the construct $-384/+176$ was also highest in the seven constructs (Fig. 5b).

These results indicated that a core functional promoter was present in the upstream region of 384 bp from the transcription initiation site (+1), consistent with previous findings that the core promoter was generally near the transcription initiation site [31, 32].

To identify positive regulatory elements among the eight species, the region from nucleotides -384 to $+64$ was analyzed by transcription factor binding sites prediction software. The result of multi-alignments showed that retinoid X receptor alpha (RXR α), Kruppel-like factor 15 (KLF15), specificity protein 1 (Sp1), cAMP responsive element-binding protein (CREB), translation elongation factor EF-1 alpha (TEF-2), TATA-box and CCAAT-box sites were conservative in domestic animals including cattle, sheep, pigs and dogs in this region (Fig. 6). RXR α is a nuclear receptor that can form a heterodimer with PPARs to regulate the transcription of genes involved in fatty acid storage and glucose metabolism [33]. It is also reported that peroxisome proliferator activated receptor alpha (PPAR α) is required but not sufficient for transcriptional activation of *FABP3* in the muscles of mice [20]. In the present study, three PPAR α transcription factor binding sites were found on the 5'-flanking sequences of the bovine *FABP3* gene.

KLF15 is a member of the Kruppel-like factors (KLFs) family that regulate diverse arrays of biological processes including cell stemness, proliferation, differentiation, apoptosis and energy metabolism [34]. KLF15, as a critical regulator, cooperates with PPAR α to regulate cardiomyocyte lipid gene expression [35]

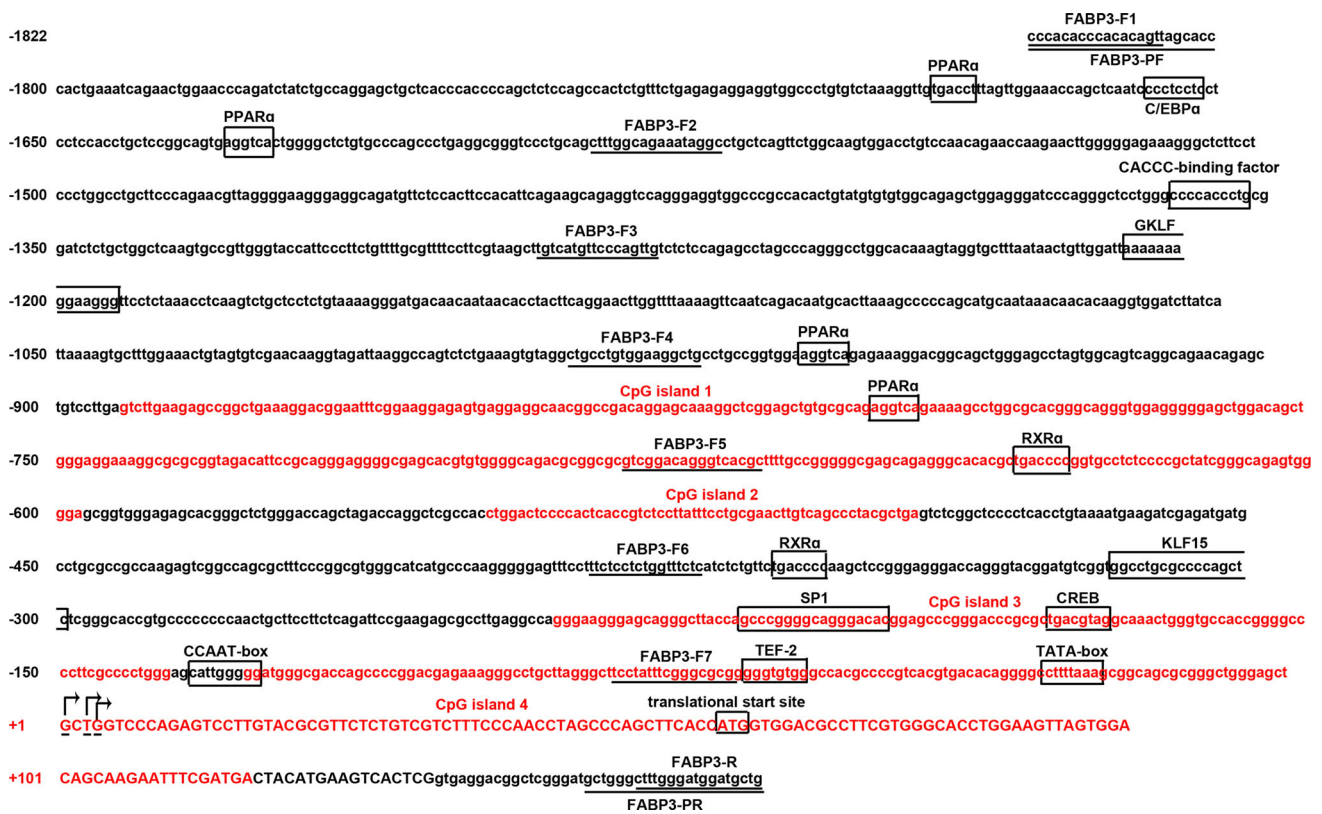
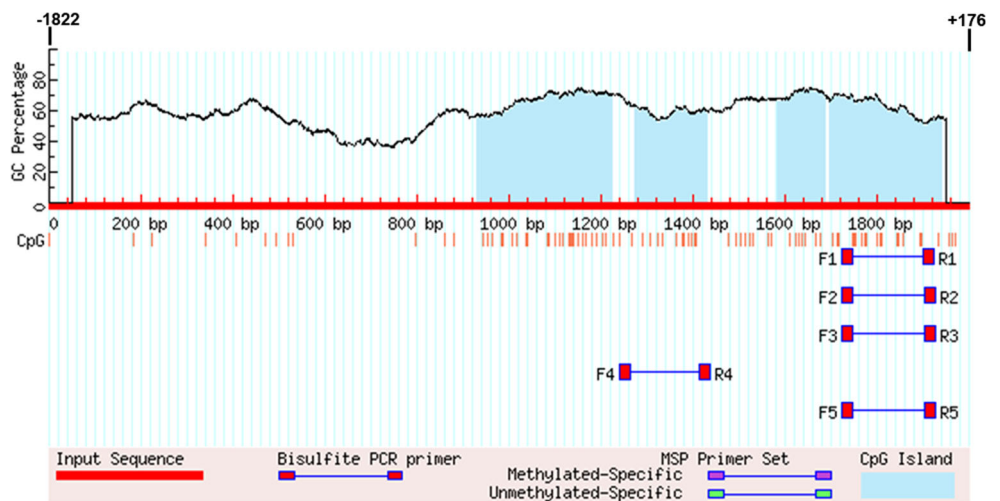


Fig. 3 Nucleotide sequences of the 5'-regulatory region of the bovine *Fatty acid binding protein 3 (FABP3)* gene. Arrows indicate the transcription initiation sites. The guanine residue (G) is designated as

+1, other nucleotides are numbered relative to it. The transcription factor binding sites are indicated with boxes. The PCR primers are underlined. Four CpG islands are indicated in red. (Color figure online)

Fig. 4 The methylation level analysis of the 5'-regulatory region of the bovine *Fatty acid binding protein 3 (FABP3)* gene. Four CpG islands are indicated in blue. (Color figure online)



and plays an important role through regulating the expression of PPAR γ in transcriptional regulation of adipogenesis [36].

Sp1 is a zinc finger transcription factor that binds to GC-rich motifs of many promoters; it is involved in multiple cellular processes such as cell differentiation, cell growth and apoptosis [37, 38].

CREB is a member of the leucine zipper family of DNA binding proteins, which can form both homo- and heterodimers with related factors to regulate the transcription of adipocyte-specific genes for *C/EBP β* , *FABP4*, and *ADIPOQ* in 3T3-L1 cells [39–41].

In addition, it is reported that Sirt1 can also regulate the expression of *FABP3* gene partly through the PPAR γ in pig

Fig. 5 Analysis of bovine *Fatty acid binding protein 3 (FABP3)* gene promoter activities. 7-serial deletion constructs in pGL3-basic were transfected into C2C12 myoblasts or myotubes (a) and 3T3-L1 cells (b). Error bars indicate the SD (n = 3). The significance levels of T-tests in comparison to the construct -384/+176 are indicated with *P < 0.05 or **P < 0.01

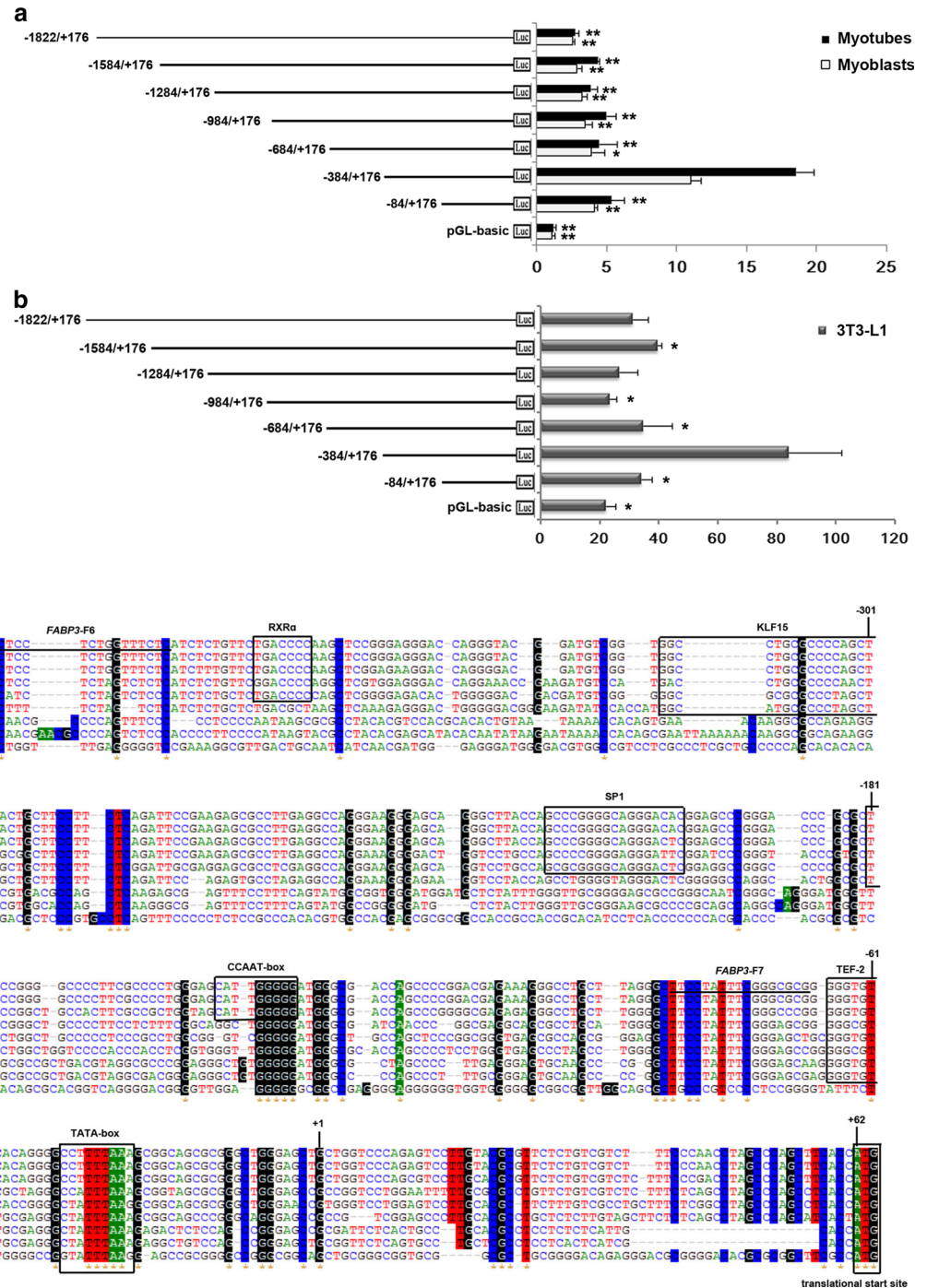


Fig. 6 Multi-alignments sequence analysis of core functional promoter of *Fatty acid binding protein 3 (FABP3)* in mammals. The transcription factor binding sites are indicated with boxes. Nucleotide

sequences are numbered in 5'-regulatory sequence of the bovine *FABP3* gene (GenBank No. KJ649748)

adipocytes [21]. Taken together, we speculate that RXR α cooperated with PPAR α to regulate the expression of *FABP3* in muscle, while KLF15 and CREB interact with PPAR α or PPAR γ to regulate the expression of *FABP3* in adipocytes.

Conclusions

In summary, this study provided important information about the expression and regulation of bovine *FABP3*. The promoter sequence of bovine *FABP3* gene was highly

similar to the homologs of sheep. It was highly expressed in heart and muscles. Its core promoter was located on the sequence from +384 to +1, where transcription factor binding sites RXR α , KLF15, CREB and Sp1 were conservative among cattle, sheep, pigs and dogs. These results provide further understanding of the function and transcriptional regulation mechanism of the *FABP3* gene.

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