# Duck *IL-2* promoter cloning and the effects of methylation status on mRNA levels in immune tissues

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**Abstract**

Interleukin 2 (IL-2), a cytokine, plays an important role in animal immune systems. To investigate the influences of epigenetic modifications on transcription of the duck IL-2 gene, the promoter region of the duck IL-2 gene was cloned. Then, the DNA methylation status of the IL-2 gene promoter (-1337 bp/-924 bp) in immune tissues of ducks was determined using the Sequenom Mass Array methylation technique, and their corresponding expression levels were determined using real-time PCR. The results showed that 2850 bp of the duck IL-2 gene promoter region were obtained. There was one CpG island (-1231 bp/-902 bp) in which 11 CpG sites were distributed. The CpG1 and CpG2 sites are located between the binding sites of NFAT and AP-1, and they had higher homology methylation patterns in different individuals and tissues. The methylation frequencies of 28.5% CpG sites showed negative correlations with the expression levels of the IL-2 mRNA, whereas 71.5% showed positive correlations. These results indicate that the transcription of duck IL-2 may be distinct from that of mammals. CpG1 (-1284 bp) and CpG2 (-1264 bp) in the duck IL-2 promoter showed a higher homology of methylation patterns, indicating a similar regulatory effect on their gene expression, and these CpG sites may be essential for the regulation of transcription of duck IL-2. The methylation pattern of the IL-2 gene promoter in duck was tissue specific.

**Key words:** CpG, duck, DNA methylation, IL-2, mRNA expression, promoter.

## Introduction

Interleukin 2 (IL-2), a cytokine, plays an important role in immune systems, and it can determine the differentiation and growth status of T cells, as well as enhancing their killing activities. Additionally, IL-2 induces the proliferation and antibody secretion of B cells and strengthens the killing activities of other types of immune cells, such as natural killer (NK) cells and monocytes [1-3].

The functions of IL-2 in immune processes have been clarified. However, little is known about the mechanism by which the expression of *IL-2* is regulated in immune processes. As an important regulatory element in gene expression, the promoter can affect the transcription levels of genes and, accordingly, influence their functions. In the mouse and human *IL-2* genes, the binding sites of many transcription factors have been identified, such as the nuclear factor of activated T cells (NFAT), nuclear factor κB (NF-κB), activating protein 1 (AP-1) families and the constitutive factors of the octamer-binding protein 1 (Oct-1).

These sites exist in the region of 300 bp upstream of the *IL-2* gene transcription start site (TSS), and they may play essential roles in inducing *IL-2* gene expression [4-6]. Rooney *et al.* (1995) showed that nucleotide mutations in the NFAT binding sites of the *IL-2* promoter region completely block the anti-CD3-inducible promoter activity in transfected AE7 cells, indicating that the NFAT site is critical for regulating *IL-2* transcription processes [7]. The increase of AP-1 activities in T cells stimulated with immobilized anti-CD3 antibodies enhances the expression and secretion of IL-2, suggesting that AP-1 is a major regulatory factor of *IL-2* gene expression in splenic T cells activated through the TCR/CD3 complex [8]. Mutations in the AP-1 site significantly decrease or abrogate the induction of the *IL-2* promoter [9]. Furthermore, NF-κB as an intracellular second messenger may play an important role in the transduction of signals from the outer cellular membranes, leading to IL-2-induced activation and growth via NF-κB [10].

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As one of the most important epigenetic modification pathways, DNA methylation can affect gene transcription via the inactivation of positive or negative regulatory elements [11]. Studies showed that the methylation levels of the region consist of a 1.3 kb segment upstream of the TSS in the human *IL-2* gene and contain 6 CpG sites, which play an important role in inducing *IL-2* gene expression [12]. Oct-1-binding followed by CpG demethylation is a key event in the epigenetic regulation of human *IL-2* expression and significantly decreases expression of the *IL-2* gene [13]. Similarly, methylation of the *IL-2* promoter inhibited *IL-2* transcription in T lymphocytes [14]. Effector CD4+ T cells stimulated with anti-CD3 and anti-CD28 resulted in a significant decrease of the average degree of methylation at each CpG site of the *IL-2* promoter and enhancer, which leads to *IL-2* transcription [15]. Therefore, the methylation level of the *IL-2* promoter is important for *IL-2* expression in both humans and mice.

The duck is an important agricultural economic animal with a greater ability to tolerate adversity than other poultry, indicating that the genetic background of the duck immune genes may differ from other species [16]. IL-2 plays an important role in immune systems and it is produced and secreted from the thymus, spleen and bursa of Fabricius [17-21]. Therefore, study of the transcriptional regulation of the *IL-2* gene in the thymus, spleen and bursa of Fabricius is important for understanding the molecular basis of the regulation of the immune response in the duck. The objective of the present study was to ex-

plore the characteristics of the duck *IL-2* promoter, then through comparing and analyzing the relationship between IL-2 promoter methylation level and the expression level of mRNA in the spleen, thymus and bursa of Fabricius to research the possible effects of the methylation status on *IL-2* gene expression in duck immune tissues. These studies may help us obtain insights into the major functions of IL-2 in immune processes and lay the foundation for future research on the transcriptional regulation of avian *IL-2* genes in immune functions.

## Material and methods

### Birds and sampling

A total of 30 one-day-old ducklings of the NongHua-P strain were randomly selected from the Waterfowl Breeding Experimental Farm of Sichuan Agricultural University. All ducks were numbered, and blood was collected from the veins. After exsanguination, the tissues of the spleen, thymus and bursa of Fabricius were isolated. All samples were ground with liquid nitrogen and stored at –80°C until needed. All procedures in the current study were approved by the Animal Welfare Committee of Sichuan province.

### Amplification and sequence analysis

Total genomic DNA was extracted from the spleen, thymus and bursa of Fabricius with the DNA Extraction Kit (TianGen Co., Beijing, China) according to the man-

**Table 1.** Primers for amplification of duck *IL-2* nucleotide sequence

| Primer name | Primer sequence (5'-3') | Product length (bp) | Tm (°C) |
|---|---|---|---|
| A1-F | CCCATAGAATAAGCCATAGAG | 745 | 56.5 |
| A1-R | TATGCGACAGACGATGAA | | |
| A2-F | CCCATAGAATAAGCCATAGAG | 829 | 53.9 |
| A2-R | TATGCGACAGACGATGAA | | |
| A3-F | CATTGCGTTTGATGGTGC | 1192 | 62.3 |
| A3-R | GCTGGTTGGGCTGTGAT | | |
| A4-F | GTTTTCAATGCTATTCGGTC | 1153 | 58.6 |
| A4-R | ACTCTTGTCAGGTGGCGTA | | |
| β-actin-F | GCTATGTCGCCCTGGATTTC | 168 | 60 |
| β-actin-R | CACAGGACTCCATACCCAAGAA | | |
| GAPDH-F | AAGGCTGAGAATGGGAAAC | 254 | 53 |
| GAPDH-R | TTCAGGGACTTGTCATACTTC | | |
| IL-2-F | GCATGAATGGGATTGATCTTGAG | 111 | 60 |
| IL-2-R | CAATTTCTTCCTCCAAGGTGACTAT | | |
| Mass ARRAY-F | AGGAAGAGAGAGATTGAGTTTTTTAGAAGTTGGGA | 414 | 62 |
| Mass ARRAY-R | CAGTAATACGACTCACTATAGGGAGAAGGCTTAAACCAAATACTCCCCCTACTACC | | |

*F, R – forward and reverse primers, respectively*

**Table 2.** Software used for sequence analysis

| Name | Website | Application |
| --- | --- | --- |
| FPROM | http://www.softberry.com/berry.phtml?topic=fprom&group=programs&subgroup=promoter | Promoter prediction |
| CpG Island Searcher | http://www.uscnorris.com/cpgislands2/cpg.aspx | CpG island analysis |
| PATCH™ public 1.0 | http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi | Analysis of core transcription factor binding sites |
| Plant Care | http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ | Typical structural domain of promoter analysis |

ufacturer's instructions. Four pairs of gene-specific primers (A1-A4, Table 1) were designed using the Primer 5.0 software (Premier Biosoft International, CA, USA) according to the predicted sequence of the *IL-2* gene in NCBI (NW_004676716.1). PCR amplification was performed using duck gDNA, and the PCR products were cloned into the pMD19-T vector (TaKaRa Co., Dalian, China) after purification with the Gel Extraction Kit (OMEGA Co., Guangzhou, China). Finally, the products were transformed into competent DH5 alpha cells, screened for positive clones and propagated. The positive clones were then sent to a biological company (Invitrogen Co., Shanghai, China) for sequencing. The results were spliced using DNASTAR software (Madison, Wisconsin, USA). The characteristics of the *IL-2* promoter and transcription factor binding sites were predicted and analyzed using the software provided. The software names, websites and applications are shown in Table 2.

## Mass ARRAY

Quantitative DNA methylation analysis of IL-2 was performed using the Mass ARRAY Compact System. This system utilized mass spectrometry (MS) for the detection and quantitative analysis of DNA methylation using homogeneous mass CLEAVE (HMC) base-specific cleavage and matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF) [22]. Briefly, 3-5 μg of genomic DNA was treated with sodium bisulfite and PCR amplified (Table 1). The bisulfite reactions were designed, which covered the region of interest, extending from -1337 bp to -924 bp relative to the translation initiation site. The reactions were in vitro transcribed and then cleaved by RNase A. The samples were quantitatively tested for DNA methylation status using matrix-assisted laser desorption ionization-time of flight mass spectrometry. Methylation data for the individual CpG sites or an aggregate of multiple CpG sites were generated with the EpiTyper v4.0 software (SEQUENOM, CA, USA). The nonapplicable reading frames and their corresponding sites were eliminated in the calculation.

## Real-time PCR

Total RNA was extracted from the spleen, thymus and bursa of Fabricius of 15 ducklings with the RNA Extraction Kit (TianGen Co, Beijing, China) according to the manufacturer's instructions, followed by synthesis of cDNA from 2 μg of RNA using the PrimeScript RT Reagent Kit (TaKaRa Co., Dalian, China). The real-time PCR primers (*IL-2*, Table 1) were designed using the Primer 5.0 software (Premier Biosoft International, CA, USA) according to the mRNA sequence of the target gene *IL-2* (JX239765.1), the endogenous reference gene β-actin (EF667345.1) and GAPDH (AY436595.1) of the duck in NCBI. The reaction was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad) using the reaction system SYBR Premix Reaction System Ex Taq II Kit (Takara, Dalian, China). The reaction program was 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s, for 40 cycles. The dissolution curves were run from 65°C to 95°C, reading for 10 s per 0.5°C. Each sample was run in triplicate.

## Data analysis

The $2^{-\Delta\Delta CT}$ method [23] was employed to calculate the relative mRNA expression of the *IL-2* gene according to the Ct value of PCR reaction, and the results were corrected using the internal control genes β-actin and GAPDH. All analyses were performed with the SAS software package, version 8.2 (SAS Institute, 1999). Results are formatted as means ± standard error (SD). Matrix Hierarchical Cluster Analysis (normalized data, Euclidean distance, average linkage clustering) was obtained with the MultiExperiment Viewer (MeV) (http://www.tm4.org/mev.html) (Dana-Farber Cancer Institute, 44 Binney St, Boston, MA, USA).

## Results

### Cloning and analysis of duck *IL-2* promoter sequence

The duck *IL-2* gene 5' flanking promoter region of 2850 bp was obtained in this study. The potential promoter sequence and the TATA-box sites were predicted to exist in the 5' flanking sequences of the *IL-2* gene in duck, chicken, human and mouse (Table 3). However, the position and number of the promoter elements in the duck *IL-2* were different from those in chicken, human and mouse. The duck *IL-2* gene promoter possessed two

**Table 3.** Prediction of *IL-2* gene promoter by FPROM software

| Species (Latin name) | Promoter position (bp) | TATA box position (bp) |
|---|---|---|
| Duck (*Anas platyrhynchos*) | -2152 | -2180 |
| | -996 | -1027 |
| Human (*Homo sapiens*) | -2026 | -2056 |
| House mouse (*Mus musculus*) | -1391 | -1421 |
| Chicken (*Gallus gallus*) | -1875 | -1905 |
| | -526 | -555 |

*The human IL-2 promoter sequence, house mouse IL-2 and chicken IL-2 promoter sequence were from Homo sapiens genome (NC_000004.12), Mus musculus genome (NC_000069.6) and Gallus gallus genome (NC_006091.3), respectively.*

TATA-box elements, located at -2180 bp and -1027 bp. By contrast, there was only one TATA-box in the *IL-2* promoter of human and mouse, located at -2056 bp and -1421 bp, respectively. In addition, although the chicken *IL-2* promoter also had two TATA-box elements, their positions were different from duck, located at -1905 bp and -555 bp in the promoter.

The bioinformatics results revealed that the duck IL-2 promoter region contains numerous transcription factor binding sites, a core-promoter structure TATA-box and two typical CpG islands (Fig. 1A). The region from -900 bp to -1500 bp upstream of the duck IL-2 transcription start site was possibly the core transcription regulation region. In this region, there was NF-κB, AP-1, NFAT, Sp-1 and two TATA-box elements. Moreover, the predicted CpG islands in the IL-2 promoter region showed that the transcription of IL-2 may be influenced by epigenetic modifications (Fig. 1B). There were 11 CpG sites in the CpG island (-1231 bp/-902 bp); However, only 7 had methylation information. CpG1 and CpG2 are located between NFAT and AP-1, and only CpG4 was located in the binding site of the transcription factor GATA-1. Moreover, there are no additional CpG sites located in the transcription factor binding sites (Fig. 1C).

## Cluster analysis for methylation levels of CpG sites

The two-way cluster results of the methylation states in the CpG islands of the duck *IL-2* promoter are listed in Fig. 2. The data showed that most individuals have the highest homology distributed in the CpG island site methylation states in spleen tissues and have relatively higher homology distributed in the thymus and bursa. The data indicate that the methylation patterns of the duck *IL-2* promoter CpG locus were tissue specific, and the spleen had

a distinct methylation pattern of the *IL-2* promoter island compared to the thymus and bursa. Additionally, the sites of CpG1 and CpG2 in different individuals and tissues had higher homology methylation patterns. There was likely a similar regulatory effect among these cluster sites for the methylation patterns on *IL-2* gene transcription.

## Comparison of CpG site methylation levels in immune tissues

Figure 3 is a comparison of methylation frequency of different CpG sites in the same tissue. The results show that the CpG2 site methylation frequency was significantly higher than that of other sites in the thymus, spleen and bursa of Fabricius ($p < 0.05$). The methylation frequency of CpG1, CpG4, CpG7 and CpG9 was significantly higher than that of CpG6 in the thymus and bursa of Fabricius ($p < 0.05$). However, the CpG6 site methylation frequency was significantly higher than that of other sites except the CpG2 site in the spleen ($p < 0.05$). These data indicate that CpG2 methylation frequency was significantly higher than that of other sites in the thymus, spleen and bursa of Fabricius ($p < 0.05$), and the methylation pattern of the CpG6 site in the spleen was different from the thymus and bursa of Fabricius.
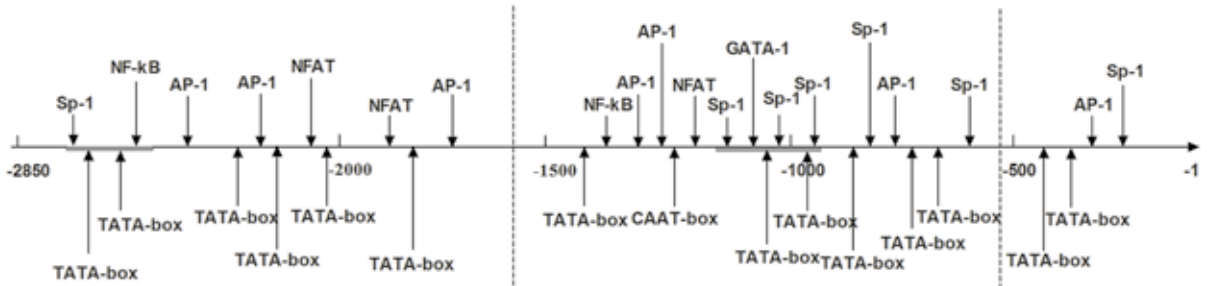
## The impact of promoter methylation states on IL-2 transcription

Figure 4A is a schematic diagram of the regulatory elements within the duck *IL-2* promoter. It lists a 1.5 kb nucleotide sequence upstream from the TSS, which contains 7 CpG sites. The results showed that the methylation levels at the same CpG site among tissues were clearly different (Fig. 4B). Specifically, the methylation frequency of CpG1, CpG9 and CpG10 in the thymus and bursa of Fabricius was significantly higher than in the spleen ($p < 0.05$). Additionally, the methylation frequency of the CpG2 site in the thymus was significantly higher than in the spleen and bursa of Fabricius ($p < 0.05$), and the methylation frequency of CpG6 in the spleen was significantly higher than in the thymus and bursa of Fabricius ($p < 0.05$). The overall methylation states and mRNA expression of *IL-2* among tissues are compared in Fig. 4C, D and show that the overall methylation states in the thymus were significantly higher than in the spleen ($p < 0.05$) (Fig. 4C). The expression of *IL-2* mRNA in the bursa of Fabricius was higher than in the spleen, and that in the spleen was higher than in the thymus. However, there were no significant differences in *IL-2* mRNA expression among the three immune tissues (Fig. 4D).
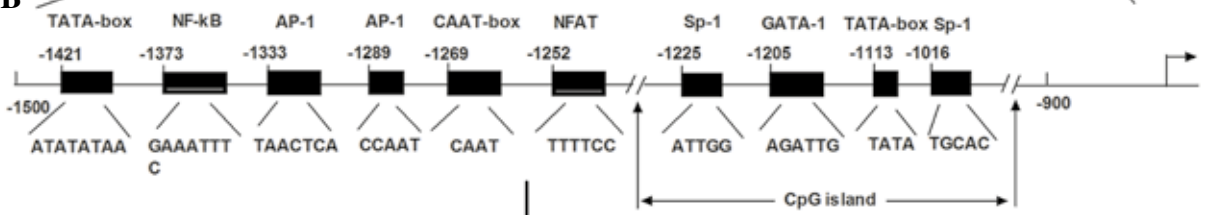
## Correlation analysis between promoter methylation states and expression levels of IL-2

The correlation analysis showed that there were no significant correlations between the methylation states of each

## A



## B



## C



**Fig. 1.** Schematic representation of *IL-2* promoter showing the regulatory elements governing gene transcription in duck. **A**) Regulatory elements predicted in the duck *IL-2* promoter. The arrow indicates the position of the core transcription factor binding sites and TATA-boxes, and the thick gray line shows the CpG islands. **B**) Transcriptional regulatory region. **C**) CpG sites in the transcriptional regulatory region. There are 11 CpG sites in this region, of which CpG3, CpG5, CpG8 and CpG11 site had no methylation data. The bases of the arrow points are CpG sites. The transcription factor binding site and the promoter structure are represented by the base sequence in the box, and the underlined sequence is the methylation detection region
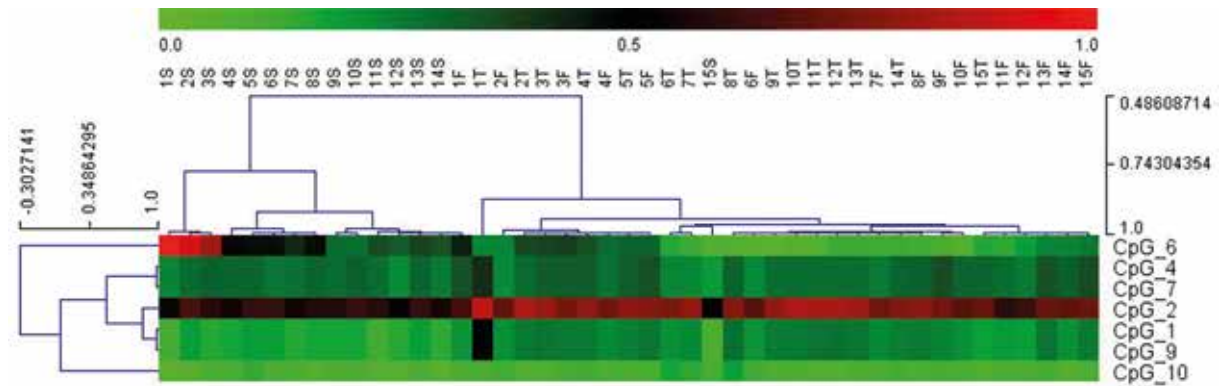
**Fig. 2.** Cluster analysis of tissues and CpG sites by *IL-2* methylation in immune tissues. Light green to deep red represents the frequency of methylation from 0 to 100%. Each row represents 1 sample, and each column represents 1 methylation site. Seven CpG sites of the *IL-2* promoter were analyzed by cluster analysis in the thymus (*n* = 15), spleen (*n* = 15) and bursa of Fabricius (*n* = 15). The molecular weights of CpG1 and 9, 4 and 7 sites are the same, and the methylation of each site is the mean value. T represents the thymus, S represents the spleen, and F represents the bursa of Fabricius
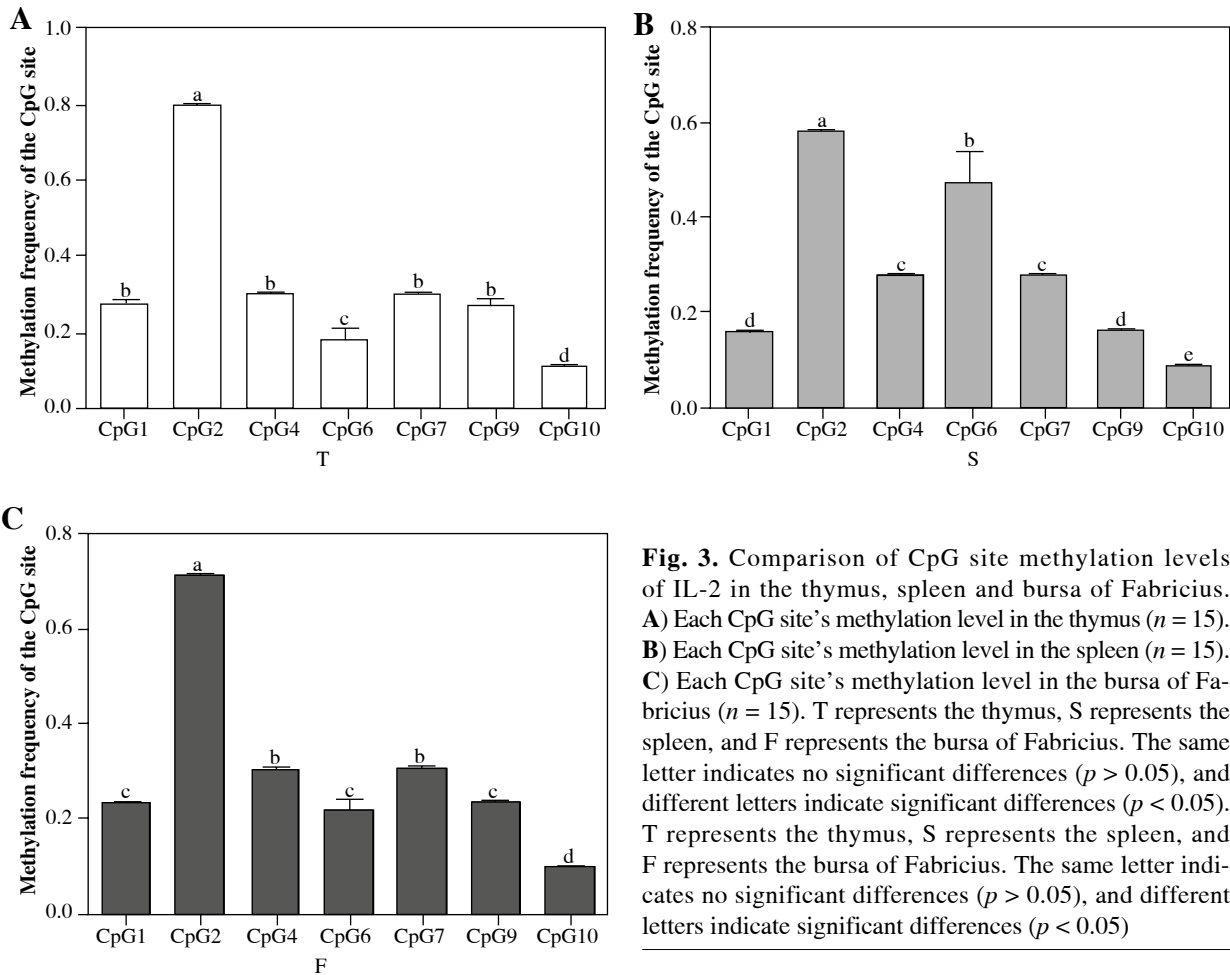




**Fig. 3.** Comparison of CpG site methylation levels of IL-2 in the thymus, spleen and bursa of Fabricius. **A)** Each CpG site's methylation level in the thymus (*n* = 15). **B)** Each CpG site's methylation level in the spleen (*n* = 15). **C)** Each CpG site's methylation level in the bursa of Fabricius (*n* = 15). T represents the thymus, S represents the spleen, and F represents the bursa of Fabricius. The same letter indicates no significant differences (*p* > 0.05), and different letters indicate significant differences (*p* < 0.05). T represents the thymus, S represents the spleen, and F represents the bursa of Fabricius. The same letter indicates no significant differences (*p* > 0.05), and different letters indicate significant differences (*p* < 0.05)
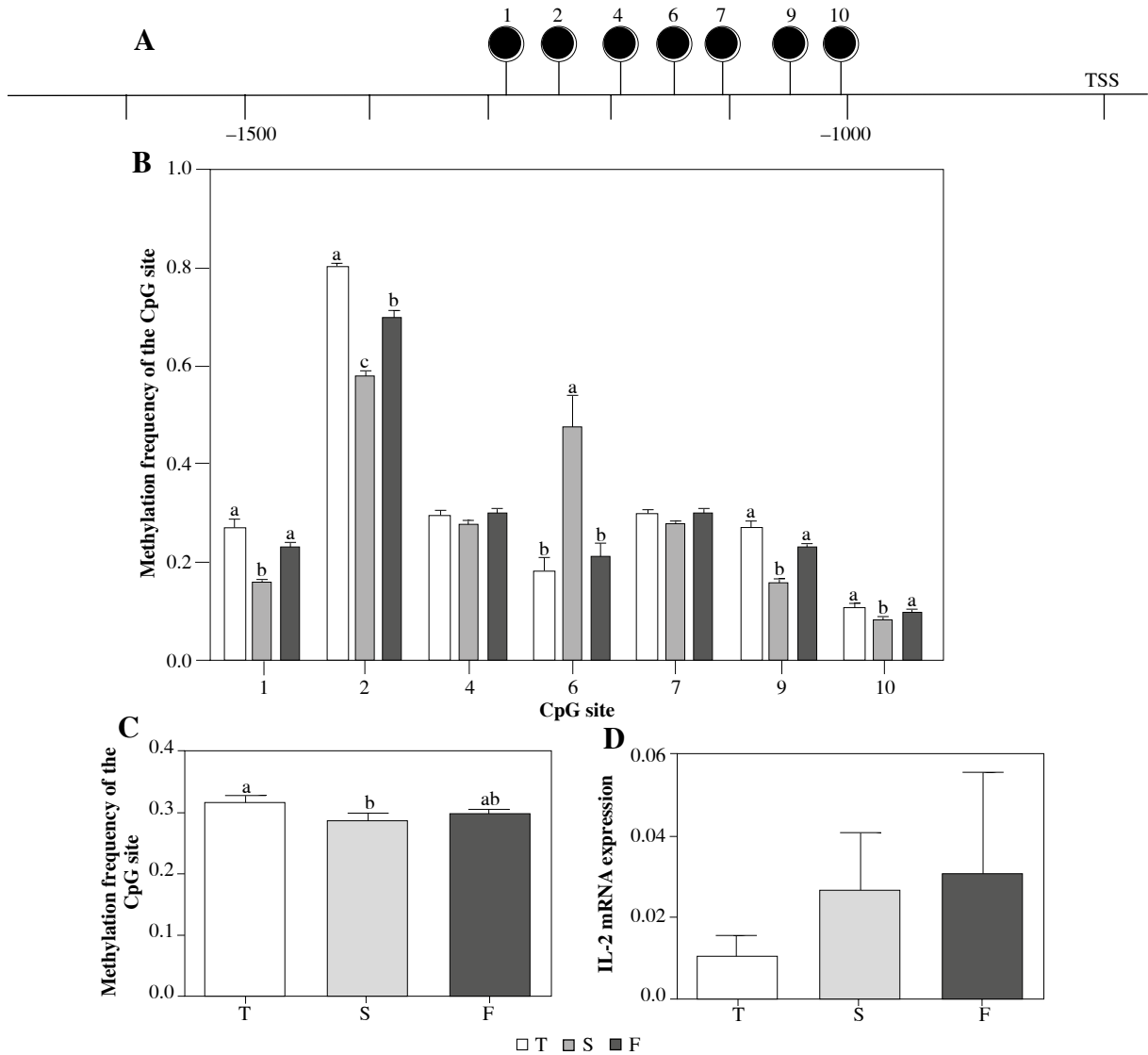
**Fig. 4.** Comparison between promoter methylation states and mRNA expression of *IL-2* in immune tissues. **A**) Schematic diagram of the regulatory elements within the duck *IL-2* promoter. The region shown includes 1.5 kb upstream from the TSS, which contains 7 CpG sites (●, methylated CpG). **B**) Methylation frequency of CpG sites of the duck *IL-2* gene promoter in the spleen, thymus and bursa of Fabricius ($n = 15$). **C**) Overall methylation frequency of *IL-2* gene promoter in the spleen, thymus and bursa of Fabricius ($n = 15$). **D**) mRNA expression of *IL-2* gene in the spleen, thymus and bursa of Fabricius. T represents the thymus, S represents the spleen, and F represents the bursa of Fabricius. The same letter indicates no significant differences ($p > 0.05$), and different letters indicate significant differences ($p < 0.05$)

**Table 4.** Correlation analysis between methylation status of CpG sites and mRNA expression

|   | CpG1 | CpG2 | CpG4 | CpG6 | CpG7 | CpG9 | CpG10 | Overall |
|---|------|------|------|------|------|------|-------|---------|
| T | −0.025 (0.931) | 0.000 (0.999) | 0.028 (0.925) | −0.007 (0.981) | 0.028 (0.925) | −0.025 (0.931) | −0.151 (0.607) | −0.068 (0.809) |
| S | 0.171 (0.543) | 0.29 (0.294) | 0.352 (0.198) | −0.062 (0.826) | 0.352 (0.198) | 0.171 (0.543) | −0.129 (0.646) | 0.106 (0.707) |
| F | 0.443 (0.098) | 0.380 (0.163) | 0.295 (0.286) | 0.210 (0.453) | 0.295 (0.286) | 0.443 (0.098) | 0.023 (0.935) | 0.406 (0.133) |

*T represents the thymus, S represents the spleen, and F represents the bursa of Fabricius. The molecular weights of CpG1 and 9, 4 and 7 sites were similar; therefore the methylation of each site was given as their mean value. P values are in parentheses. N = 15*

CpG site and IL-2 mRNA expression in the thymus, spleen and bursa of Fabricius (Table 4). We found that 28.5% of the CpG sites showed negative correlations between the methylation frequencies and the mRNA expression of IL-2, and 71.5% showed positive correlations. The methylation levels of CpG1, CpG6 and CpG10 in the thymus and CpG6 and CpG10 in the spleen were negatively correlated with the expression of IL-2 ($p > 0.05$). The other CpG sites were positively correlated with the expression of IL-2 mRNA in these tissues ($p > 0.05$).

## Discussion

Interleukin 2, which is mainly produced in the thymus and spleen, plays an important role in immune systems. The functions of IL-2 in immune processes have been clarified. However, the mechanism by which the expression of *IL-2* is regulated in immune processes has rarely been studied in birds. In addition, it had been reported that the passively acquired antibodies are released into the circulation when the yolk is digested by the embryo and can be detected as early as the 11th day of incubation, and IL2-producing cells such as those of the thymus and spleen can be detected in the late embryo [24-26], suggesting that *IL-2* expression may be triggered by stimulation of the embryonic period in immune tissues of naïve newborn (one-day-old) ducklings. On the other hand, stimulation of human CD4+ T cells induced *IL-2* expression following epigenetic changes, including active demethylation of specific CpG sites, as shown in previous documents. Therefore, to minimize the influence of external factors on the methylation status, the immune tissues of naïve newborn (one-day-old) ducklings were selected to study. In this study, the duck *IL-2* gene 5' flanking promoter region of 2850 bp was obtained. It was predicted that there was one NF-κB, two AP-1, one NFAT, and other core transcription factor binding sites distributed from -900 bp to -1500 bp, upstream of the duck *IL-2* transcription start site (Fig. 1B). In addition, there were two TATA-boxes and one CAAT-box typical core promoter structure. The NFAT family, AP-1 and NF-κB are important transcription factors inducing *IL-2* transcription in humans and mice, and all regulate the transcription of *IL-2* [27-31]. The data indicate that this region may play a key role in regulating *IL-2* transcription. Studies in mice and humans revealed that 300 bp upstream of the transcription start site of the *IL-2* gene was the core regulatory region [5], revealing that the transcriptional regulation of the *IL-2* promoter in ducks may be different from that in mammals. Furthermore, the duck *IL-2* promoter had two CpG islands, of which one (-1231 bp/-902 bp) is located in the core of the transcribed region (Fig. 1B). This indicates that duck *IL-2* gene transcription is possibly regulated by methylation modification and distinct from that of mammals.

The epigenetic modification of a gene promoter is an important method of regulating gene transcription. As

a main type of epigenetic modification, DNA methylation plays an important role in the transcription of genes. Several studies have indicated that methylation of promoters inhibits the transcription of genes by blocking transcription factor binding to its promoter regions [32-34]. In the present study, the CpG island (-1231 bp/-902 bp) was important for the gene expression of duck *IL-2* because there were more transcription factors predicted to exist in this area. Therefore, the CpG sites were detected in this region, and the relative position of the CpG sites and the core transcription factor binding sites were analyzed. The results showed that there were 11 CpG sites distributed in the CpG island (-1231 bp/-902 bp). Furthermore, CpG1 and CpG2 were located between NFAT and AP-1 (Fig. 1C), and CpG2 methylation frequency was significantly higher than that of other sites in the thymus, spleen and bursa of Fabricius ($p < 0.05$) (Fig. 3). In addition, we found that CpG1 and CpG2 in different individuals and tissues had higher homology methylation patterns (Fig. 2), suggesting that there was a similar regulation effect among these cluster sites for the effects of methylation patterns on *IL-2* gene transcription. Some studies have confirmed that cooperative interactions between NFAT and AP-1 were essential for *IL-2* gene expression induction [35, 36]. Therefore, our data suggest that these CpG sites may be essential for the regulation of transcription of duck *IL-2*.

Further studies have been performed to assess the effect of DNA methylation on the expression of *IL-2* in duck immune tissues. First, a two-way cluster analysis was used to determine the methylation frequency of the 7 CpG sites of the *IL-2* promoter in the thymus, spleen and bursa of Fabricius. The results revealed that the methylation level of the *IL-2* gene in the spleen tissue was different from the thymus and bursa of Fabricius (Fig. 2), suggesting that the methylation pattern of the *IL-2* gene promoter in the duck was tissue specific. Similar results were reported by Ma *et al.*, who found that the degree of DNA methylation differed between porcine fat and muscle tissue in pigs [37]. Fan *et al.* found that the frequency of methylation is different in human tissues [38]. This may be because the methylation pattern of the CpG6 site was different from the thymus and the bursa of Fabricius, leading to the methylation pattern of the *IL-2* gene in the spleen tissue being different from the thymus and bursa of Fabricius. Second, the correlation of *IL-2* promoter methylation states and the levels of *IL-2* gene expression were analyzed. The results showed that the expression of *IL-2* mRNA was different in the thymus, spleen and bursa of Fabricius in Fig. 4D ($p > 0.05$). A study indicated that the strength of the immune response is regulated largely by the amount of IL-2 available for T-cell growth [39]. Therefore, these results suggested that the strength of the immune response may be different in young duck immune tissue. This conclusion was in agreement with a previous study by Longenecker *et al.* (1966), who reported that the immune function of the thymus was weak. In chicken,

the spleen is of primary importance in the development of immunity to this strain of *Plasmodium lophurae*, the bursa is of secondary importance and contributes something to the development of the immune mechanism before 18 days of age [40], and there were differences in the immune function of the thymus and bursa of Fabricius [41]. In addition, the results showed that 28.5% of the CpG sites showed negative correlations between the methylation frequencies and the mRNA expression of *IL-2*, and 71.5% showed positive correlations (Table 4). For the methylation level of all CpG sites, the methylation level in the thymus was significantly higher than that in the spleen ($p < 0.05$) (Fig. 4C). Moreover, the expression of *IL-2* mRNA in the spleen was higher than that of the thymus ($p > 0.05$). These data show that the methylation frequency may be negatively correlated with expression of the IL-2 gene in duck immune tissue. The conclusion is consistent with the study by Marie-Chloé *et al.*, who found that arginine methylation of Tat negatively regulates its transactivation activity [42]. The DNA methylation level of the *Six1* promoter core region was negatively correlated with *Six1* gene expression *in vivo* [43]. However, whether DNA methylation has an effect on the expression of duck IL-2 has to be determined by some *in vitro* transcription experiments such as reporter plasmids carrying *IL-2* promoter/enhancer region or mutants, or using the promoter-targeted shRNAi method under stimulation conditions.

## Conclusions

The duck *IL-2* gene 5' flanking promoter region of 2850 bp was obtained in this study. A total of 11 CpG sites were predicted in the CpG island (-1231 bp/-902 bp) of the duck *IL-2* promoter. The transcription of duck *IL-2* may be distinct from that of mammals. CpG1 (-1284 bp) and CpG2 (-1264 bp) in the duck *IL-2* promoter showed a higher homology of methylation patterns, indicating a similar regulatory effect on their gene expression, and these CpG sites may be essential for the regulation of transcription of duck *IL-2*. The methylation pattern of the *IL-2* gene promoter in the duck was tissue specific.

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