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Promoter methylation negatively correlated with mRNA expression but not tissue differential expression after heat stress

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ABSTRACT. DNA methylation plays a central role in gene expression. In this study, we detected the promoter methylation pattern of the chicken heat shock protein 70 (*HSP70*) gene and its association with messenger RNA (mRNA) expression before and after heat shock. The results showed that mRNA expression increased in response to heat stress and peaked at 3 h before dropping. Hypomethylation of the *HSP70* promoter occurred in all of the groups studied, but the difference between groups within tissue type was not significant. The DNA methylation level of the control and the 6-h treatment groups was slightly higher than that of the 3-h treatment group in brain tissue and leg muscle. Correlation analysis between mRNA expression and DNA methylation of *HSP70* showed that DNA methylation was negatively associated with mRNA expression in leg muscle ($P = 0.0124$), indicating that DNA methylation may be negatively associated with the expression of *HSP70*, although the difference was not significant. We concluded that the expression of *HSP70* is heat inducible and tissue dependent and that heat induction

may correlate with DNA methylation pattern in the *HSP70* promoter, whereas tissue dependence is unrelated to DNA methylation pattern.

Key words: *HSP70*; DNA methylation; Heat inducible

INTRODUCTION

Heat shock protein 70 (HSP70) is a member of the heat shock protein family. As a molecular chaperone, it is required for the correct folding of newly synthesized proteins and the degradation of misfolded proteins (Hartl, 1996; Mayer and Bukau, 2005). HSP70 is also involved in apoptosis inhibition and cell cycle regulation (Kregel, 2002). More important, HSP70 plays a critical role in the regulation of the heat shock response and the acquisition of thermotolerance (Mayer and Bukau, 2005). In view of these important roles, HSP70 is a proposed biomarker for the study of environmental stress in chickens (Maak et al., 2003).

The expression of *HSP70* has been demonstrated to be heat inducible and occur rapidly after transient heat stress (Wang and Edens, 1998), but the expression of *HSP70* differs according to individuals and tissue type. Compared with normally feathered birds, heat-resistance birds (naked-neck Label Rouge) display lower HSP70 levels in hepatic cells when gradual heat stress is induced (Mazzi et al., 2002). Guerreiro et al. (2004) measured brain and hepatic HSP70 protein levels using Western blot after gradual heat stress and found that brain HSP70 levels are 3-4 times higher than those in the hepatic tissue of heat-stressed birds reared at thermoneutrality. Much research has been performed to illuminate the cause of HSP70 expression differences in individuals and tissue-dependent expression. Mahmoud (2000) has analyzed *HSP70* in birds submitted to heat stress and found that more resistant birds have only 1 *Pst*I *HSP70* allele upstream from the coding region, whereas the other breeds had 2 different alleles for that gene. Zhang et al. (2002) also detected polymorphism in regulatory and coding regions of *HSP70* in chickens with various heat tolerance capabilities using polymerase chain reaction (PCR) single-strand conformation polymorphism analysis and sequencing. Mazzi et al. (2003) analyzed the promoter region and the beginning of the coding region of *HSP70* in chickens with various heat tolerance capabilities and found 2 single nucleotide polymorphisms (SNPs) - A+258G and C+276G - both of which were silent mutations. Zhen et al. (2006) tested the chicken *HSP70* mRNA expression of various genotypes at polymorphism sites C+276G and A+258G in liver and leg muscle tissue using real-time quantitative PCR (qPCR). The results showed that the heterozygote has a higher expression level than that of other genotypes at these polymorphism sites.

These studies are illuminative but still fail to illustrate why *HSP70* is heat inducible and why its expression is tissue dependent, because SNPs in the chicken *HSP70* identified to date are not localized to the heat shock element (HSE), CAAT box, SP1 binding sites, TATAA box, or any other cis-elements in the regulatory region, and they have not been found to affect the binding activity of heat-shock transcription factors (HSFs). Therefore, the aim of the present study was to determine the cause of *HSP70* differential expression from another angle - epigenetics. DNA methylation is an epigenetic modification that plays an important role in the modulation of gene expression. Aberrant DNA methylation is associated with gene silencing or downregulation (Bird, 2002; Dai et al., 2003). Studies of human diseases have revealed that complex diseases such as cancer are often associated with sudden hypermethylation of

tumor suppressor genes or hypomethylation of cancer-related genes (Robertson and Wolffe, 2000; Brena et al., 2006). Methylation of the CpG island within the promoter prevents protein binding at DNA methylation sites, directly inhibiting gene transcription because when double-stranded DNA forms a three-dimensional structure, the methyl of methylated cytosine may be highlighted in the major groove, blocking the interaction between transcription factors and genes (Kuroda et al., 2009). Normally the gene CpG island is unmethylated (Straussman et al., 2009). Xu et al. (2007) measured the genome-wide DNA methylation level of 3 chicken strains, and the average incidence of DNA methylation was approximately 29.4% in muscle, 27.2% in liver, 27.2% in heart, and 26.1% in kidney, indicating that the methylation level of the chicken genome is low. However, when the organism is subjected to certain stimuli, the methylation level may change to a certain extent. Xing et al. (2011) fed betaine to broilers and found that mRNA expression of the lipogenesis gene decreased significantly, possibly owing to the upregulation of DNA methylation in some CpG sites within the lipogenesis gene promoter. Yossifoff et al. (2008) found that transferred 3-day-old birds to 37.5°C for 24 h caused transient changes in the expression of brain-derived neurotrophic factor that coincided with changes in the CpG methylation pattern in the avian brain-derived neurotrophic factor promoter region. Our preliminary research has uncovered a CpG island with high GC content in the 5'-flanking region of *HSP70*. DNA methylation may occur more easily in regions with high GC content (Watanabe and Maekawa, 2010).

Given the results of previous studies and our preliminary findings, we hypothesized that DNA methylation may play a role in the heat-inducible and tissue-dependent expression of chicken *HSP70*. To verify the hypothesis, we subjected 8-day-old female chicks to acute heat stress (40°C), and liver, brain, and leg muscle tissue were sampled for RNA and DNA preparation at various time points. The *HSP70* mRNA and DNA methylation levels of the tissues were analyzed, and correlations between mRNA expression and DNA methylation were explored to determine whether the methylation pattern of the *HSP70* promoter is associated with the differential expression of *HSP70*.

MATERIAL AND METHODS

Chicks and experimental design

Eighty female chicks (Dwarf Yellow Chicken) were obtained from the Guangdong Wens South China Poultry Breeding Company (Guangdong, China) on the day of hatching and raised in an adequate environmental temperature under continuous artificial illumination and *ad libitum* access to food and water. At 8 days of age, 64 chicks were transferred to an artificial climate chamber (LH-100-RDCT, Japan) in which the temperature was 40°C and the humidity was 80% for 6 h. The remaining 16 chicks served as controls and were left in a thermoneutrality room (ambient temperature, 30°C). After heat stress for 1, 2, 3, and 6 h, 16 chicks were caught randomly for peripheral blood and sample collections.

Tissue collection, RNA, and DNA preparation

Peripheral blood from all of the chicks was collected from the heart, and the chicks were killed via cervical dislocation. Liver, brain, and leg muscle tissue were sampled for DNA

and RNA preparation and immediately placed in liquid nitrogen. Tissue RNA was extracted using the TRI reagent (TaKaRa, Japan) and tissue DNA was extracted using a TaKaRa Universal Genomic DNA Extraction Kit 3.0 (TaKaRa) according to manufacturer instructions. Genomic DNA was extracted from blood using the phenol-chloroform method.

Real-time qPCR

First-strand complementary DNA was synthesized from total RNA using a ReverTra Ace qPCR RT Kit (TOYOBO, Japan). Samples were then analyzed with real-time qPCR using SYBR Green Real-Time PCR Master Mix (TOYOBO). Real-time qPCRs were performed in a final volume of 20 μL according to manufacturer instructions. Expression was determined by comparing the *HSP70* mRNA levels with those of β-actin, and the final expression level was standardized through comparison to the expression level of the liver tissue in the control group. The following primers were used for real-time qPCR: *HSP70*: F: 5'-GCGCCAGGCC ACCAAAGATG-3'; R: 5'-GCCCCCTCCCAAGTCAAAGATG-3'; β-actin: F: 5'-CTCCCCCA TGCCATCCTCCGTCTG-3'; R: 5'-GCTGTGGCCATCTCCTGCTC-3'.

Amplification of the *HSP70* promoter region

The promoter region of a gene is normally positioned between 2500 bp upstream and 200 bp downstream of nucleotide A in the initiator consensus sequence. The promoter region sequence was downloaded using the EBI Ensembl Tool (http://www.ensembl.org/index.html). Primers designed to amplify the promoter region were as follows: F: 5'-AATCGACACCCTT CCCATCCA-3'; R: 5'-TGGTGTTGGTGGGGTTCATTG-3'. The amplification product was 2020 bp.

Analysis of the *HSP70* promoter region CpG islands

The Online software (http://www.urogene.org/methprimer/index1.html) was used to analyze the CpG islands in the promoter region of *HSP70*.

DNA methylation assay of the CpG island

Quantitative high-throughput DNA methylation analysis was performed using the MassARRAY system as described elsewhere (Ehrich et al., 2005). Two bisulfite reactions (A1 and A2) for detecting the methylation level of the CpG island in the *HSP70* promoter were designed, covering 31 and 39 CpGs. The following primers were used for the reactions: A1, F: 5'-ACGTTTTTGATTGGGTAGGAGGTAA-3'; R: 5'-AACAATAATCTCCACTTTACCA TAC-3', covering a region of 365 bp. A2, F: 5'-ATTTTTTATTTTGAGGGAAAGGGGT-3'; R: 5'-ACCTCCTACCCAATCAAAAACGT-3', covering a region of 496 bp. The methylation levels of the control, 3-, and 6-h treatment groups were analyzed.

Expression vectors and luciferase reporter assay

Because the CpGs in the *HSP70* gene promoter are too numerous for us to study, we

focused on finding the core promoter of *HSP70* and analyzing the CpGs within it. A PGL3 basic vector (Promega, USA) was double-restriction enzyme digested and used to ligate the truncation amplification products of the *HSP70* promoter to determine the core promoter. Seven expression vectors called 1F, 2F, 3F, 4F, 5F, 6F, and 7F were constructed; the primers used to amplify the *HSP70* promoter are shown in Table 1. The PGL3 basic vector served as a negative control, and the PGL3 promoter vector served as a positive control. The PCR products were ligated into the pGL3 basic vector between the *Mlu*I and *Xho*I site. The plasmids were then transfected into chicken embryo fibroblasts at a density of 1.5 x 10^5 cells/well. A 24-well plate was used with 500 µL medium containing 5% serum for each well. A total of 1 µg plasmid pGL3 vector and 500 ng pRL-TK internal control vector (Promega) were co-transfected into cells using Lipofectamine™ 2000 (Invitrogen, USA). After 6 h, the medium was changed to fresh medium with 10% fetal bovine serum. The cells were then incubated for 48 h, and the luciferase assay was performed according to manufacturer instructions (Promega). Luciferase activity was normalized using pRL-TK activity. Each experiment was performed 6 times.

Table 1. List of primers used in the truncation amplification of the promoter region of *HSP70*.

Primer	Sequences (5'→3')
P1F	*gcg*ACGCGTATTTCTCCAAGGTCAA
P2F	*gcg*ACGCGTGCTGCACTTTTAACAT
P3F	*gcg*ACGCGTTTAGCACAACAGACAA
P4F	*ata*ACGCGTCTGGCATAGAGGGAGC
P5F	*ata*ACGCGTCCCCCGAGAGGCAGTG
P6F	*ata*ACGCGTGAGGGTTGGGCTAGAG
P7F	*gcg*ACGCGTGAGGAATCTATCATCA
PR	*at*CTCGAGTGGTGTTGGTGGGGTTCA

Nucleotides in lower case letters are protective bases; underlined bases are restriction enzyme recognition sequences: ACGCGT is recognized by *Mlu*I and CTCGAG is recognized by *Xho*I.

Statistical analysis

The relative expression levels of *HSP70* were determined with $2^{-\Delta\Delta Ct}$, for which ΔCt is $Ct_{target\ gene}$ minus $Ct_{\beta\text{-}actin}$ and ΔΔCt is ΔCt minus $\Delta Ct_{standard\ value}$. Differences between the groups in all the biochemical studies were examined using SAS 8.2 with a general linear model procedure. Correlation analysis between methylation and mRNA expression levels of *HSP70* was carried out using SAS 8.2 with a CORR procedure. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

HSP70 mRNA expression

Real-time qPCR analysis revealed that compared to expression in control tissues, *HSP70* mRNA expression increased in response to heat stress and peaked at 3 h at levels 40 times higher in liver tissue, 19 times higher in brain, and 2.8 times higher in leg muscle, then

dropped at 6 h (Figure 1 A-C). The expression levels in liver and brain in chicks treated for 3 h were significantly different than those in other groups ($P < 0.01$), whereas the expression levels in leg muscle in chicks treated for 3 h were significantly different than those of other groups ($P < 0.01$) with the exception of the 6-h treatment group ($P = 0.08$). *HSP70* expression was higher in leg muscle, followed by brain and liver tissue.

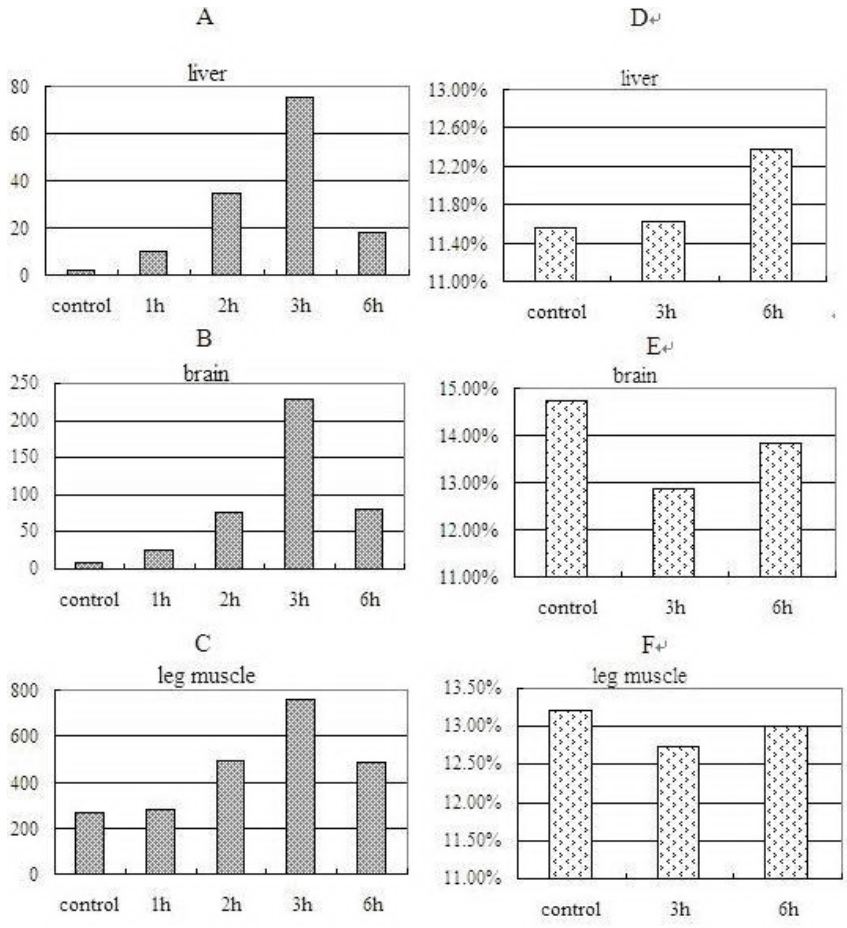

Figure 1. A. B. C. mRNA expression level of *HSP70* in different tissues at different times. **D. E. F.** Promoter methylation level of *HSP70* in different tissues at control, 3 and 6 h.

DNA methylation of the *HSP70* promoter CpG island

The CpG island region of the *HSP70* promoter was 1068 bp according to the Online software analysis (see Figure 2). The total average methylation incidences of the control, 3-, and 6-h treatment groups were 11.56 ± 1.42, 11.62 ± 1.59, and 12.38 ± 2.08%, respectively, in liver tissue (see Figure 1D); 14.73 ± 1.68, 12.87 ± 1.33, and 13.82 ± 2.05%, respectively, in brain tissue (see Figure 1E), and 13.22 ± 4.77, 12.74 ± 2.46, and 13.03 ± 1.69%, respectively,

in leg muscle (see Figure 1F). The differences within tissue groups were not significant (P > 0.05), and correlation analysis showed that methylation level was negatively associated with mRNA expression in leg muscle (P = 0.0124).

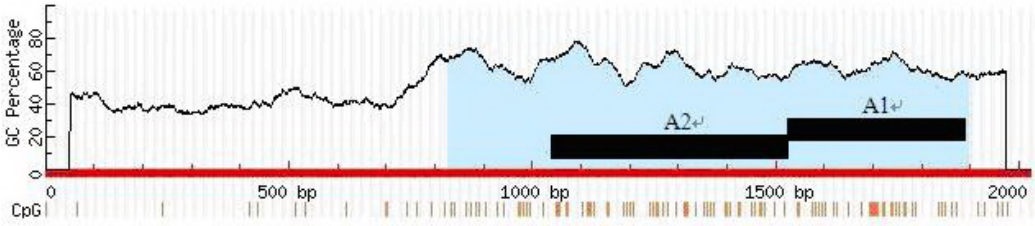

Figure 2. CpG island of *HSP70* promoter. The light blue region is the CpG island of *HSP70*, the black thick lines marked by A1 and A2 are the methylation detection regions.

Luciferase reporter assay of core promoter activity

The dual-luciferase reporter assay showed similar values in vectors 5F and 6F, with slightly higher values in 5F, which had higher values than any other expression vectors, suggesting that compared with 5F, the missing fragment of 7F could be the core promoter (-546 to -15 bp). The expression value of 2F was higher than that of 1F, suggesting that compared with 1F, the missing fragment of 2F may contain transcription factors that inhibit the transcription of *HSP70* (Figure 3).

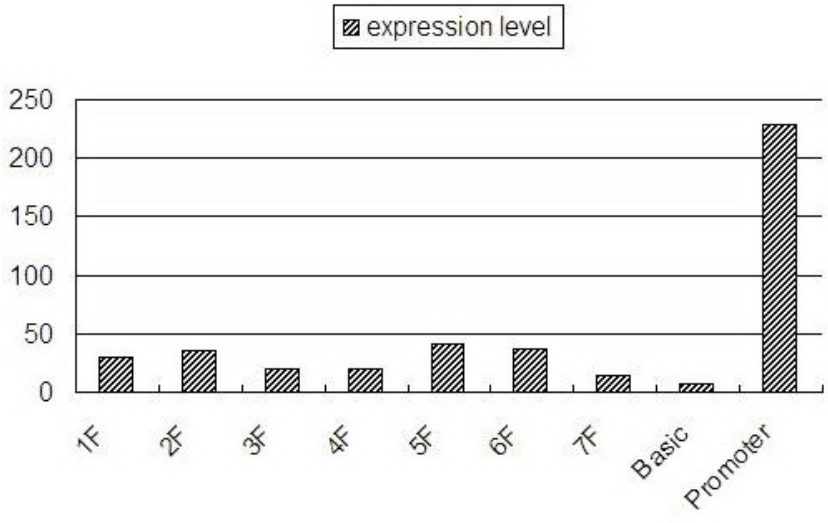

Figure 3. Dual-luciferase reporter assay result. 1F, 2F, 3F, 4F, 5F, 6F, and 7F represent different expression vectors. Basic is the negative control and promoter is the positive control.

Methylation levels of core promoter transcription sites

The transcription factors of the *HSP70* core promoter were analyzed using the Online software (http://www.genomatix.de/en/index.html). As shown in Figure 4, the CpG sites at positions -426, -419, and -413 formed a CpG cluster. The analysis revealed that this region was an SP1 transcription factor, and the methylation levels of this region in the control, 3-, and 6-h treatment groups were 25.00 ± 3.89, 22.88 ± 2.95, and 26.25 ± 3.49%, respectively, in liver tissue; 27.43 ± 3.60, 25.50 ± 5.73, and 23.29 ± 4.07%, respectively, in brain tissue, and 25.86 ± 6.72, 24.25 ± 3.20, and 26.25 ± 2.20%, respectively, in leg muscle. None of the differences within tissue groups was significant ($P > 0.05$). The CpG sites at positions -243, -239, and -232 formed another CpG cluster. Online software analysis showed that this region was also an SP1 transcription factor, but the methylation levels of this region in the control, 3-, and 6-h treatment groups showed no consensus with those above (data not shown). In addition, a CpG site at position -172 in the HSE, which is the HSF binding site, displayed hypomethylation at a level 6% lower than that in any of the groups (data not shown). Correlation between the DNA methylation of the transcription factor CpGs and *HSP70* expression was not significant (data not shown).

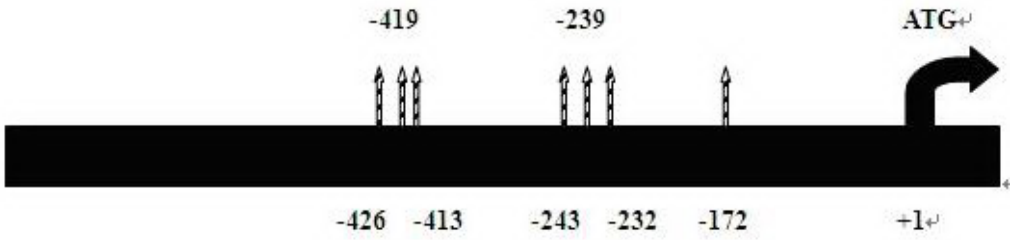

Figure 4. Schematic chart depicting the CpG locations upstream of the *HSP70* coding region. The analyzed methylated sites are marked by the number in slash arrow. Positions are numbered with nucleotide A at the initiator element.

DISCUSSION

In the present study, the mRNA expression of chicken *HSP70* in different tissues under various treatments was analyzed, revealing that *HSP70* is heat inducible. The results coincide with those of Gabriel et al. (1996) and Dionello et al. (2001). In addition, mRNA expression of chicken *HSP70* was tissue dependent, which agrees with the results of Givisiez et al. (2003). Because no intron is present within *HSP70*, it can transcribe quickly after heat shock, and it increases sharply with gradual heat stress. Given the results of previous studies of gene polymorphisms that fail to explain clearly the heat-inducible and tissue-dependent nature of *HSP70*, other mechanisms likely play a role in this phenomenon.

DNA methylation studies in the literature have uncovered profound results (Esteller, 2008; Ushijima and Asada, 2010). Higher methylation levels of the CpG island within the gene promoter region are known to correlate with gene silencing or downregulation; DNA methylation represses transcription directly by inhibiting the binding of specific transcription factors (Esteller, 2007; Lopez-Serra and Esteller, 2008) and indirectly by recruiting methyl-CpG-

binding proteins and their associated repressive chromatin remodeling activities (Kuroda et al., 2009). In our study, the methylation level of chicken *HSP70* promoter CpG island was detected using the MassARRAY system, and it revealed hypomethylation of this region in the control, 3-, and 6-h treatment groups in various tissues. The low level of methylation agreed with current data showing that in normal somatic cells, CpG islands are unmethylated or hypomethylated (Strichman-Almashanu et al., 2002; Song et al., 2005). The results of DNA methylation analysis indicated that regardless of tissue type, the chicken *HSP70* promoter showed a similar methylation incident, meaning that the tissue-dependent expression of chicken *HSP70* was not associated with the DNA methylation pattern, but interestingly, the methylation level may be negatively associated with mRNA expression because the methylation level of the control and 6-h treatment groups was slightly higher than that of the 3-h treatment group (except in liver tissue), whereas mRNA expression was higher in the 3-h treatment group, and correlation analysis showed that the methylation level was negatively associated with the mRNA level of *HSP70* in leg muscle ($P = 0.0124$). These results indicated that the heat inducibility of *HSP70* may be associated with DNA methylation pattern, as it has been demonstrated that heat treatment can change the DNA methylation pattern of certain genes (Yossifoff et al., 2008) However, in the present study, only leg muscle showed a significant negative association between the methylation and mRNA levels, so additional studies are needed to determine the complete relationship between DNA methylation and mRNA expression before and after heat stress.

Methylation levels of CpG sites within the core promoter were also analyzed. CpG sites at positions -426, -419, and -413 formed a CpG cluster that was identified as an SP1 transcription site, since none of the differences within tissue groups was significant, but these values were negatively associated with mRNA expression. SP1 is widely expressed in various cell types and plays an important role in the regulation of many housekeeping genes such as those related to nucleic acid metabolism (Safe and Abdelrahim, 2005) and oxidative phosphorylation (Zaid et al., 1999). This transcription factor can also activate gene transcription by preventing the methylation of CpG islands (Samson and Wong, 2002). In the CpG site at position -172, which was contained in the HSE, the methylation level was no more than 6%. The HSE is known to play an important role in the transcription of the *HSP70*. During heat stress, HSFs bind to the HSE to start the transcription of *HSP70* and initiate the heat shock response, in which *HSP70* is rapidly transcribed and translated. This process may not only be due to the absence of introns in *HSP70* but also be associated with the low level of methylation of the HSE, which allows easy access of HSFs to the HSE.

In conclusion, we explored the relationship between promoter CpG island methylation and *HSP70* mRNA expression and demonstrated that the tissue-dependent expression of chicken *HSP70* has no relationship with the promoter CpG island methylation pattern. However, the results hinted that DNA methylation may play a role in the heat-inducible nature of *HSP70* because of its negative association with mRNA expression of *HSP70*. Additional studies should be carried out to confirm this phenomenon and indentify the mechanism through which DNA methylation affects the expression of *HSP70*.

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