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cDNA Cloning, Expression Analysis of Slc24a5 and Its Relationship With Melanin Deposition in Chicken*

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Abstract Melanin is a kind of biopolymer, composed of a complex quinine/indole-quinone derived mixture which is produced in melanocytes from tyrosine. More than 100 genes related to melanin deposition have been found in mouse. Slc24a5 (solute carrier family 24 , member 5) is a novel gene first cloned in zebrafish. And it has been confirmed that the Slc24a5 is involved in controlling the melanin deposition in zebrafish. Here the full length of the chicken slc24a5 mRNA sequence, its expression profile and a discussion of its relationship to melanin deposition in chicken were reported. Chicken Slc254a5 has a CDS of 1 269bp, predicting a protein of 423 amino acids which is about 80 amino acids shorter than in human and mouse. It has 9 exons and 8 introns and spans more than 11kb of genome sequence. The quantitative real-time PCR confirmed that the chicken Slc24a5 was highly expressed in the eye of White Leghorn and Beijing Fatty Chickens, and in the eye, skin and muscle of Silky. The relative expression in Silky eye is more than two times that of White Leghorn. The relative expression in Silky skin is about 70 times that of White Leghorn and about 15 times that of Beijing Fatty Chickens. The relative expression in Silky muscle is about 15 times that of White Leghorn and about 3 times that of Beijing Fatty Chickens. And the expression result is in accordance with the melanocyte and melanin deposition in these tissues.

Key words Slc24a5, chicken, melanin

The skin and hair color of animals is mainly caused by the deposition of melanin. Melanin is a kind of biopolymers, composed of a complex quinine/ indole-quinone derived mixture. There are two different kinds of melanin: eumelanin, which is black or brown; and pheomelanin, which is yellow or red[1,2]. Melanin is produced in melanocytes which are dentritic cells derived from neural crest[3]. Tyrosine is oxidized by tyrosinase and other enzymes to turn into the melanin[4,5]. Genes that can influence the melanin deposition are involved in many biological pathways, such as melanocyte development, components of melanosomes and their precursors, melanosome construction/protein routing, melanosome transport, eumelanin and pheomelanin and systemic effects. Until the year 2003, more than 800 phenotypic alleles located at 127 identified color loci had been found in mouse[6].

Slc24a5 (solute carrier family 24 , member 5) is a cation (sodium/potassium/calcium) exchanger which was first cloned in zebrafish[7]. It was confirmed to control the phenotype of *golden* in zebrafish. And the phenotype of golden is characterized by hypopigmentation of skin melanophores and retinal pigment epithelium[8]. A coding SNP (rs1426654) within SLC24A5 was found in the International Haplotype Map (HapMap)[9] and this coding region variantion in African-American and African-Cribbean populations indicated its function in human skin colour[7,10,11]. This single SNP with G and A alleles encoded alanine or threonine at amino acid 111 in the third exon of SLC24A5. The allele frequency for the Thr111 variant ranged from 98.7% to 100% among several European-American population samples, and for Ala111 variant the frequency ranged from 93% to 100% in African, Indigenous American, and East Asian population samples[7,9,12]. And the SNP in human SLC24A5 can be considered ancestry informative marker[13].

The Slc24a5 gene in birds has not yet been cloned. The chicken has been as an important

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experimental system for biological study for a long time in the field of development biology, immunology and microbiology. In recent years it has been used as a model to study gene function in large-scale[14].

Using chicken as a model to study Slc24a5 can give some information of this gene in bird. The chicken breed Silky is characterized by melanin deposition in the epidermis and dermis, and in the connective tissues, the mesenteries, the peritoneum, and in the meninges of the brain and spinal chord[15~18]. Silkies hold a particular promise for understanding pigment patterning and have used to study the characterization of melanocytes[19~22]. Here we use the Silky to study the Slc24a5 gene to provide information about melanin deposition at the molecular biological level.

1 Materials and methods

1.1 Total RNA and cDNA preparation

Three breeds of chicken (White Leghorn, Silky and Beijing Fatty Chickens) were used. Organs obtained from animals were immediately frozen in liquid nitrogen and stored at −70℃. Total RNA was prepared with Trizol reagent (Tiangen, China) from chicken E12 (embryo day 12), E16 (embryo day 16) and 1d (1d after birth) tissues (brain, eye, skin, muscle, heart, liver, kidney and lung). About 1μg of total RNA was used for reverse transcription. The reverse transcription reaction was done by MMLV reverse transcriptase (Promega, USA) and the oligo (dT) 18 primer. The mix without MMLV and RNasin was heated to 70℃ for 5 min to melt secondary structures of RNA and cooled immediately on ice for 2 min and collected by brief centrifugation. After adding MMLV and RNasin, reaction was carried out for 120 min at 37℃ and for 15 min at 72℃ in a 25 μl reaction volume.

1.2 RT-PCR analysis of chicken Slc24a5 transcripts

RT-PCR was performed on cDNA templates derived from chicken E12 and 1d total RNA of different tissues (brain, eye, skin, muscle, heart, liver, kidney and lung) to identify the expression profile of Slc24a5 in chicken. Two breeds of chicken (White Leghorn and Silky) were selected. In all PCRs, a housekeeping gene (GAPDH), was used to monitor genomic DNA contamination and equality of sample loading. Primer sequences of Slc24a5 and GAPDH are as follows. Slc24a5: Forward 5′ TGGAGGAATTGGTTCATGGT 3′, Reverse 5′ ACGTTGGATCCCACAATGTT 3′; GAPDH: Forward 5′ GACGTGCAGCAGGAACACTA 3′, Reverse 5′ CATCCACCGTCTTCTGTGTG 3′. PCRs were performed as follows: 35 cycles of 94℃ for 30 s, 60℃ for 30 s, 72℃ for 30 s.

1.3 3' and 5' RACE

A BLAST search was carried out using the protein sequences of Slc24a5 of human (NP_995322) and mouse (AAH94232) on NCBI to find a predicted chicken Slc24a5 mRNA sequence (NM_001038497). This sequence (NM_001038497) was then used to do a BLAST search in chicken EST databases. Several ESTs (BU324199, AJ728070, DR431117, CN231016, CK985412) which were very similar to NM_001038497 were assembled into a contig. Using the sequences derived from the contig, a pair of primers (Forward 5′ GATGATTACTTCCTACCGT 3′, Reverse 5′ ATTGTTCCCTATGATGCCA 3′) were designed to amplify a 1.2 kb fragment of Slc24a5. After the sequences were confirmed by sequencing, primers for 3′,5′ RACE were designed from the 3′ and 5′ end of the 1.2 kb fragment. Primers for 5′RACE are as follows. Forward primers: UAP, 5′ CUACUACUACUAGGCCACGCGTCGACTAGTAC 3′, AAP, 5′ GGCCACGCGTCGACTAGTACGGGIIGGIIGGGIIG 3′; Reverse primers (first 5′ RACE): 5R1, 5′ GCCAGGCAGTCTCTAAACAGCG 3′, 5R2, 5′ AAGATTGTAGATGGCTGATCCA 3′, 5R3, 5′ CTCCTTTTGTCACGAAGACTCC 3′; Reverse primers (second 5′ RACE): 5R1′, 5′ GGTAGGAAG TAATCATCGCA 3′, 5R2′, 5′ TGGACGCGGCCAAAAACATG 3′, 5R3′, 5′ CTCCATCCTTTCTCTCCTGC 3′. 3′RACE: Forward primers, 3R1, 5′ ATGTCTAACATTGTGGGATC 3′, 3R2, 5′ GCCTTCATAAATACATCCG 3′; Reverse primers AP, 5′ GGCCACGCGTCGACTAGTAC(T)17 3′, AUAP, 5′ GGCCACGCGTCGACTAGTAC 3′. After the sequences were confirm by sequencing, primers for 3', 5' RACE experiment were designed from the 3′ and 5′ end of the 1.2 kb fragment of Slc24a5. 5′ RACE was performed using the 5′ RACE Kit (Invitrogen, USA). 5′ RACE were performed twice. PCR was performed twice in one round of 5′ RACE. In the first round, 5′ RACE 5R1 was used to amplify the first strand of the cDNA and in the second round 5′ RACE 5R′ was used. The two times of PCRs were performed as follows: 35 cycles of 94℃ for 1 min, 55℃ for 45 s, 72℃ for 1.5 min. The PCR product was recycled and cloned into pMD-19 T-vector (Takara, China) and sequenced. More than

four clones were used to confirm the sequences.

When doing the 3′ RACE, the AP primer was used to amplify the first strand of the cDNA from the polyA site. Then two rounds of PCR were used to collect the 3′ end sequences of Slc24a5 which were the same as in the 5′ RACE procedure.

1.4 Quantitative real-time PCR

The first strand of cDNA was obtained as described in **1.1** (total RNA and cDNA preparation). Primers for Slc24a5 were designed to amplify the part common for all transcripts (Forward 5′ TGGAGG-AATTGGTTCATGGT 3′, Reverse 5′ ACGTTGG-ATCCCACAATGTT 3′). The housekeeping β-actin gene was used as a reference (Forward 5′ GCCC-ATCTATGAAGGCTACG 3′, Reverse 5′ TCCTTG-ATGTCACGCACAAT 3′). Quantitative real-time PCR was performed using ABI Prism 7900 Sequence Detection System (Applied Biosystems, USA). SYBR@ GREEN PCR Master Mix (Applied Biosystems, USA) was used for detection. PCR was performed in the following conditions: precycling hold at 95℃ for 10 min, cycles: 95℃, 15 s, 60 ℃, 1 min, up to 40 cycles. ΔΔCT method was used for quantity calculations[23]. The slope of the validation curve was <0.1, which ensures, that target and reference efficiencies were approximately equal. Excel was used to collect the data of the quantitative real-time PCR and to draw the histogram of the relative expression of Slc24a5 in different tissues and breeds.

1.5 HE (hematoxylin and eosin) staining

For the three breeds of chicken, four tissues (eye, skin, muscle and heart) from E16 were collected. The skin was removed from the back of the chicken and the muscle was from the leg. All the collected tissues were fixed in 4% paraformaldehyde, and embedded in paraffin. 7-μm sections were stained with hematoxylin and eosin, and examined by light microscopy (Olympus, Japan). Images were captured by a DP70 CCD camera (Japan).

1.6 Melanin deposition analysis

Photoshop was used to detect the melanin deposition in different tissues and Excel was used to analyze the results obtained from Photoshop.

Photoshop was used to open the HE staining image, and the magic wand tool was used to select the black areas (melanin deposition sites) (shown in Figure 1a). The histogram tool is used to calculate the pixels of the selected areas (show in Figure 1b,c). The pixels were recorded in Excel. And the pixels of the total HE staining image is collected using the same way.

The melanin deposition ratio was calculated using the formula below. $MR=SP/TP$. *MR*: melanin deposition ratio, *SP*: pixels of selected region, *TP*: pixels of the total HE staining image.

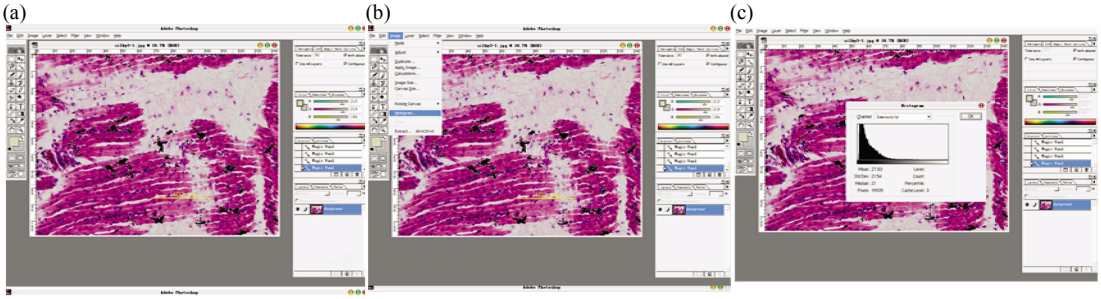

Fig. 1 Flowchart of using the Photoshop to detect the melanin deposition in different tissues

(a) Selection of the black regions. The selected regions are highlighted by white broken line; (b), (c) Using the histogram to calculate the pixels of the selected areas.

2 Results

2.1 Characterization and genomic analysis of chicken Slc24a5

The complete chicken Slc24a5 cDNA was obtained using a combination of bioinformatics tools, PCR cloning, and 3′ and 5′ rapid amplification of cDNA ends (RACE) analysis (described in **Materials and methods**).

The known mammalian Slc24a5[7] sequences were used to do a BLAST search in the chicken genome database on NCBI. Homologous sequences were used to design PCR degenerate primers to amplify the cDNA of chicken Slc24a5. A PCR fragment of about 1.2 kb was obtained. After sequencing, the sequence of the 1.2 kb was compared with sequences in GenBank. This sequence matched other known mammalian

Slc24a5 and the predicted chicken Slc24a5. 3′ and 5′ RACE primers were designed based on the 3′ and 5′ sequence of the amplified 1.2 kb. Poly(A)+ RNA was purified from chicken (White Leghorn) eye. In the 3′ RACE experiment, a PCR fragment of 350 bp was obtained and in the first 5′ RACE, a PCR fragment of about 450 bp was obtained. In the second 5′ RACE, a PCR fragment of about 200 bp was obtained. After sequencing and analyzing, the full length of chicken Slc24a5 cDNA was obtained. A conceptual fusion of the result of 3′ and 5′ RACE is presented in Figure 2. The chicken Slc24a5 cDNA is composed of an open reading frame of 1 269 bp and 84 bp of 3′ untranslated sequence with a potential polyadenylation signal sequence (AATAAA) (GenBank Accession No. DQ_915178). A translation product of 423 amino acids is predicted, with a molecular mass of 46 345.2 u, and an isoelectric point of 4.88. The comparison of chicken Slc24a5 protein with other organisms (human, mouse and zebrafish) indicated that the protein is conserved among different organisms as there are many large regions of conserved sequences (Figure 3). The resulting protein of chicken Slc24a5 is about 80 amino acids shorter than those of human, mouse and zebrafish.

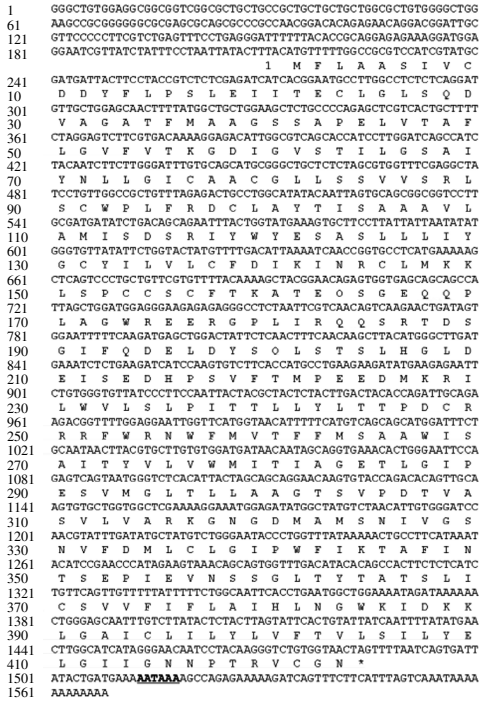

Fig. 2 Nucleotide sequence, deduced amino acid sequence of chicken Slc24a5

And the coding region are highlighted by underlining. The polyadenylation signal sequence is highlighted by underlining and bold.

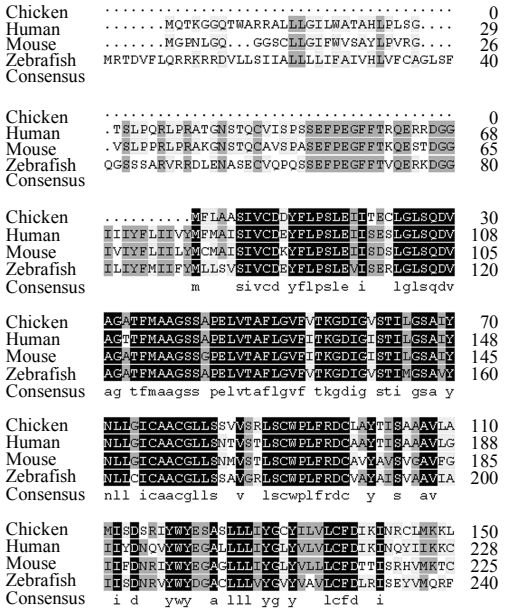

Fig. 3 Comparison of different Slc24a5

Human SLC24A5: NP_995322; Mouse Slc24a5: NP_778199; Zebrafish Slc24a5: NP_001025451.

With the completion of the Chicken Genome Project, we were able to obtain chicken genome sequences from the Chicken Genome Database. A BLAST search was done using the cloned full length cDNA sequence of the chicken Slc24a5. The BLAST result shows that the chicken Slc24a5 has 9 exons and 8 introns and spans more than 11 kb of genome sequence on chicken Chr.10 (UCSC chicken genome database, website: http://genome.ucsc.edu). The splice donor/acceptor boundaries of chicken Slc24a5 agree with the GT/AG rule.

2.2 Analysis of chicken Slc24a5 transcripts by RT-PCR and quantitative real-time PCR

The analysis of the expression of chicken Slc24a5 embryo (embryo day 12, E12) and adult (1 day after birth, 1d) tissues was carried out by RT-PCR (Figure 4). A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as a control to monitor genomic DNA contamination and the equality of sample loading. Two different breeds of chicken (White Leghorn, and Silky) having different skin color were used in this experiment. The RT-PCR of GAPDH indicated that the loading of samples were almost the same and that none of the RNA samples had genomic DNA contamination. Slc24a5 is expressed in different tissues (including brain, eye, skin, muscle, heart, liver, lung, gut and kidney) and in different stages of chicken development (embryo stage, E12 and adult stage, 1d).

The expression level however, is different in different tissues. The expression tendency in both the embryo and adult stages are similar. A relatively high expression of Slc24a5 can be seen in the eye of White Leghorn. And in other tissues of White Leghorn, the expression of Slc24a5 is in similar level which is lower than that in the eye. In Silky, the expression of Slc24a5 in eye, skin and muscle are higher than those in other tissues. The expression of Slc24a5 in skin and muscle of Silky is higher than the White Leghorn in both embryo and adult stages.

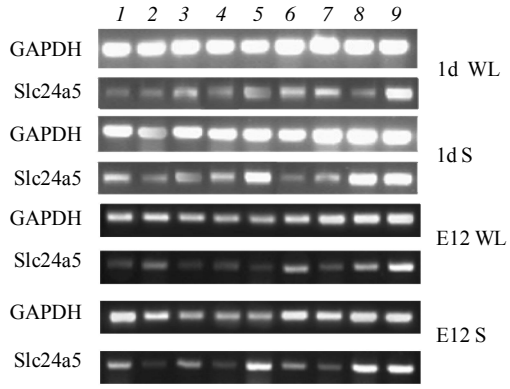

Fig. 4 Slc24a5 expression in 1d (1 day after birth) and E12 (embryo 12 days) chicken

WL: White Leghorn; S: Silky. *1*～*9*: Kidney, heart, brain, gut, muscle, lung, liver, skin, eye.

We can see different expression in different tissues of White Leghorn and Silky from the RT-PCR result. The expression level in skin and muscle between White Leghorn and Silky is different. A quantitative real-time PCR was performed to confirm the different expression levels of Slc24a5. Three breeds of different skin-colored chicken (white-skinned White Leghorn, yellow-skinned Beijing Fatty Chickens, and black-skinned Silky) were used in this experiment. Comparisons were made in tissues of E16 chickens. From the results of the quantitative real-time PCR (Figure 5), we confirmed relatively high expression of Slc24a5 gene in the eye of all the three breeds of chicken. The expression in Silky eye was extremely high with its relative expression reaching up to more than two times that of White Leghorn. The relative expression in Silky skin and muscle was also very high. The relative expression in Silky skin is about 70 times that of White Leghorn and about 15 times that of Beijing Fatty Chickens. The relative expression in Silky muscle is about 15 times that of White Leghorn and about 3 times that of Beijing Fatty Chickens. The expression of Slc24a5 in other tissues is almost the same in the three breeds of chicken.

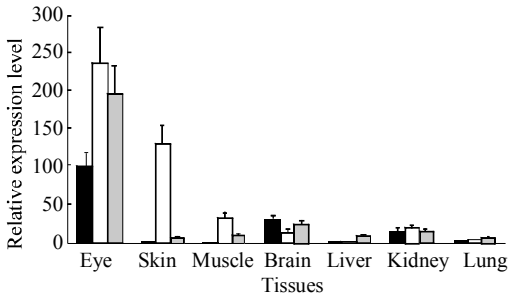

Fig. 5 Quantitative real-time PCR results

■: White Leghom; □: Silky; ▨: Beijng Fatty Chickens.

2.3 HE staining

Tissues (eye, skin and muscle) of E16 in the three breeds of chicken (White Leghorn, Silky and Beijing Fatty Chickens) were used. Skin of the back and muscle of the leg were removed from E16. We can see the melanocyte and the melanin deposition from the HE staining result. A large amount of melanin was identified in the eyes of three species of chicken. But the amount of melanin was the highest in the eye of Silky with melanin depositing in the membranes (Figure 6).

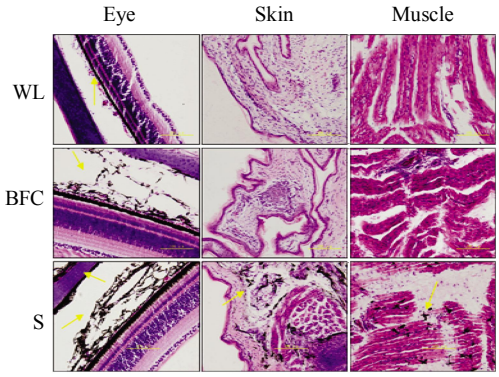

Fig. 6 HE staining results of eye, skin and muscle

WL: White Leghorn; BFC: Beijing Fatty Chickens; S: Silky. Yellow arrow points at the melanocyte and melanin deposition.

As in the skin and muscle, a large amount of melanocytes and melanin was seen only in the Silky (Figure 6). In the skin of Silky the melanocytes and melanin are located in the base of the epidermis, in the dermis and in the connective tissue (Figure 6). In addition many melanocytes with heavy deposition of

melanin were found among the muscle fiber and the connective tissue of Silky (Figure 6). But the melanocytes and melanin deposition could not be detected in the skin and muscle of White Leghorn and Beijing Fatty Chickens (Figure 6).

2.4 Melanin deposition analysis

The HE stained slices were used to determine the relative quantity of melanin deposition and were transferred into TIFF images. Photoshop and Excel were used to analyze the results. The eye slices of three breeds of chicken (White Leghorn, Silky and Beijing Fatty Chickens) and the skin and muscle samples of Silky were used. Each tissue was collected from five chickens per breed. For each tissue, six slices were used to calculate the relative amount of melanin deposition.

The results indicate that melanin deposition was highest in the eye of the Silky, about four times of the melanin deposition in the eye of White Leghorn and two times of Beijing Fatty Chickens (Figure 7). The melanin deposition in the eye of Silky was the highest among all tissues, about 10 times that of the muscle and about 5 times that of the skin (Figure 7).

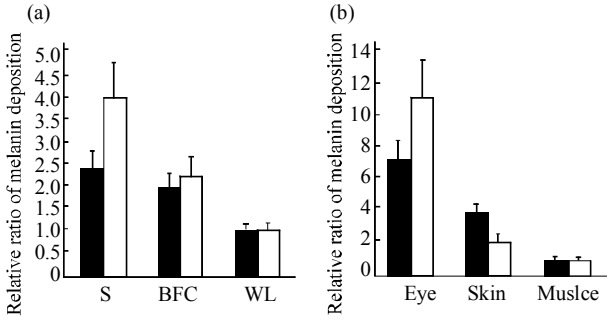

Fig. 7 Melanin deposition analysis and its accordance with real-time results

(a) Relative ratio of melanin deposition and real-time result of relative expression level in the eye of White Leghorn (WL), Beijing Fatty Chickens (BFC) and Silky (S). (b) Relative ratio of melanin deposition and real-time result of relative expression level in the eye, skin and muscle of Silky. ■: Real-time result of relative expression level. □: Relative ration of melanin deposition.

3 Discussion

To obtain the utmost 5′ end sequence of chicken Slc24a5 mRNA, 5′ RACE was performed twice. The second 5′ RACE primers were based on the first 5′ RACE sequence. But the sequence of chicken Slc24a5 mRNA we obtained from the second 5′ RACE is shorter than the human and mouse Slc24a5 in the 5′ end. To determine whether the sequence we obtained from the RACE experiment is the full length CDS of Slc24a5. Primers were designed encompassing the initial sequence of the 5′ RACE result based on the genome sequence. This sequence is predicted to be in the same exon of the initial sequence of the 5′ RACE, and these primers were used to carried out a RT-PCR. The primers failed, however, to generate a positive band from the cDNA. A comparison of the predicted proteins of different organisms demonstrated that the sequence is conserved in the 5′ end from the first amino acid of chicken Slc24a5. The region encompassing the first 80 amino acids of human, mouse and zebrafish which is the lacking region of chicken Slc24a5 protein, it is not so conserved(Figure 3). Thus, we concluded that the obtained maximized sequence contained the full CDS of the chicken Slc24a5.

The HE staining of the tissues confirmed that the melanin deposition in the eyes of all the three breeds was the highest. The quantitative real-time PCR results also showed that the expression of Slc24a5 was the highest compared with all other tissues. Tissues from the Silky which had the most deposition of melanin expressed the highest level of Slc24a5. The same tissues in White Leghorn and Beijing Fatty Chickens that had no deposition of melanin expressed lower amounts of Slc24a5. This result was similar to the result of quantitative real-time PCR in mouse[7]. The HE staining analysis confirmed these results(Figure 7). The quantitative real-time PCR indicates that the expression of Slc24a5 in the eye of Silky was the highest, the second in the eye of Beijing Fatty Chickens, and the lowest in the eye of White Leghorn. The same order of the melanin deposition in the eye was observed (Figure 7a). Comparison of real-time and melanin-deposition analysis results confirmed the relationship between Slc24a5 expression and melanin deposition in the skin and muscle of Silky(Figure 7b).

Melanin was deposited in the melanocytes and the keratinocytes nearby the melanocytes[2,24]. No melanin deposition was observed in the keratinocytes in our experiment(Figure 6). So the melanin deposition sites in our experiment were the melanocyte location sites. From the results of our experiments a conclusion was made that the Slc24a5 is highly expressed in the melanocyte containing tissues in chicken which was also that of the mouse[7,10]. The expression differences of Slc24a5 can be related to the different deposition of

melanin and the different dispersion of the melanocytes.

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鸡 Slc24a5 基因的 cDNA 克隆、表达分析及其与黑色素沉积的关系研究 *

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摘要　黑色素是一种由醌 / 吲哚 - 醌来源的混合物组成的生物高分子化合物．目前在小鼠中已发现的和黑色素沉积相关的基因已经超过了 100 个．Slc24a5 (solute carrier family 24 , member 5)基因是在斑马鱼中克隆的一个新基因．研究表明，Slc24a5 基因可以调控斑马鱼中的黑色素沉积．鸡作为一种模式动物，已经广泛地被应用于实验研究．为了研究 Slc24a5 基因在鸡中的情况，克隆了鸡 Slc24a5 基因全长 CDS，并且分析了其与黑色素沉积的关系．鸡 Slc24a5 基因全长 CDS 为 1 269 bp，编码一个 423 氨基酸残基的蛋白质．该蛋白质比哺乳动物中的少大约 80 个氨基酸残基．基因全长超过 11 kb，包含 8 个内含子和 9 个外显子．RT-PCR 结果显示，鸡 Slc24a5 基因在多处组织表达(眼、脑、皮、肉、心、肝、肾和肺)．通过荧光实时定量 PCR 对不同鸡种中的 Slc24a5 基因表达量进行检测，发现其在白莱杭，乌骨鸡和北京油鸡的眼睛中表达量很高．并且在乌骨鸡的皮肤和肌肉中 Slc24a5 基因也有很高的表达．乌骨鸡眼睛中的 Slc24a5 基因表达量为白莱杭的 2 倍．而在乌骨鸡皮肤中，Slc24a5 基因表达量为白莱杭的 70 倍，为北京油鸡的 15 倍．Slc24a5 基因在乌骨鸡肌肉中的表达量为白莱杭的 15 倍，为北京油鸡的 3 倍．同时通过对这 3 个鸡种中黑色素在各组织中沉积量进行分析，发现黑色素沉积越多的地方，Slc24a5 基因表达量越高．这些结果表明鸡 Slc24a5 基因的表达与鸡中黑色素的沉积相关．

关键词　Slc24a5，鸡，黑色素

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