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Identification of methylation pattern in the partial promoter of acetyl CoA carboxylase beta (*ACACB*) gene in White Leghorn line

C H SHIVAPRASAD[1]✉, R VINOO[2], R N CHATTERJEE[3], M MURALIDHAR[4], D NARENDRANATH[2], K ASWANIKUMAR[2], T K BHATTACHARYA[5], G SUSHMA[6], M KANAKACHARI[3] and A R PRASAD[7]

ICAR-Directorate of Poultry Research, Rajendranagar, Hyderabad, Telangana 500 030 India

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ABSTRACT

The present study was initiated with an objective of identifying methylation pattern in the partial promoter of Acetyl CoA carboxylase beta (*ACACB*) gene in White Leghorn IWK line. To understand the epigenetic regulation of gene expression, birds with highest and lowest expression at 18th week and 40th week age were chosen. Epigenetic profile of the minimal promoter indicated that there were seven CpG islands in this region, positioned at 75, 87, 155, 269, 284, 311 and 426 positions of the 555 bp promoter region. Among these, except the position 75, the remaining positions showed cis-acting transcription factors. There was negative relationship between highest and lowest expressed birds with the per cent methylation in White Leghorn IWK line. The mean methylation per cent was found to be 42.85 and 21.42 at 18th and 40th weeks of age, respectively. The results indicated decrease in methylation per cent with age. The overall mean methylation per cent of 32.14 was observed.

Keywords: *ACACB* gene, Methylation, Partial promoter, White Leghorn

The acetyl-coenzyme A carboxylase beta (*ACACB*) is a candidate gene that influences fatty acid composition. The word epigenetics was used more broadly to categorize all of the developmental events leading from the fertilized zygote to the mature organism (Waddington 1953). Many scientists defined epigenetics in different ways, as ´the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence' (Riggs *et al*. 1996, Riggs and Porter 1996). Epigenetics describes mechanisms that lead to changed, heritable structural and activation states of the chromatin without changes to the primary nucleotide sequence (Knippers and Nordheim 2015).

A portion of the variability of complex traits is affected by interactions with the environment, through epigenetic phenomena (Feil and Fraga 2012, Skinner 2015). Thus, besides genetic variability, external influences that affect early life stages can have enormous consequences on the adult phenotype (Guerrero and Skinner 2012). Among the environmental influences acting on parental animals, several factors are reported to affect epigenetic processes during early development of the offspring, such as endocrine disrupting chemicals (Susiarjo *et al*., 2013), inorganic chemicals (Kile *et al*. 2014), nutritional compounds (Dolinoy *et al*. 2007, Guerrero *et al*. 2008) and stressful conditions (Fagiolini *et al*. 2009). Altering epigenetics states in some genomic regions can have drastic phenotypic consequences such as changes in the coat colour (Dolinoy *et al*. 2007), and increased disease susceptibility (Guerrero and Skinner 2012, Guerrero and Jensen 2015). Recently, epigenetic reprogramming of gene expression was shown to impact, in the long term, the phenotypes of birds. Notably, several studies have investigated epigenetic marks that might be implicated in behaviour (Nätt *et al*. 2009, Verhulst *et al*. 2016), adaptation (Yossifoff *et al*., 2008, Kisliouk and Meiri 2009) and the impact of nutrition on chicken performance (Li *et al*. 2016). In recent years, a growing number of studies have revealed that epigenetic modifications could be transmitted across generations (Miska and Ferguson 2016), in domesticated birds transgenerational inheritance until the F3 generation (Leroux *et al*. 2017), parent-to-offspring transmission in bird species and impact of early stressors on behaviour (Carlos *et al*. 2018).

DNA methylation is a stable epigenetic modification found in most of the eukaryotes that plays a crucial role in many biological processes, including gene expression regulation, gene imprinting and transposon silencing in mammals and plants (Bird 2002, Goll and Bestor 2005, Zhang *et al*. 2006). DNA methylation involves the addition

Present address: [1]College of Veterinary Science, PVNRTVU, Rajendranagar, Hyderabad, Telangana. [2]NTR College of Veterinary Science, Gannavaram, Andhra Pradesh. [3]ICAR-Directorate of Poultry Research, Rajendranagar, Hyderabad, Telangana. [4]College of Veterinary Science, Garivide, Andhra Pradesh. [5]ICAR-National Research Centre on Equines, Hisar, Haryana. [6]College of Veterinary Science, Tirupati, Andhra Pradesh. [7]College of Veterinary Science, Mamunoor, Telangana. ✉Corresponding author email: prasadshivach21@gmail.com

of a methyl group to the fifth position of cytosine in the context of CpG dinucleotides, which are underrepresented in DNA. Clusters of CpGs, called CpG islands, are often found in association with genes, most often in the promoters and first exons (Jones and Takai 2001, Takai *et al*. 2001). In mammals, cytosine DNA methylation occurs mostly at the CpG dinucleotides except for the CpGs in CpG islands (Weber *et al*. 2005). Although many genome-wide DNA methylation profiles and their functional analysis have been reported, there is little knowledge about the DNA methylation patterns in birds (Eckhardt *et al*. 2006, Zilberman *et al*. 2007, Lister *et al*. 2008, Gehring *et al*. 2009, Hsieh *et al*. 2009). Perusal of literature revealed that the identification of methylation pattern in the partial promoter of Acetyl CoA carboxylase beta (*ACACB*) gene in White leghorn line was not done in chicken. Hence the present study is aimed at epigenetic regulation of *ACACB* gene in chicken.

MATERIALS AND METHODS

Experimental birds: The present study was conducted on IWK lines of the White Leghorn breed maintained at ICAR-Directorate of Poultry Research (ICAR-DPR), Hyderabad. IWK line is being subjected to selection for higher egg production up to 64 weeks of age as well as for higher egg weight at 28 weeks of age.

Sample collection: Breast muscle tissues were collected to study the trend of *ACACB* gene expression. Before on set of egg production (i.e. 18th WK) 4 birds, during 40th week, 4 birds were slaughtered to collect breast muscle tissues under aseptic conditions following the approved slaughter protocol of ethical committee of ICAR-Directorate of Poultry Research, Hyderabad. Breast muscles were collected in 1.5 ml DNAse, RNase free sterile polypropylene tubes under aseptic conditions using 0.1% Diethyl Pyrocarbonate (DEPC) treated sterile instruments. Samples were immediately chilled on ice to minimize RNA degradation and transferred to lab on ice in ice pack box and kept in -80°C until further use.

RNA extraction: Total RNA was isolated from embryo and muscle tissues using Trizol (Amresco), according to the manufacturer's protocol. Resulted RNA pellet was re-suspended in 50 µl nuclease-free water and the concentration and quality were determined using respective Genova plus NanoDrop and 1.2% formaldehyde agarose gel. The RNA sample showing the OD_{260}:OD_{280} values in between 1.8 to 2.2 were considered as good quality and were used further.

First strand cDNA synthesis: Each sample of RNA was treated with DNaseI (Fermentas) and converted in to cDNA using Verso cDNA synthesis kit (Thermo Scientific, #00775881). This reverse transcription was carried out in thermocycler (Himedia) using the components 5× cDNA Synthesis Buffer (4 µL), Anchored Oligo dT (0.25 µL), Random Hexamer (0.75 µL), dNTP Mix (2 µL), RT Enhancer (1 µL), Verso Enzyme Mix (1 µL), RNA template (RNA) (2 µg) and Nuclease-free H_2O (make up the volume to 20 µL) under the condition 42°C @ 30 min and 95°C @ 2 min. The resulted cDNA was confirmed by agarose gel electrophoresis and stored at -20°C until further use. The *ACACB* and *GAPDH* genes were amplified by using cDNA as a template in RT-PCR with gene specific primers and were then confirmed by 1% agarose gel electrophoresis. The *GAPDH* gene was used as internal housing keeping gene for quantification of *ACACB* expression in real-time PCR (Bhattacharya *et al.* 2011).

Real-time quantitative PCR (qPCR): Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control for normalizing different amounts of input RNA. Intron-spanning primers used for qPCR expression were designed using the IDT Primer Quest software (Table 1). The mRNA expression levels of the target (*ACACB*) and reference (*GAPDH*) genes were quantified by using thermal cycler Applied Biosystems® Step One Real-time PCR (Life Technologies) with BrightGreen 2× qPCR MasterMix-ROX (abm). All the PCR reactions were performed in three biological replicates with a final volume of 10 µL containing 5 µL of BrightGreen 2× qPCR MasterMix-ROX, 0.5 µL of each forward and reverse primer (10 µM), 1 µL of cDNA and 3µl of nuclease-free water. Thermal cycling conditions followed were initial denaturation at 95°C for 10 min followed by 40 cycles of PCR stage (95°C for 15 sec and 58°C for 1 min) and melting stage (95°C for 15 sec, 60°C for 1 minute and 95°C for 15 sec) was performed at the end of the qPCR to check the specificity of amplification.

Identification of epigenetic signatures in ACACB gene: The collected breast muscle tissues were used so as to determine the methylation profile of *ACACB* promoter sequence. The IWK line birds were selected based on their expression level (i.e. the one showing highest and lowest expression levels (40-ΔCt)) at 18th week and 40th week of age and the genomic DNA was isolated from breast muscle tissue using Phenol-Chloroform extraction method (Sambrook and Russell 2001) with slight modification.

Bisulfphite modification of genomic DNA: Bisulfphite treatment was done as per the manufacturer's protocol for the above isolated DNA samples in free 200 µL PCR tubes with the help of Nucleo-pore® DNA methylation kit (Genetix NP-5005D, New Delhi). The resulted eluted DNA was kept at -20°C till use.

Table 1. Primer details for amplification of genes

Gene symbol	Primer sequences (5'-3')	Amplicon size (bp)	Annealing temp (°C)
ACACB	F: GCTCCTGCTGCCCATATATTA R: GTCCGTGATGACACCTTTCT	94	58
GAPDH	F:ATGGGAAGCTTACTGGAATGG R:TCATCATACTTGGCTGGTTTCT	97	58

Amplification of bisulphite modified DNA: The primers were designed based on the sequence upstream to the transcription start site of *ACACB* gene. The region was selected based on the binding of transcription factors for the expression of gene. It was assumed that all unmethylated cytosine gets converted into uracil that is being read as thymine in PCR amplification. The principle behind bisulfite modification is conversion of cytosine to uracil that is being read as thymine in following amplification and sequencing. The methylated and unmethylated cytosines in the sequence can be identified easily as the former ones are resistant to conversion, i.e. immune to bisulfite modification (Frommer *et al.* 1992). CpG island of respected promoter sequence was predicted by Methyl primer express software v1.0 (Applied Biosystem).

The sequence upstream to the transcription start site of *ACACB* gene was divided into two fragments of 322 bp and 233 bp size. Bisulphite PCR primers (Table 2) for the two fragments of each were designed following the detailed description of primer guidelines (Li and Dahiya 2002).

Table 2. Primers used for methylation of *ACACB* gene promoter

Primer name	Primer sequence (5'-3')	Size (bp)
ACACB-M1	F: GTGAGAGGTTTGGTTGTTTA R: TCCTTATAAAACAAAAATACCAT	322
ACACB-M2	F: TTAGGTGAGTTTTATGGTATTG R: CAACCAAACCTCTCACATTC	233

Table 3. PCR reaction mix of methylation of *ACACB* gene promoter

Component	Amount (µl)	Final concentration
10× Dream Taq Buffer	2.5	1×
2.5 mM dNTP mix	0.5	50 µM
Forward primer (20 ng/µL)	1.5	20 ng
Reverse primer (20 ng/µL)	1.5	20 ng
Taq DNA Polymerase (5U/µL)	0.2	0.5 units
Bisulfite modified DNA	2.0	80-100 ng
Nuclease-free water	16.8	---
Total	25	---

To amplify the respective fragments, the final PCR reaction mix is mentioned in Table 3. Above PCR reaction mix was used to amplify the fragments by using the PCR conditions mentioned in Table 4. The positive amplification was visualized by electrophoresis of the product on 1.5% agarose gel in a submarine horizontal electrophoresis.

Gel elution of amplified product: The amplified products of all the fragments were run on 1% agarose gel electrophoresis and the desired bands were excised from the gel with a clean, sharp scalpel. The sliced gel pieces of different fragments were purified as per the manufacturer's (Thermo Scientific Gene Jet Gel Extraction Kit (#K0691)) protocol.

Sequencing: The respective amplified and gel eluted products of 18th week and 40th week of age showing highest and lowest expression were sequenced by means of automated dye-terminator cycle sequencing method of ABI PRIZM 377 DNA sequencer (Perkin-Elmer), ScieGenom lab with gene specific primers.

Statistical analysis for methylation data: Methylation data were studied by using a web tool, Methylation plotter (Mallona *et al.* 2014) where the tool aids in summarizing the status of every CpG site and for every sample in lollipop or grid style. The proportion of methylation was calculated by using the formula as

$$\text{Proportion of methylation} = \frac{\text{No.of methylated CPG sites}}{\text{No.of methylated CpG sites + No. of unmethylated CpG sites}}$$

The statistical analysis of methylation data was carried out by Univariate GLM procedure (SPSS software 17.0) with the following model:

Model I (methylation level analysis):

$$Y = \mu + L + A + L \times A + e$$

Model II (expression level analysis) :

$$Z = \mu + L + A + L \times A + e$$

where, Y = *ACACB* methylation level; Z = *ACACB* expression level; μ = Overall population mean; L = Line (layer line selected for higher and lower trait) as fixed effect; A = Fixed effect of the age; $L \times A$ = As interaction of line by age; e = Random error.

The two groups and more than two groups were compared by using two samples independent t-test and Duncan's significant difference test respectively.

RESULTS AND DISCUSSION

The most studied epigenetic mark associated with major changes in gene expression was DNA methylation (Egger *et al*. 2004, Bock 2012). DNA methylation is one of the heritable epigenetic marker engaged in deactivation of a gene and in regulation of tissue-specific transcription (Popiela *et al*. 2004, Tost 2009, Schübeler 2015). These events are essential for cell differentiation, embryonic development and to avoid growth disorders. Epigenetic markers regulates the gene expression by hampering the transcription factor activated-promoter sites (Suzuki and Bird 2008). Understanding epigenetic profile will help in summarizing the patterns of DNA methylation in conjunction with their functional aspects (Eckhardt *et al*.

Table 4. PCR conditions of methylation for *ACACB* gene promoter

Fragment	Initial denaturation at 95°C for 10 min	Thermal cycling conditions Cycles (35)			Final Extension at 72°C for 10 min
		Denaturation	Annealing	Final Extension	
ACACB-M1		95°C for 30 sec	52°C for 35 sec	72°C for 35 sec	
ACACB-M2		95°C for 30 sec	56°C for 30 sec	72°C for 30 sec	

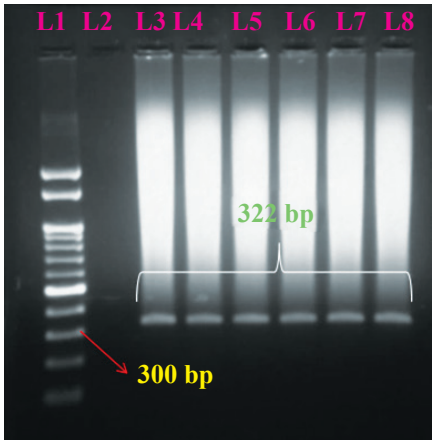

Fig. 1. Agarose gel electrophoresis (1.5%) showing PCR amplified ACACB-M1 (322 bp) fragments of *ACACB* gene promoter from bisulfite treated DNA. Lane 1: 100 bp DNA ladder Plus; Lane 2: Negative control; Lane 3 to 8: ACACB-M1 fragments.

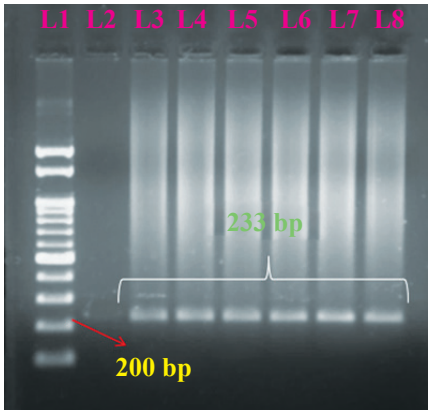

Fig. 2. Agarose gel electrophoresis (1.5%) showing PCR amplified ACACB-M2 (233 bp) fragments of *ACACB* gene promoter from bisulfite treated DNA. Lane 1: 100 bp DNA ladder Plus; Lane 2: Negative control; Lane 3 to 8: ACACB-M2 fragments.

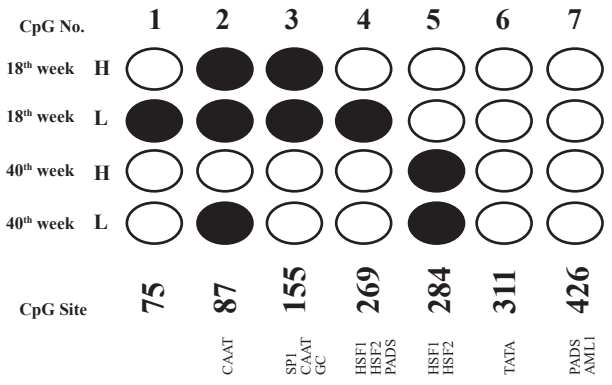

Fig. 3. Lollipop style representation of methylation data during post-hatch period in IWK. Each line represents one individual sample of the corresponding expression group (H-highest, L-Lowest) and each circle represents one single CpG dinucleotide. Filled (black) circles correspond to methylated, unfilled (white) circles correspond to unmethylated. Location of putative cis acting transcription factors in the CpG dinucleotide sites are mentioned at the bottom.

Table 5. Expression (40-ΔCt) value and methylation per cent of *ACACB* gene.

Age	Expression level	IWK		
		Expression (40-Δ Ct)	Methylation (%)	Mean
18th week	Highest	31.51	28.57	
	Lowest	27.63	57.14	42.85
40th week	Highest	32.72	14.28	
	Lowest	31.33	28.57	21.42
Overall mean				32.14

2006, Zilberman *et al*. 2007, Lister *et al*. 2008, Gehring *et al*. 2009, Hsieh *et al*. 2009). The DNA methylation pattern is unexplored for *ACACB* gene promoter and hence in the present study, the methylation pattern of minimal promoter region in *ACACB* gene is attempted using bisulfite sequencing.

The upstream region of 555 bp in the transcription start site of *ACACB* gene was amplified using bisulfite treated DNA with specific primers. The 555 bp fragment was divided into two non-overlapping smaller fragments of 322 and 233 bp. These fragments were bisulfite treated, amplified and visualized in 1.5% agarose gel electrophoresis. The PCR products are presented in Fig. 1 and Fig. 2. In the present study, seven CpG islands were observed upstream of *ACACB* gene (555 bp). The transcription binding factors like SP1, which is present in the minimal promoter, is known to be involved in DNA methylation. The methylation percent results revealed that highest expression birds showed the lower methylation (Table 5). The methylation of DNA in gene promoters is usually negatively associated with gene expression (Razin and Cedar 1991)

The mean methylation per cent was found to be 42.85 and 21.42 at 18th and 40th weeks of age, results indicating the decrease in methylation per cent with age. The overall mean methylation per cent observed was 32.14. On the whole, a differential methylation pattern was noticed in high and low expressing groups of 18th and 40th weeks of age at CpG dinucleotide sites with 5 (75, 87, 155, 269 and 284) positions being methylated (Fig. 3). The CpG island at 311 and 426 positions were unmethylated. The DNA methylation is influenced by other mysterious factors (Rakyan *et al*. 2010) like diet, environment, chemicals etc. The negative relationship between different lines of chicken was also observed for other genes in chicken like *Myostatin* (Liu *et al*. 2010, Wen *et al*. 2014), *FGF2* (Lu *et al*. 2013). Nätt *et al.* (2012) studied heritable genome wide variation of gene expression and promoter methylation between Red Jungle Fowl and White leghorn chicken in thalamus and hypothalamus. It was observed that there were significantly higher proportions of differentially expressed genes between the wild and domestic chicken and also large differences were observed in the methylation pattern in the promoter region. Interestingly these differences were observed to be heritable even after eight generations.

It is important to explore the methylation pattern in

relationship with spatial gene expression pattern involving different tissues so that the functional role of methylation in the promoter region of *ACACB* gene can be explored. The study could establish epigenetic profile of the promoter region of *ACACB* gene.

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