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Original article

Identification of a differentially expressed gene, *ACL*, between Meishan × Large White and Large White × Meishan F1 hybrids and their parents

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Abstract – ATP-citrate lyase (ACL), one of the lipogenic enzymes, catalyses the formation of acetyl-coenzyme A (CoA) involved in the synthesis of fatty acid and cholesterol. In pig, very little is known about the *ACL* gene. In this work, the mRNA differential display technique was used to analyse the differences in gene expression between Meishan and Large White pigs and the F1 hybrids of both direct and reciprocal crosses. Our results show that among the differentially expressed genes *ACL* is up-regulated in the backfat of the F1 hybrids. After cloning and analysing the full-length cDNA and the 870 bp 5′-flanking sequence of the porcine *ACL* gene, a *C/T* mutation at position −97 bp upstream of the transcription site was detected. Luciferase activity detection showed that this mutation changed the transcriptional activity. In F1 hybrids, the heterozygous genotype *CT* was more frequent than the homozygous genotypes *CC* and *TT*. Real-time PCR analysis showed that in Meishan pigs, *ACL* mRNA expression was more abundant in individuals with genotype *CT* than in those with genotype *CC* or *TT* or in Large White pigs. These results indicate that the *C/T* mutation affects *ACL* mRNA expression, probably *via* the activator protein 2.

differential gene expression / ATP-citrate lyase / promoter / mutation / pigs

1. INTRODUCTION

Significant phenotypic differences exist between Chinese indigenous Meishan pigs and western commercial Large White pigs. The latter present higher growth rate, carcass lean meat percentage and feed to body weight conversion ratio,

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whereas Chinese indigenous pigs have higher prolificacy, superior meat quality and strong resistibility. Offspring produced by crossbreeding distantly related breeds frequently display greater vigour, size, resistance, *etc*., than the respective parents. Since phenotypic variances mainly result from genotypic differences, it is necessary to provide experimental evidence for the genetic basis to differences between hybrids and their parents. In our laboratory, using suppression subtractive hybridization (SSH) and mRNA differential display, we have detected significant differences in mRNA quantities and expression patterns for several genes between porcine F1 hybrids and their parents [12,17,19]. Thus, cloning and characterizing genes that are differentially expressed between hybrids and their parents should provide further insights into the genetic basis of phenotypic differences. In this work, we show that, in backfat, the gene ATP-citrate lyase (*ACL*) is differentially expressed between Meishan × Large White and Large White × Meishan F1 hybrids and their parents.

ACL is a cytosolic enzyme that catalyses the formation of acetyl-coenzyme A (CoA) and oxaloacetate from citrate and CoA, with the hydrolysis of ATP to ADP and phosphate [7]. Since the acetyl-CoA produced by ACL is involved in the synthesis of fatty acid and cholesterol, ACL is considered as one of the lipogenic enzymes like fatty acid synthase and acetyl CoA carboxylase [3]. In mammals, the activity of ACL is regulated by diet regimen and insulin [6]. It is generally believed that changes in ACL activity in terms of the *de novo* lipogenesis state are due to alterations in the rate of its biosynthesis [4]. In rat, it has been shown that changes in ACL activity correlate with modifications of its mRNA concentration and transcription rate, and that *ACL* mRNA amounts begin to decrease when the level of hepatic lipogenesis is low [5]. Other studies have reported that *ACL* expression in liver is regulated at the transcriptional level by SREBP-1 [18] and that lipid biosynthesis rates and *ACL* mRNA expression increase when fasted mice are fed a carbohydrate-rich diet [16]. These findings strongly suggest that ACL activity is regulated at the transcription level. However, very little is known about *ACL* and its expression pattern in pig. In this paper, we describe the cloning and expression profile of the porcine *ACL* gene. In addition, we present the characterization of its transcriptional activity and the detection of a mutation in its promoter region that alters transcriptional activity.

2. MATERIALS AND METHODS

2.1. Animals

Fifty-eight Large White pigs, 33 Meishan pigs, 81 Large White × Meishan pigs and 48 Meishan × Large White pigs maintained at the Huazhong Agricultural

University Jingpin Pig Station were fed the same diet and sampled at the age of six months.

2.2. Differential display of mRNA

Three boars and three sows for each Meishan × Large White and Large White × Meishan hybrid and their parents *i.e.* a total of 24 pigs were sampled. Total RNA was isolated from the backfat of these 24 pigs and for each breed and each hybrid, the RNA from six individuals was pooled into one tube, respectively. Total RNA samples were treated with DNase I (Promega, USA) to eliminate any contaminating genomic DNA. Subsequently, cDNA sequences were synthesized with M-MLV reverse transcriptase and an oligo (dT)15 anchored primer (Promega, USA). Differential display PCR was carried out as described by Ren *et al*. [17]. After differential display, cDNA fragments were re-amplified, cloned and sequenced. The sequences were compared with those available in GenBank using BLAST.

2.3. Reverse transcription PCR analysis

Semi-quantitative RT-PCR was used to evaluate *ACL* expression in the backfat of F1 hybrids and their parents. The primer pair, GHF and GHR, was synthesized to amplify specifically the housekeeping gene, glyceraldeyhyde-3-phosphate dehydrogenase (*GAPDH*), as an internal control (all primer sequences used in this study are presented in Tab. I). The differentially expressed cDNA fragment, EST39, was detected with the gene-specific primers EST39F and EST39R. *GAPDH* and EST39 were amplified in separate tubes and electrophoresed on 1.5% agarose/ethidium bromide gels. Densitometry values were measured using the BandScan software (www.Glyko.com). RT-PCR values are presented as ratios between the EST39 signal in the selected exponential amplification cycle and the *GAPDH* signal. Each sample was amplified eight times. In addition, semi-quantitative RT-PCR was used to identify porcine *sp1*, *SREBP-1* and *SREBP-2* expression in backfat of F1 hybrids and their parents.

2.4. Cloning of the *ACL* cDNA and its 5′-flanking sequence

Switching Mechanism At 5′ end of the RNA Transcript (SMART) cDNA was synthesized using the SMART PCR cDNA Synthesis Kit (Clontech, USA) for RACE-PCR. The 5′ and 3′ ends of *ACL* cDNA were obtained with primer pairs Smart5′/GSP1 and Smart3′/GSP2, respectively.

Table I. Primers used in the present study.

Primers	Sequences (from 5′ to 3′)	Annealing temperature (°C)	Gene amplified
GHF	ACCACAAGTCCATGCCATCAC	58	*GAPDH*
GHR	TCCACCACCCTGTTGCTGTA		
EST39F	CCCTTTGCCATTGTTATA	57	*EST39*
EST39R	TCAGAGGTCGGTCAAACG		
sp1F	ACGGGCAATACCCTCTGG	55	*sp1*
sp1R	AGGACTCGTCGGGAAGCA		
SREBP-1F	CCACCAGTCCTGATGCCA	54	*SREBP-1*
SREBP-1R	AGCCTTCAAGCGGGGAG		
SREBP-2F	CAAGCTCTTGAAAGGCATCG	58	*SREBP-2*
SREBP-2R	AGAGGGCTTCCTGGCTCA		
Smart5′	AACGCAGAGTACGCGGG	57	*ACL*
GSP1	CAGCCAAGGGTGGTCCTGC		
Smart3′	CAGAGTACTTTTTTTTTTTTTTTT	57	*ACL*
GSP2	AGCAGGGGCTGTATCGTC		
R1	NGTCGASWGANAWGAA		
R2	GTNCGASWCANAWGTT		
R3	WGTGNAGWANCANAGA		
R4	NCAGCTWSCTNTSCTT		
ACSE	CTGCTCTCTACGAAAGGCCGTGC		
ACSF	CCCAACTCGCCGCCTACCTTCC		
ACSG	TCGCCGCCTACCTTCCGGAGCGC		
RGHF	ACCACAAGTCCATGCCATCAC	58	*GAPDH*
RHGR	TCCACCACCCTGTTGCTGTA		
RACLF	TCTGGGAGGTGTCAACGAG	58	*ACL*
RACLR	GGTCTTGGCATAGTCATAGGT		
AC996F	GCTACGCGTTCAGCACTATCAGATCGGG		
AC756F	GCTACGCGTCCTTCCTAGCCCCACCT		
AC698F	GCCACGCGTATCTATTAGCCTCGTCCCAC		
AC486F	GATACGCGTCAGCCCGCCACATCTCAG		
AC374F	GATACGCGCATAGCCCAGCCCATCTC		
AC216F	GATACGGCGAATTGGGAGGAAGCC		
AC169F	GATACGCAATCGCCGGGCGGCTCGC		
AC158F	GATACGCGGCTCGCACGGTGTGCC		
ACR	GTACTCGAGCTGCTCTCTACGAAAGGCC		

The 5′-flanking sequence of the *ACL* gene was amplified by genome walking based on Thermal Asymmetric Interlaced PCR (TAIL-PCR) [11]. R1, R2, R3 and R4 were used as arbitrary primers and ACSE, ACSF and ACSG as gene-specific primers. All PCR products were cloned into pMD-18T vector (Takara, Japan) and sequenced commercially.

2.5. Mutation detection and genotyping

The 5′-flanking region of the *ACL* gene was amplified by PCR from genomic DNA of three Meishan and three Large White pigs with primer pairs AC996F and ACR, and sequenced to identify novel mutations. Allele frequencies were then determined in the different pig populations.

2.6. SYBR Green RT-PCR analysis of *ACL* expression

Relative quantitative RT-PCR was performed as follows: denaturation at 95 °C for 2 min followed by 45 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 18 s (ABI, USA). Porcine *GAPDH* and *ACL* genes were amplified with primers RGHF/RGHR and RACLF/RACLR, respectively.

For spatial expression analysis, total RNA was also isolated from various Meishan pig tissues including backfat tissue, *Longissimus dorsi*, heart, liver, spleen, lung, kidney, stomach, uterus, ovary and small intestine. Each sample was repeated four times and the comparative C_t ($\Delta\Delta C_t$) value method [13] was used to compute relative quantifications. Expression levels were considered as undetectable when the C_t value of the targeted gene exceeded 35 in the sample tissue.

2.7. Plasmid construction

A 1014 bp DNA fragment was amplified by PCR from porcine genomic DNA with primers AC996F and ACR as sense and anti-sense oligonucleotides, respectively. *Mlu I* and *Xho I* restriction sites were introduced in the 5′ ends of AC996F and ACR, respectively. The PCR product was double-digested by *Mlu I* and *Xho I*. The *Mlu I*/*Xho I* (−853/143 bp) fragment was subcloned into the pGL3-Basic vector (Promega, USA) to yield construct −853 bp. Constructs −613, −555, −343, −231, −73, −27 and −15 bp were also produced using the forward primers AC756F, AC698F, AC486F, AC374F, AC216F, AC169F and AC158F in combination with the reverse primer ACR, respectively. The constructs were identified by double-digestion and sequencing. This method refers to Butta *et al*. [1].

2.8. Cell culture, transient transfection and luciferase assay

Pig kidney cells (PK-15) purchased from China Center for Type Culture Collection were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) bovine calf serum (Gibco, USA) and maintained at 37 °C in 5% CO_2.

Cells were seeded into 24-well plates at an initial density of 60–80% and cultured overnight to ensure adhesion and spreading. Co-transfections were then performed using 2 μL of Lipofectamine 2000 reagent (Invitrogen) with 3 μg of the firefly luciferase plasmid DNA, and 0.6 μg of pRL-TK plasmid DNA (Promega, USA) as an internal control. For co-transfection analyses, the levels of reporter plasmids were kept constant. The pGL3-Control vector (Promega, USA) was used as a positive control. After 6 h, the transfection medium was removed and replaced with growth medium.

Transfected cells were collected by rocking the plates for 15 min with 1 X passive lysis buffer (PLB, Promega, USA). Firefly and *Renilla* luciferase activities were measured at 48 h post-transfection using the Dual-Glo Luciferase Assay System (Promega, USA) and a TD20/20 luminometer (Turner Designs). In each case, transfection efficiencies were normalized using the *Renilla* luciferase activity levels and each construct was tested in triplicate in a minimum of three independent experiments. In addition, a *t*-test was performed to compare the transcriptional activities between these recombinants.

3. RESULTS

3.1. Identification of EST39, an up-regulated gene, in F1 hybrids

One band, designated as EST39 and visualized only in the Large White × Meishan and Meishan × Large White F1 hybrids, was isolated from the differential display gel (Fig. 1A) and re-amplified. Semi-quantitative RT-PCR analysis showed that the expression level of EST39 in backfat was higher in the F1 hybrids than in their parents (Fig. 1B).

3.2. Cloning and analysis of porcine *ACL* gene

The differentially expressed EST39 shares 88% sequence identity with the human *ACL* gene. A 3463 bp contig was constructed by *in silico* cloning using the GenBank ESTs database. We obtained a 4378 bp full-length porcine *ACL* cDNA (GenBank Accession No. EU073662) by 5′ and 3′ RACE-PCR. Porcine *ACL* gene contains a 3231 nucleotide (nt) open reading frame. We inferred that the ATG codon at nt residue 134–136 is the true start site of translation, because it begins the longest reading frame and is preceded by one in-frame stop codon in the 5′ untranslated region [8].

Analysis of a 870 bp sequence in the 5′-flanking region (GenBank Accession No. EU073663) of the porcine *ACL* gene obtained by TAIL-PCR showed no TATA-like elements but a high G + C content in the proximal promoter region. Potential binding sites for the transcription factors were predicted using

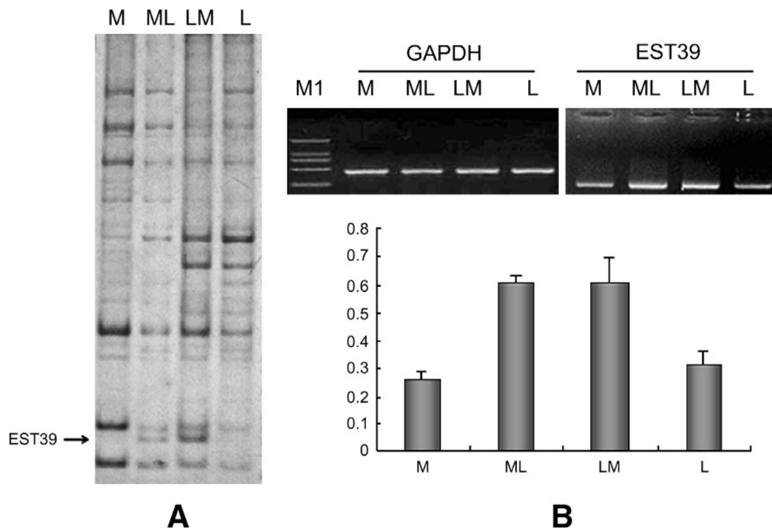

Figure 1. Identification of EST39 an up-regulated gene in the backfat of F1 hybrids as compared with their parents. (A) Silver staining of mRNA differential display. The arrow points to EST39. M, ML, LM and L represent Meishan, Meishan × Large White, Large White × Meishan and Large White pigs, respectively. (B) Semi-quantitative RT-PCR analysis of EST39 and the bar graph of the percentage of EST39/*GAPDH*.

the software "Searching Transcription Factor Binding Sites" with an 85 threshold score (TFSEARCH program, Version 1.3). The following transcription factors were investigated: stimulating protein 1 (sp1), GATA (GATA-binding factor) family, heat shock factor, upstream stimulating factor, cap (cap signal for transcription initiation), activator protein 4 (AP-4) and activator protein 2 (AP-2).

3.3. *Xho I* PCR-RFLP polymorphism in the 5′-flanking region of porcine *ACL* gene

A *C*/*T* mutation was found at position −97 bp from the transcription site. The forward (5′-CGCCTTCCTAGCCCCACCT-3′) and reverse (5′-CGCC-GCCTACCT-TCCGGAG-3′) primers amplify a 711 bp product. The *ACL* C-97T introduces a *Xho I* recognition site in the presence of *T*, resulting in the digestion of the 711 bp fragment into two 518 bp and 193 bp fragments, consequently forming three genotypes *CC*, *CT* and *TT*. In addition, the transition from *C* to *T* results in the absence of a binding site for AP-2 as predicted by TFSEARCH.

We have genotyped 58 Large White, 33 Meishan, 81 Large White × Meishan and 48 Meishan × Large White pigs for the *Xho I* PCR-RFLP polymorphism

Table II. Genotype and allele frequencies in F1 hybrids and their parents.

Breed	Number	Genotype frequency			Allele frequency	
		CC	*CT*	*TT*	*C*	*T*
Large White	58	0.776	0.224	0.000	0.888	0.112
Meishan	33	0.182	0.667	0.152	0.515	0.485
Large White × Meishan	81	0.346	0.568	0.086	0.630	0.270
Meishan × Large White	48	0.292	0.521	0.188	0.522	0.478

and calculated genotype and allele frequencies (Tab. II). No genotype *TT* was detected in Large White pigs. Among the four pig populations, allele *C* was more frequent than allele *T*. The homozygous genotype *CC* was preponderant in Large White pigs, whereas genotype *CT* was the most frequent genotype in Meishan pigs and F1 hybrids.

3.4. Expression profile of porcine *ACL*

Real-time analysis was performed to further reveal the differential expression of *ACL* between F1 hybrids and their parents. *GAPDH* was used to normalize the expression level of *ACL*. The relative quantitative results showed that *ACL* mRNA in backfat was up-regulated in F1 hybrids in comparison with their parents (Fig. 2A).

To isolate total RNA from backfat, we selected six Meishan and Large White pigs with genotypes *CC*, *CT* and *TT*, respectively (no genotype *TT* was detected in Large White pigs). RT-PCR results showed that *ACL* mRNA expression was more abundant in Meishan pigs with genotype *CT* than in those with genotype *CC* or *TT* or in Large White pigs (Fig. 2B).

We have also determined the spatial expression of *ACL* in various porcine tissues (Fig. 2C). The highest level of porcine *ACL* mRNA expression was observed in the uterus, followed by the ovary, small intestine, lung, spleen, liver, kidney, backfat and stomach, whereas expression in skeletal and cardiac muscles was weak.

In addition, we analysed the mRNA expression of porcine *sp1*, *SREBP-1* and *SREBP-2* genes in backfat and the results showed no significant difference between F1 hybrids and their parents (Fig. 3).

3.5. Features of the 5′-flanking region of porcine *ACL* gene

To identify the location of the promoter region in the porcine *ACL* gene, we have studied the transcriptional activity of recombinants with progressively

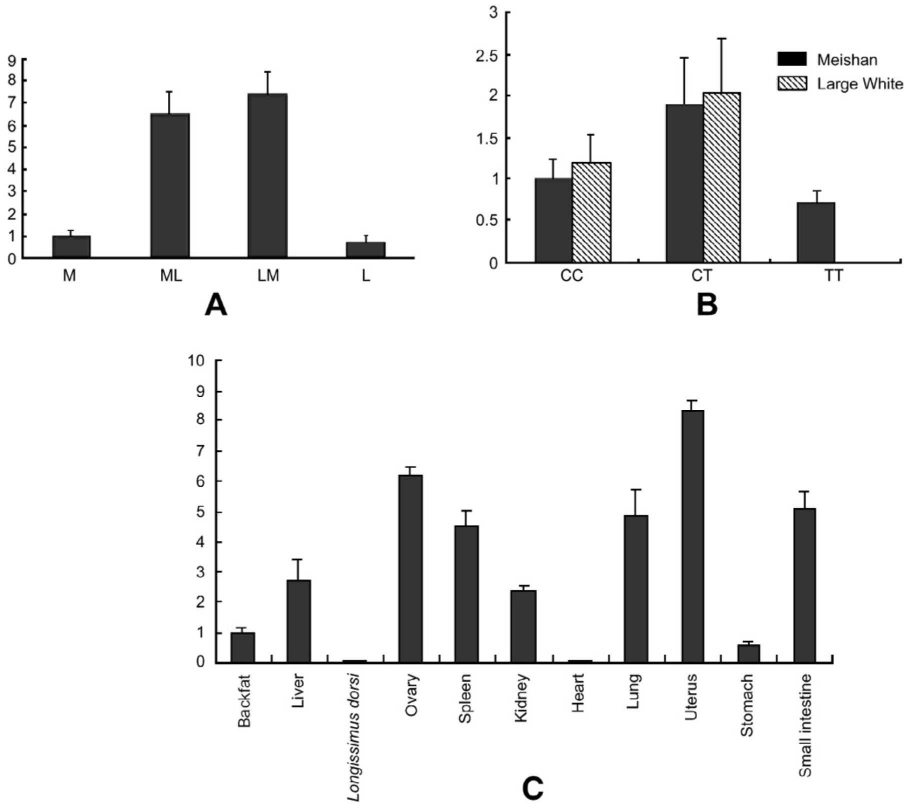

Figure 2. mRNA expression of three isoforms of porcine *ACL* by RT-PCR. Error bars indicate the SD ($n = 4$) of relative *ACL* mRNA expression levels to *GAPDH*, determined by RT quantitative PCR. The values were normalized to the housekeeping gene *GAPDH* expression. (A) Porcine *ACL* mRNA expression in backfat between F1 hybrids and their parents. The value of *ACL* in Meishan pigs was arbitrarily set to 1. (B) Porcine *ACL* mRNA expression in Meishan and Large White pigs with genotypes *CC*, *CT* and *TT*. The value of *ACL* in Meishan pigs with genotype *CC* was arbitrarily set to 1. (C) The tissue distribution of porcine *ACL* including backfat, liver, *L. dorsi*, ovary, spleen, kidney, heart, lung, stomach, small intestine and uterus. The value of *ACL* in backfat was arbitrarily set to 1.

5′-deleted DNA fragments (from −853, −613, −555, −343, −231, −73, −27, −15 to +143 bp, respectively) subcloned into the pGL3-Basic reporter plasmid (Fig. 4A). Recombinants, carrying a C at position −97 bp instead of a T, were transiently transfected into PK-15 cells. Detection of the luciferase relative activity showed that transcriptional activity was not significantly different between recombinants −15, −27 and pGL3-Basic. Activity was detected from construct

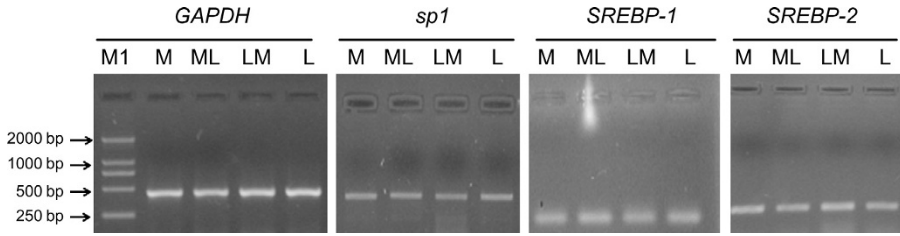

Figure 3. RT-PCR analysis of *sp1, SREBP-1* and *SREBP-2* genes. M1 corresponds to the DNA molecular size marker and M, ML, LM and L to Meishan, Meishan × Large White, Large White × Meishan and Large White pigs, respectively.

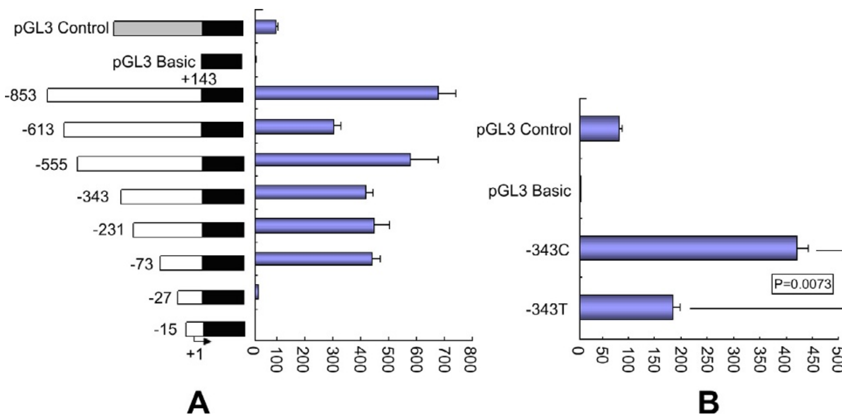

Figure 4. Transient transfection of deletion mutants of the 5′-flanking region of the porcine *ACL* gene. Luciferase activity was corrected for transfection efficiency with the values obtained with *Renilla*. The results are means ± SD of three experiments performed in duplicate. (A) Transcriptional activity of eight recombinants. (B) Comparison of luciferase activity between constructs −343C and −343T.

−73 and increased in constructs from –73 bp to –853 bp with a little fluctuation. Thus, these experiments show that the basal promoter activity is located within the –73 bp to +143 bp region, while the region from –853 bp to +143 bp confers maximal transcriptional activity.

To investigate whether the *C*/*T* mutation alters transcriptional activity, we constructed recombinants −343C and −343T, corresponding to alleles *C* and *T*. Results from transient transfection experiments showed that the transcriptional activity of construct −343T was significantly lower than that of construct −343C ($P < 0.01$) (Fig. 4B).

4. DISCUSSION

Many methods have been used to reveal differential gene expression, such as SSH, cDNA-RDA (cDNA representational differential analysis), SAGE (serial analysis of gene expression) and DNA microarray. Compared with these methods, the advantage of the mRNA differential display technique is that more than two samples can be displayed simultaneously, as shown in our experiments. However, its shortcoming is that it gives a high percentage of false positives and thus, the differentially expressed ESTs displayed in a gel need further identification. In the present study, we have isolated a cDNA fragment (EST39) that is present in the backfat of F1 hybrids but not in the parents. Subsequently, we have confirmed mRNA differential expression of EST39 between F1 hybrids and their parents by both semi-quantitative and RT-PCRs.

Both porcine and rat *ACL* mRNAs are expressed in most tissues [2]. In fact, in human, rat and pig the promoter region of the *ACL* gene has no TATA box, which is usually a feature of housekeeping genes. However using RT-PCR, we have shown that the porcine *ACL* gene is differentially expressed between the F1 hybrids and their parents. Thus, our results suggest that upstream transcription factors or other proteins regulate this differential expression. It has been found that sp1 is one of the most important transcription factors for the *ACL* promoter to produce basal and induced transcription by low fat/high carbohydrate diet [15]. Sterol regulatory element-binding proteins (SREBPs), especially SREBP-1, regulate ACL enzyme activity at the transcriptional level, whereas NF-Y binding is a prerequisite for activating the *ACL* promoter [14]. Diet and various hormones also regulate ACL activity [5,6]. In rats, the concentration of ACL enzyme is increased 20- to 39-fold when they are subjected to fasting and then re-feeding. The promoter sequence of porcine *ACL* gene is highly conserved with those of rat and human, suggesting that regulation at the transcription level is similar in these three species. Based on the above findings, we have analysed the mRNA expression of porcine *sp1*, *SREBP-1* and *SREBP-2* genes between F1 hybrids and their parents and have found no significant difference. Thus, other mechanisms are responsible for the differential expression. BLAST analysis between the *ACL* promoter sequence from Meishan pigs and Large White pigs detected a *C*/*T* mutation at position −97 bp. A transition from C to T in the promoter region is predicted to result in the absence of a binding site to AP-2. Thus, we have constructed recombinant plasmids −343C and −343T, corresponding to −97C and T, and the results have shown that the former has a significantly higher transcriptional activity ($P < 0.01$). A similar effect has been reported for other genes. Kroeger [9] has reported that in PMA-stimulated Jurkat and U937 cells, the −308A allelic form of the tumour

necrosis factor-alpha gene increases two-fold the transcription level as compared with the −308G form. Furthermore, in the *CD14* promoter, a C/T transition at position −159 increases the transcriptional activity and decreases the affinity of Sp1 protein binding [10]. Thus, in the *ACL* promoter, a C/T transition at position −97 probably decreases the affinity of AP-2 protein binding and depresses the transcriptional activity. Accordingly, the transcriptional activity of porcine *ACL* promoter in heterozygotes *CT* or homozygotes *CC* should be higher than in homozygotes *TT* regardless of a dominant or additive effect. In addition, our results show that *ACL* mRNA expression is more abundant in Meishan pigs with the genotype *CT* than in those with genotype *CC* or *TT* or in Large White pigs. Thus, we speculate that the transcriptional activity of the porcine *ACL* promoter in heterozygotes *CT* is higher than in homozygotes *CC* or *TT*, which should help to understand the genetic basis of the differences between F1 hybrids and their parents.

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