Screening the optimal activity region of the dopachrome tautomerase gene promoter in sheep skin melanocytes

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\textbf{ABSTRACT}

To determine the optimal activity region of the dopachrome tautomerase gene (DCT) promoter in sheep skin melanocytes, five eukaryotic expression vectors were constructed containing DCT promoter fragments of different lengths (441 bp, 652 bp, 834 bp, 1155 bp, and 1504 bp) ligated to the pLV.ExSi.P/Puro-mouse\textsuperscript{f}DCT-eGFP vector. These constructs were transiently transfected into sheep skin melanocytes using liposome transfection technology. GFP (Green Fluorescent Protein) expression varied among the groups transfected with different constructs as follows: 652 bp > 834 bp > 441 bp > 1504 bp > 1155 bp. GFP expression was significantly higher in the 652 bp and 834 bp groups than in the 1155 bp group (p < 0.01), while expression in the 652 bp group was significantly higher than in the 1504 bp group (p < 0.05). Additionally, we identified elements participating in the initiation of gene transcription (\textit{a} CAAT box), cell type-specific expression (M-box), and the regulation of gene expression (E-box) in the sheep DCT promoter. In conclusion, the optimal active region of the sheep DCT promoter is located between –593 bp and + 41 bp.

1. Introduction

Melanin is produced by melanosomes within melanocytes, and is responsible for the mammalian coat colour (Zhang et al. 2017). Several enzymes are involved in catalyzing the synthesis of melanin, such as tyrosinase (TYR), dopachrome tautomerase (DCT or TYRP2), and tyrosinase-related protein 1 (TYRP1) (Woods and Bishop 2011). The absence of tyrosinase leads to an albino phenotype, while mutations of \textit{DCT} and \textit{TYRP1} alter the type of pigment produced (Beermann et al. 2004). For example, the absence of \textit{DCT} leads to a dark grey coat colour (Guyonneau et al. 2004). \textit{DCT} is first expressed in the embryonic retinal pigmented epithelium, then the telecephalon and melanoblasts (Woods and Bishop 2011).

Mouse \textit{DCT} is around 60 kb, and contains eight exons. An M-box located at –150 bp to –140 bp of mouse \textit{DCT} is the transcription initiation site, while the equivalent in human \textit{DCT} is located at –135 bp to –125 bp. \textit{TYR} and \textit{TYRP1} mouse promoters also have M-boxes at similar positions (Budd and Jackson 1995). In humans, a 32-bp element spanning from –268 bp to –56 bp and a proximal region located at –447 bp to –415 bp are necessary for pigment cell-specific expression of \textit{DCT} (Yokoyama et al. 1994). Moreover, melanogenesis and melanocyte cellular differentiation are regulated by the microphthalmia-associated transcription factor (MITF) and sex determining region \textit{Y-box} 10 (SOX10), which have been verified as transcription factors that bind the \textit{DCT} promoter (Ludwig et al. 2004).

Based on existing knowledge of the \textit{DCT} promoter in mice and humans, we hypothesized that an optimal melanin cell-specific \textit{DCT} promoter for sheep skin melanocytes could be developed. To obtain an efficient \textit{DCT} promoter region for use in sheep skin, five segments of the \textit{DCT} promoter were cloned, analyzed, inserted into eukaryotic expression vectors, and transfected into sheep melanocytes. Our results indicated that the region –593 bp to + 41 bp is the most efficient for expression in sheep skin melanocytes.

2. Materials and methods

2.1. Sample collection

All animal procedures followed the Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments. Five healthy 2-year-old small fat-tailed sheep were selected for sample collection from a sheep farm in Jixiu, Shanxi Province, China. Five pieces of skin (10 × 10 mm\textsuperscript{2}) from the ears were obtained through punch skin biopsy under local anesthesia and immediately stored in liquid nitrogen.

2.2. Plasmids

Genomic DNA was extracted from the skin of sheep ears using phenol–chloroform extraction. Five pairs of primers were designed based on the sheep \textit{DCT} sequence (GenBank: NC_019467) from –2000bp to + 297 bp, including exon 1, which is regarded as the promoter region. All downstream primers were located in exon 1. \textit{SnaB1} and \textit{SalI} restriction enzyme sequences were added to the 5’ ends of upstream
Five fragments of the sheep DCT promoter were PCR-amplified using 2 × Taq MasterMix (Dye) (CWBIO, Beijing, China) with a thermal PCR programme consisting of pre-denaturation at 94°C for 2 min, then 35 cycles of 94°C for 30 s, 58.1°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were cloned into the pUC-T vector (CWBIO), digested with SnaB I and Sal I (TAKARA, Dalian, China), and purified. Meanwhile, the pLV.ExSi.P/Puro-mouse DCT-eGFP vector was digested by SnaB I and Sal I and purified. Then, purified PCR products and pLV.ExSi.P/Puro-eGFP were linked using T4 DNA ligase (TAKARA) to construct pLV.ExSi.P/Puro-sheep DCT-eGFP.

### 2.3. Sequence analysis

BLAST sequence analysis (https://www.ncbi.nlm.nih.gov/) was used to analyze sequence homology with reference sequences. The motifs of the DCT promoter were predicted by Primer 5.0 software based on the longest fragment.

### 2.4. Sheep skin melanocyte culture and transfection

Sheep skin melanocytes were donated by the Laboratory of Alpaca Biology, Shanxi Agricultural University, China, and cultured in MelM medium (ScienCell Research Laboratories, Carlsbad, CA, USA).

Based on the manufacturer’s instructions, eight groups with different ratios of plasmids (pMSCV PIG vector (Addgene, Cambridge, MA, USA)) and transfection reagents

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**Table 1.** PCR primers for the DCT 5′ regulatory region.

| Gene     | Sequence (5′-3′)                  | PCR product |
|----------|----------------------------------|-------------|
| DCT (1155) | TACGTATCTTGTACGTCAGCCCTCA         | 1155 bp     |
|          | GTCCGACCTGTGTGCTGTTGTGCACAT       |             |
| DCT (1504) | TACGTAAAAACCTGCTGACAGATGCG        | 1504 bp     |
|          | GTCCGAC GTGCGGCAAGGCACTAAGGA       |             |
| DCT (834)  | TACGTAAAGCAGAACTCAGCTGACATTC      | 834 bp      |
|          | GTCCGACAGAAGCCGCCCCACCAGAGAG      |             |
| DCT (652)  | TACGTATCTCAGGCGCTTACACAAAC        | 652 bp      |
|          | GTCCGACCAGACAGGCGACAGAAAG         |             |
| DCT (441)  | TACGTAAAAGGCTGTTTTTTAACCAGGGA     | 441 bp      |
|          | GTCCGACAGAAGCCGCCCCACCAGAGAG      |             |

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**Figure 1.** Flow chart of eukaryotic expression vector construction, using the 834 bp fragment as an example.
were incubated with melanocytes in 24-well plates (Table 2). The medium was replaced with fresh serum-free medium (MelM) 6 h after transfection, then cells were incubated for an additional 48 h. GFP (Green Fluorescent Protein) expression was then observed using an inversion fluorescence microscope (Leica, Wetzlar, Germany) to select the optimal volume of plasmids and transfection reagents.

Based on these optimization conditions, five recombinant plasmids (441 bp, 652 bp, 834 bp, 1155 bp, and 1504 bp) were transfected into sheep skin melanocytes using the X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland). pMSCV PIG vector was used as control. Forty-eight to 72 h after transfection, GFP expression was observed and measured by Image Pro Plus 6.0 software. Each experiment was repeated three times for each group and three fields were selected to detect GFP expressions.

2.5. Statistical analysis

Differences in GFP expression among melanocytes transfected with different DCT promoter fragments were analyzed using SPSS software.

3. Results

3.1. Amplification of sheep DCT promoter fragments

PCR amplification produced five fragments that were about 441 bp, 652 bp, 834 bp, 1155 bp, and 1504 bp in length (Figure 2).

3.2. Analysis of sheep DCT promoter

Sequencing results showed that the homology between 1504 bp, 1155 bp, 834 bp, 652 bp, and 441 bp fragments with the reference sequence was 97%, 96%, 96%, 96%, and 96%, respectively (data not shown). This indicated that all five recombinant plasmids were constructed successfully. The transcription start site was predicted to be located at −508 bp based on the mouse DCT sequence (Figure 3). The five fragments contained sequences from −396 bp to + 27 bp (441 bp), −593 bp to + 41 bp (652 bp), −789 bp to + 27 bp (834 bp), −929 bp to + 206 bp (1155 bp), and −1247 bp to + 237 bp (1504 bp) (Figure 4).

Primer 5 software identified motifs within the 1504 bp fragment, including a CAAT box, M-box, and E-box (Table 3; Figure 3).

3.3. The optimal ratio of plasmid to transfection reagents

GFP expression was highest when the ratios of plasmids to transfection reagents were 2:3 and 2:4 (Figure 5). Taking the cytotoxicity of transfection reagents into account, we selected the ratio of 2:3 for further experiments. This corresponded to 1 μg plasmid and 1.5 μL transfection reagent per well of a 24-well plate.

3.4. Expression activity of sheep DCT promoter

GFP expression varied among the different transfection groups, with the following trend: 652 bp > 834 bp > 441 bp > 1504 bp > 1155 bp. GFP expression in the 1155 bp group was significantly lower than in the 652 bp and 834 bp groups (p < 0.01), while GFP expression in the 1504 bp group was significantly lower than in the 652 bp group (p < 0.05) (Figure 6). This was observed from the analysis of fluorescent images (Figure 7). These findings indicated that the entire DCT promoter region from −1247 bp to + 237 bp can initiate transcription, but that the optimal active region is located between −593 bp and + 41 bp.

4. Discussion

In the present study, we showed that the sheep DCT promoter contains a CAAT box, M-box, and E-box. The CAAT box signals

Table 2. Volume of DNA and transfection reagents used in cell transfection.

| Ratio | Plasmid (μg) | Transfection reagents (μL) |
|-------|-------------|---------------------------|
| 1:3   | 0.5         | 1.5                       |
| 1:4   | 0.5         | 2                         |
| 1:5   | 0.5         | 2.5                       |
| 1:6   | 0.5         | 3                         |
| 1:3   | 1           | 1.5                       |
| 2:4   | 1           | 2                         |
| 2:5   | 1           | 2.5                       |
| 2:6   | 1           | 3                         |

Figure 2. PCR products detected by agarose gel electrophoresis. Marker: DL2000 marker; lanes 1 and 2: 441 bp PCR product; lanes 3 and 4: 652 bp PCR product; lanes 5 and 6: 834 bp PCR product; lanes 7 and 8: 1155 bp PCR product; lanes 9 and 10: 1504 bp PCR product.
Figure 3. Motifs of sheep DCT promoter and location of different cloned DCT fragments.

Figure 4. Sequence of sheep DCT promoter. Primers are in boldface. Nucleotides on the 5′ side of ATG are indicated by negative numbers. The transcription start site is indicated by a box. The ATG codon for initiating methionine is indicated by a double underline and is marked as +1. The CAAT box sequence and M-box are indicated by underline and undulated line, respectively.
the binding site for RNA transcription factors, and is typically accompanied by a conserved consensus sequence. Its function is to enable gene transcription at sufficient quantities (Dolfini et al. 2009). The M-box is the most striking feature of the promoter region of TYR family members, and is located between –160 bp and –40 bp. We found that the sheep DCT M-box was located at –139 bp to –129 bp upstream of the transcription start site, which is similar to the location in human DCT (Budd and Jackson 1995). The M-box is the binding site of MITF, and the SOX10 binding site is located in its immediate vicinity (Ludwig et al. 2004).

MITF, a basic helix–loop–helix leucine zipper transcription factor, regulates melanocyte cellular differentiation and the transcription of melanogenic enzymes such as TYR, TYRP1, and DCT (Levy et al. 2006). SOX10 has been shown to regulate MITF expression in melanocytes (Britsch et al. 2001), although dog DCT lacks SOX10 binding sites (Jakubczak et al. 2016; Kowalczyk et al. 2016). The E-box binds transcription factors to imitate transcription (Chaudhary and Skinner 1999), and the E-box of TYR family promoters is also the binding site of MITF (Miccadei et al. 2008).

A previous study of human DCT revealed two regulatory regions, a 32-bp element from –447 bp to –415 bp and the proximal region from –268 bp to –56 bp, that are required for pigment cell-specific expression (Yokoyama et al. 1994). However, we did not find similar regions in the sheep DCT promoter. Instead, we identified E-box within the two regulatory regions.

GFP expression did not differ significantly different after transfection of 441 bp, 652 bp, and 834 bp fragments of the DCT promoter, especially between 652 bp and 834 bp groups. This indicated that –593 bp to +41 bp is the optimal region of the DCT promoter, and that the region between –593 bp and –396 bp might contain positive regulatory elements. GFP expression in cells transfected with the 1155 bp fragment was significantly lower than in the 652 bp and 834 bp groups (p < 0.01). GFP expression in the 1504 bp group was significantly lower than in the 652 bp group (p < 0.05). Each experiment was repeated three times for each group. Values represent the mean ± SE from three independent experiments. * significant difference, ** extremely significant difference.

### Table 3. Motifs of the sheep DCT promoter.

| Name | Motif       | No. | Pos              |
|------|-------------|-----|------------------|
| CAAAT | CCAAT       | 1   | –1105            |
| M-box | GGTCATGTGCT | 1   | –647             |
| E-box | CANNTG      | 3   | –644, –522, –250 |

### Figure 5. Transfection efficiencies with different ratios of plasmids and transfection reagents. The efficiency increased when the ratio increased from 1:3 to 1:6, and was highest at ratios of 2:3 and 2:4. The efficiency decreased with excess transfection reagents.

### Figure 6. GFP expression in different transfection groups. Highest and lowest GFP expression occurred in the 652 bp group and 1155 bp group, respectively. GFP expression in the 1155 bp group was significantly lower than in the 652 bp and 834 bp groups (p < 0.01). GFP expression in the 1504 bp group was significantly lower than in the 652 bp group (p < 0.05). Each experiment was repeated three times for each group. Values represent the mean ± SE from three independent experiments. * significant difference, ** extremely significant difference.
The shortest fragment of 441 bp (−396 bp to +27 bp) initiated transcription in sheep skin melanocytes. This compares with minimal regions of just 150 bp upstream of the initiation codon for the melanocortin receptor 1 promoter (Miccadei et al. 2008), 270 bp upstream of the transcription start site for the TYR promoter (Kluppel et al. 1991), and between −44 bp to +107 bp for the TYRP1 promoter (Lowings et al. 1992).

5. Conclusion
The entire region of the sheep DCT promoter from −1247 bp to +237 bp can initiate transcription, but the optimal active region is located between −593 bp and +41 bp.

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Disclosure statement
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Figure 7. GFP expression in sheep skin melanocytes transfected with different fragments of the sheep DCT promoter. A: melanocytes under white light. B: melanocytes transfected with the 441 bp fragment. C: melanocytes transfected with the 652 bp fragment. D: melanocytes transfected with the 834 bp fragment. E: melanocytes transfected with the 1155 bp fragment. F: melanocytes transfected with the 1504 bp fragment.

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Figure 7. GFP expression in sheep skin melanocytes transfected with different fragments of the sheep DCT promoter. A: melanocytes under white light. B: melanocytes transfected with the 441 bp fragment. C: melanocytes transfected with the 652 bp fragment. D: melanocytes transfected with the 834 bp fragment. E: melanocytes transfected with the 1155 bp fragment. F: melanocytes transfected with the 1504 bp fragment.
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