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Cloning and Expression Analysis of Goat cAMP-Responsive Element Modulator

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Abstract: cAMP Responsive Element Modulator (CREM) as a cAMP-responsive member of the bZIP family of transcription factor has been demonstrated to play an important role in male fertility. In the present study, researchers cloned the goat CREM from testis by RT-PCR, 3' and 5' RACE. The full-length cDNA was 1183 bp with a 960 bp Opening Reading Frame (ORF) encoding 319 amino acids which shares 84.7-99.4% similarity with the human, sheep, dog, mouse and cattle. The deduced amino acid sequence contained two Gln-rich regions, a PKA phosphorylation site and a DNA-binding domain. Goat CREM mRNA was detected in all tissues investigated in this study. The highest expression level of CREM mRNA was observed in testis whereas the lowest level was found in lung. The expression level of CREM mRNA in testis was increased with the age. This expression pattern was similar to that in mouse and human. Furthermore, immunohistochemistry study showed CREM mainly expressed in round spermatids but not in spermatogonium and spermatocyte. These results indicated that CREM may play a key role in maintain normal physiological functions and development in goat, especially in spermatid maturation.

Key words: cAMP-responsive element modulator, goat, cloning, expression, spermatid maturation

INTRODUCTION

The cAMP Responsive Element Modulator (CREM) belonged to the cAMP-Responsive Element Binding protein (CREB) family and played important roles in regulating transcription in response to various stresses, metabolic and developmental signals (Hummler *et al*., 1994; Sassone-Corsi, 1995). CREM transcription factors were involved in many physiological systems including cardiac function (Muller *et al*., 2003; Isoda *et al*., 2003), circadian rhythms (Foulkes *et al*., 1997) pituitary function (Struthers *et al*., 1991) memory and long-term potentiation (Silva *et al*., 1998), brain development (Maldonado *et al*., 1999; Diaz-Ruiz *et al*., 2008) cholesterol synthesis in the liver (Acimovic *et al*., 2008) and spermatogenesis (Sassone-Corsi, 1998).

To date, CREM was notable for its pivotal roles in spermatogenesis. As a master-switch, CREM regulated the expression of key testis-specific genes (Kimmins *et al*., 2004). The post-meiotic expression of known genes such as RT7 (Delmas *et al*., 1993) CYP51 (Rozman *et al*., 1999), transition protein-1 (Kistler *et al*., 1994), calspermin (Sun *et al*., 1995) and PHGPx (Tramer *et al*., 2004) have been proved to be targets of CREM-mediated transactivation. CREM can be acted as activator and repressor by alternative splicing of exons and alternative start sites of translation (Lalli and Sassone-Corsi, 1994; Habener *et al*., 1995). Among the repressors, it was worth mentioning that an early response CRE-binding factor designated as ICER was transcribed from a cAMP-inducible alternative intronic P2 promoter of the *CREM* gene (Vouk *et al*., 2005). The *de novo* synthesized ICER protein could repress its own promoter and thus generate a feedback regulation loop during the process of cAMP-responsive transcription (Lamas *et al*., 1996). This kind of autoregulation played an essential role in spermatogenesis during both sexual maturation and the repeated cycles of germ cell development (Walker and Habener, 1996). The activator isoforms CREMτ protein was specifically and highly expressed in haploid spermatids (Delmas *et al*., 1993). CREM deficient mouse was sterile due to post-meiotic arrest at the first step of spermiogenesis (Blendy *et al*., 1996; Nantel *et al*., 1996; Peri and Serio, 2000).

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Infertile men with round spermatid maturation arrest resulted in substantial decrease or complete shortage of both CREM protein (Weinbauer *et al*., 1998) and CREM mRNA (Steger *et al*., 1999). So, far *CREM* gene has been cloned from many species. The distribution of CREM protein in different tissues has also been studied in dog (Uyttersprot and Miot, 1997) and non-human primates (Behr and Weinbauer, 2000). However, goat *CREM* gene has not been identified. Thus, researchers cloned the nucleotide sequence of CREM cDNA from goat testis and characterized its deduced amino acid sequence. We also examined the relative expression of CREM mRNA in various tissues and in testis of different age. The cellular locations of CREM in adult goat testis were also studied by immunohistochemistry analysis. The study will be useful for further investigation of function and regulation mechanism of goat CREM in the process of spermatogenesis.

MATERIALS AND METHODS

Animal and tissue collections: Male Taihang black goat used in this study was maintained at Licheng Sheep Breeding Center in Shanxi province, China. The use of goats in this study was approved by the Institutional Animal Care and Use Committee of Shanxi Agricultural University. Five adult male bucks were used to investigate the transcription analysis of CREM mRNA in normal goat tissues. The testis, heart, lung, kidneys, liver, muscle, brain and spleen from each goat were collected and stored at -80°C for the cloning and tissue-specific expression analysis. To identify expression of CREM mRNA in different developmental stages of the testis, bucks were castrated at 1 (n = 5), 2 (n = 5), 4 (n = 5), 6 (n = 5), 8 (n = 5), 12 (n = 5) and 20 (n = 5) weeks old, respectively. Half of the testis was frozen in liquid nitrogen for total RNA extraction and the other half was fixed in a 4% paraformaldehyde solution.

Total RNA isolation: Total RNA was extracted from all collected tissues with trizol reagent protocol (Invitrogen, Carlsbad, CA, USA) according to method provided by the manufacturer and quantified using ND-1000 spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies Inc., USA). Total RNA integrity was confirmed by running the RNA sample on a 1% formaldehyde agarose gel with ethidium bromide.

Cloning the full-length cDNA of goat testis CREM: The testicular total RNA was used to reverse transcription by PrimeScript™ RT reagent kit (Takara, Japan) following the manufacturer's instructions. Specific primers (Table 1) were designed using highly conserved regions by comparing all known CREM sequences using the Blast program. Using these primer pairs, goat CREM cDNA was amplified from the testis with a Taq polymerase (TAKARA Ex Taq, Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. PCR was carried out as follows: 94°C for 3 min; 35 cycles of 94°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec and finally 72°C for 10 min. Amplified products were run on a 1% agarose gel and the corresponding DNA bands were purified and cloned into the pMD18-T vector (Takara, Japan) for sequencing.

The 5'-and 3'-cDNA RACE PCR were performed using the SMARTTM RACE cDNA Amplification kit (Clontech, USA). Gene Specific Primers (GSP) were designed based on the above partial sequence. The 3'-end was cloned by using GSP-CREM1 and 10x Universal Primer A Mix (UPM). PCR was performed as follows: 94°C for 2 min; ten cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 2 min; 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min and finally 72°C for 10 min. For 5' RACE, the 5'-end was amplified with UPM and GSP-CREM2. PCR was carried out as follows: 94°C for 2 min; ten cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 2 min; 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min and finally 72°C for 10 min. PCR products were purified, recovered and cloned into the pMD18-T vector (Takara, Japan) for sequencing. The full-length cDNA of CREM was obtained using DNAStar Lasergene 7.1 Software after 5'-and 3'-RACE were assembled.

Table 1: Sequences of primers used in the study

Primers	Sequence (5'-3')
RT-PCR (SP1)	
SP1F	GGCTCGTCGTCCCACCTCCT
SP1R	GCTGCTGGGGACTGTGCAGG
Primers for 3' RACE	
3' GSP-CREM outer primers	GCAGCACAGTAGTAGTAGATGG TACCGTCGTTCCACTAGTGATTT
3' GSP-CREM inner primers	AGCTGCTAAAGAATGTCGACGTCG CGCGGATCCTCCACTAGTGATTTC ACTATAGG
Primers for 5' RACE	
5' GSP-CREM outer primers	CATGGCTACATGCTGACAGCCTA
5' GSP-CREM inner primers	GGCTCGTCGTCCCACCTCCT CGCGGATCCACAGCCTACTGATGA TCAGTCGATG
GAPDH	
F	TCCACGGCACAGTCAAGG
R	TCAGCACCAGCATCACCC
CREM	
F	TGCTGCCACTGG CGACATGC
R	TGCTGGGGACTGTGCAGGCT

Sequence analysis: Nucleic acid and amino acid sequence of CREM cDNA were analyzed using DNAMAN sequence analysis programs (DNAMAN Version 5.2.2). Open Reading Frame (ORF) was predicted with DNA star software package. Multiple sequence alignment of different species accessible from NCBI databases was performed using the CLUSTALW multiple alignment program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Needle program (http://www.ebi.ac.uk/Tools/emboss/align/) was used to analyze the identities of *CREM* gene among different species.

Real-time PCR analysis: Quantitative real-time PCR was conducted to determine the relative CREM mRNA expression levels in different samples with GAPDH as an internal standard. All PCR reactions were performed using Mx3000P real-time PCR system (Stratagene, USA) and SYBR® Permix Ex Taq™ kit (Takara, Japan) following the manufacturers' instructions. About 1 μg total RNA from different samples was reverse transcribed into cDNA with a PrimeScript™ RT reagent kit. All the primer sequences of Q-PCR are shown in Table 1. Q-PCR was performed at 95°C for 10 sec; 40 cycles of 95°C for 10 sec, 62°C for 20 sec and a following cycle of 62°C for 30 sec and 95°C for 15 sec and reaction specificity was determined when dissociation curves were only specific peak. PCR efficiencies were detected using relative standard curve derived from diluted cDNA reaction mixture (2-fold serial dilution with six levels).

PCR efficiencies were between 90 and 110% and R^2 values for all standard curves were between 0.998 and 0.999. The Threshold Cycle (CT) values were used to quantify the PCR product. The ΔCT for each sample was subtracted CT (housekeeping gene: *GAPDH*) from CT (target gene). The relative expression level of CREM was calculated by $2^{-\Delta\Delta CT}$. Data of real-time PCR was statistically analyzed using SPSS software by one-way ANOVA and t-test to compare the difference in mean values.

Immunohistochemistry assay: Immunolocalization was performed to determine CREM localization in testis using commercial immunostaining kit (Boster, China). The testis were fixed and embedded in paraffin wax using standard techniques. Paraffin sections were deparaffinized, rehydrated and then treated with 3% H_2O_2 at 37°C for 10 min.

The deparaffinized sections were then blocked with 2% Bovine Serum Albumin (BSA) for 30 min at 37°C, washed in Phosphate Buffer Saline (PBS, 0.01 M pH 7.2) for 20 min and incubated with Polyclonal rabbit anti-CREM primary antibody (Abcam, USA) overnight at 4°C. Having been washed with PBS, slides were treated with secondary antibody (Boster, China) and red fluorescence (Cy3) under the instruction of manufacturer.

Negative controls were performed by using no primary antibody and replacing with PBS. The sections were viewed under Leica DMIRB microscope (Leica, Germany).

RESULTS AND DISCUSSION

Cloning and sequencing of CREM cDNA: The full length of CREM cDNA was determined by combining the CREM partial cDNA, 5' and 3' fragment obtained in this study. Goat CREM cDNA was 1183 bp long and contained the entire open reading frame of 960 bp encoding 319 amino acid residues with two Gln-rich regions, a PKA phosphorylation site and a DNA-binding domain with leucine zipper structure. The deduced amino acid sequence of the goat CREM cDNA cloned in this study was shown to have high identities with cattle (99.4%), mouse (96.2%), dog (86.8%), sheep (85.9%) and human (84.7%) (Fig. 1).

Expression analysis of CREM mRNA in different tissues: As shown in Fig. 2, CREM mRNA can be detected in testis, heart, liver, spleen, lung, kidney, muscle and brain. The highest expression level of CREM mRNA was observed in testis while the lowest was detected in lung. In liver, the expression level of CREM mRNA was similar with kidney. Moreover, the level of CREM mRNA showed no difference between heart, spleen and muscle.

Expression analysis of CREM mRNA in testis of different age: The expression level of CREM mRNA in testis can be found in 1, 2, 4, 6, 8, 12 and 20 weeks old of goat and it was increased with the age. The highest expression level was detected in 20 weeks old testis.

Analysis of goat CREM cDNA sequence found a potential RNA-destabilizing signal (ATTTA). The ATTTA motif had a short half-life and it was associated with the rapid turnover of mRNA (Shaw and Kamen, 1986; Wahab *et al*., 1998). The deduced amino acids contained two Gln-rich regions, a PKA phosphorylation site and a DNA-binding domain with leucine zipper structure. These functional region were highly conserved with human (Masquilier *et al*., 1993), mouse (Foulkes *et al*., 1991) and dog (Uyttersprot and Miot, 1997), suggesting their fundamental role in the transactivation process. Comparison with mouse, human orthologs and dog revealed the goat CREM sequence lack of the second DNA-binding domains. The results showed that we cloned and sequenced goat CREM cDNA corresponding to the CREMτα splice variant (Fig. 3).

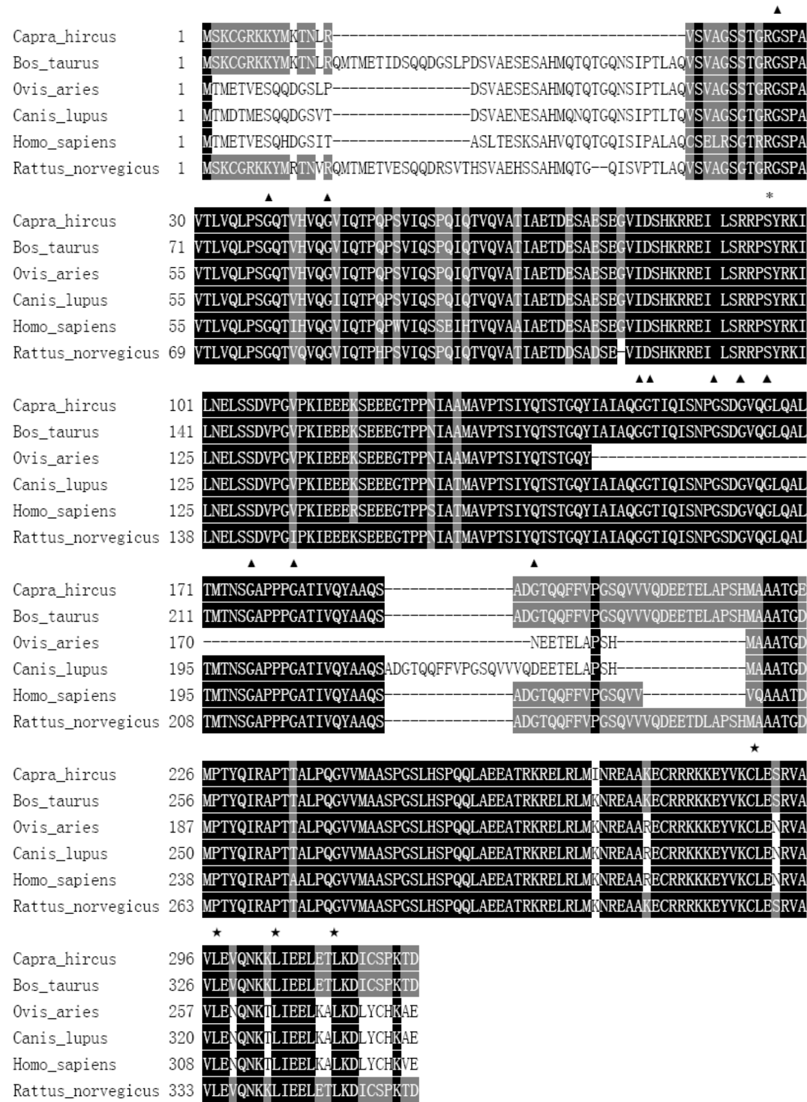

Fig. 1: Multiple sequence alignment of CREM from different species. Abbreviations are as follows: *Capra_hircus* (GenBank Accession No.: HM802260.1), *Bos taurus* (GenBank Accession No.: NM_001034710.2), *Ovis aries* (GenBank Accession No.: NM_001104935), *Canis lupus* (GenBank Accession No.: X99115), *Homo sapiens* (GenBank Accession No.: S68271.1) and *Rattus norvegicus* (GenBank Accession No.: BC078899.1). Identical residues are shown in black shaded and similar amino acids are gray shaded. The closed triangle indicates conserved glutamic residues. Asterisks represent serine residue highly conserved in all CREM. Leucine residus involved in the leucine zipper structure of DNA-binding domains are indicated by pentagons. Dashes represent amino acids absent among sequences

The results of CREM mRNA expression in adult goat tissues were shown in Fig. 2a. The expression level of CREM mRNA in testis was higher than other tissues and the lowest was detected in lung. The similar results were reported by De Cesare *et al*. (2000) who observed the high-level expression of CREM in testis compared with other tissues. Also, Northern-blot analysis showed that the CREM transcripts were dominant in the testis and very low in lung in dog (Uyttersprot and Miot, 1997). However, Behr and Weinbauer (2000) found CREM can only be detected in testis and other organs such as liver, heart, kidneys, cortex and cerebellum could not be detected in the primate.

These different results showed CREM expression was in a species-specific manner. In addition, researchers assume that distribution pattern of CREM-isoforms

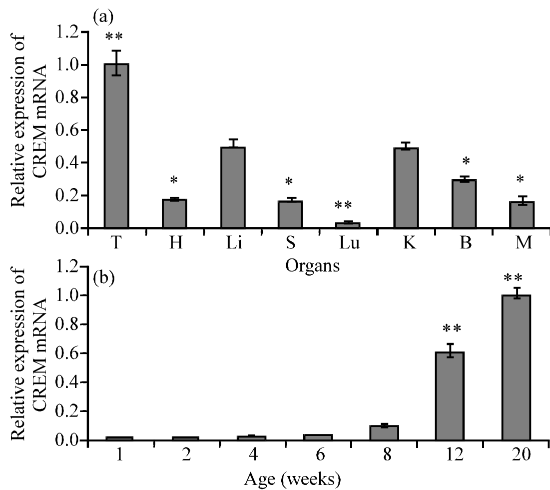

Fig. 2: a) Relative expression levels of CREM mRNA in different tissues and b) different ages of testes. Results are means±SEM (n = 5) and normalized by GAPDH. Testes (T), Heart (H), Liver (Li), Spleen (S), Lung (Lu), Kidney (K), Muscle (M), Brain (B). *means $p<0.05$ and **means $p<0.01$

among species also caused the different results. Therefore, further study is warranted to focus on CREM-isoforms expression in different tissues, especially in testis of goats.

In the following study, researchers examined the CREM mRNA expression in 1, 2, 4, 6, 8, 12 and 20 weeks old of goat testis. The results showed that relative expression of CREM mRNA showed no difference between 1, 2, 4, 6 and 8 weeks old testis, increased at 12 weeks and reached the highest level in 20 weeks old testis (Fig. 2b). It was demonstrated that CREM was an age-dependent protein in testis. The results were similar with Blocher *et al.* (2003) who found CREM mRNA was expressed in mid and late-pachytene spermatocytes as well as in round spermatid. Sexual maturity of goat was about 20 weeks old. So, CREM protein was probably acted as repressors before puberty. The cellular locations of CREM in adult goat testis (20 weeks old) showed CREM was only detected in round spermatids of the convoluted seminiferous tubules (Fig. 4). Similar observations were also observed in human

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1       TCCCAGTGGGGCTCGTCGTCCCACCTCCTCGCGTCCGTAAGCAGTGACGAGGTCCGCTAC

61      GTAAATCGCTTCGCTGCGGGATAAATAGAGAAGAAAACAGGAAGGAGCAAAGCATTGATT

121     ACTAATGTCTTAATAATGAGCAAATGTGGCAGGAAAAAATATATGAAGACAAATCTAAGA
 1                      M  S  K  C  G  R  K  K  Y  M  K  T  N  L  R

181     GTTTCTGTAGCTGGATCCAGCACCGGAAGAGGCTCCCCAGCTGTAACTCTAGTACAGTTA
 16      V  S  V  A  G  S  S  T  G  R  G  S  P  A  V  T  L  V  Q  L

241     CCTTCCGGCCAGACCGTCCACGTCCAGGGAGTAATTCAGACACCACAGCCATCCGTTATT
 36      P  S  G  Q  T  V  H  V  Q  G  V  I  Q  T  P  Q  P  S  V  I

301     CAGTCACCACAAATACAAACTGTTCAGGTAGCAACCATTGCAGAAACAGATGAGTCTGCA
 56      Q  S  P  Q  I  Q  T  V  Q  V  A  T  I  A  E  T  D  E  S  A

361     GAATCAGAAGGTGTAATTGATTCTCATAAACGTAGAGAAATCCTTTCACGAAGACCCTCT
 76      E  S  E  G  V  I  D  S  H  K  R  R  E  I  L  S  R  R  P  S

421     TACAGAAAAATACTGAATGAACTTTCCTCTGATGTGCCTGGTGTACCCAAGATTGAAGAA
 96      Y  R  K  I  L  N  E  L  S  S  D  V  P  G  V  P  K  I  E  E

481     GAAAAATCAGAGGAAGAGGGAACACCGCCTAACATCGCTGCTATGGCAGTACCGACTAGC
116      E  K  S  E  E  E  G  T  P  P  N  I  A  A  M  A  V  P  T  S

541     ATATATCAGACTAGCACGGGGCAATACATTGCTATAGCCCAAGGTGGGACAATCCAGATT
136      I  Y  Q  T  S  T  G  Q  Y  I  A  I  A  Q  G  G  T  I  Q  I

601     TCTAACCCAGGATCTGATGGTGTTCAGGGACTGCAGGCATTAACAATGACAAATTCAGGA
156      S  N  P  G  S  D  G  V  Q  G  L  Q  A  L  T  M  T  N  S  G

661     GCTCCTCCACCAGGTGCTACAATTGTGCAGTACGCAGCACAGTCAGCAGATGGCACACAG
176      A  P  P  P  G  A  T  I  V  Q  Y  A  A  Q  S  A  D  G  T  Q

721     CAGTTCTTTGTCCCAGGCAGCCAGGTGGTGGTTCAAGATGAGGAAACTGAACTTGCCCCA
196      Q  F  F  V  P  G  S  Q  V  V  V  Q  D  E  E  T  E  L  A  P

781     AGTCACATGGCTGCCGCCACTGGCGAGATGCCGACTTACCAGATCCGAGCTCCAACCACT
216      S  H  M  A  A  A  T  G  E  M  P  T  Y  Q  I  R  A  P  T  T

841     GCTTTGCCACAGGGAGTGGTGATGGCTGCCTCCCCCGGAAGCCTGCACAGTCCCCAGCAG
236      A  L  P  Q  G  V  V  M  A  A  S  P  G  S  L  H  S  P  Q  Q

901     CTAGCAGAGGAAGCGACACGCAAACGGGAGCTGAGGCTAATGATAAACAGGGAAGCTGCT
256      L  A  E  E  A  T  R  K  R  E  L  R  L  M  I  N  R  E  A  A

961     AAAGAATGTCGACGTCGAAAGAAAGAATATGTCAAGTGTCTGGAGAGTCGAGTCGCAGTG
276      K  E  C  R  R  R  K  K  E  Y  V  K  C  L  E  S  R  V  A  V

1021    CTGGAAGTTCAAAACAAGAAGCTTATAGAGGAACTGGAAACCTTGAAAGACATTTGCTCT
296      L  E  V  Q  N  K  K  L  I  E  E  L  E  T  L  K  D  I  C  S

1081    CCCAAAACAGATTAGTAGAAATATTTAACTATGAACTGATTACAACCTGTACAGTTGCTT
301      P  K  T  D  *

1141    TTGAAGGCAATACAATATATAGCCGGCAAGAAAAAAAAAAAA
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Fig. 3: Nucleotide and the predicted amino acid sequences of goat. The start codon and stop codon are in the black boxes. Gln-rich regions are underlined and PKA phosphorylation site is dotted underlined. DNA-binding domains are in the dotted boxes and leucines involved in the leucine zipper structure in this domain are also in the dotted boxes. The arrows noted the sites of alternative splicing. The ATTTA motif was double underlined

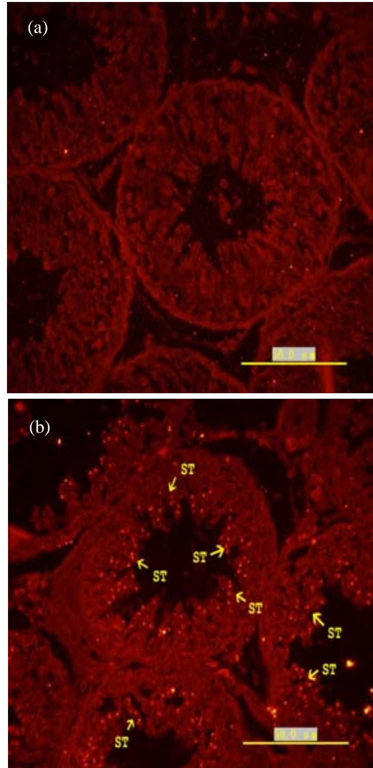

Fig. 4: Cellular location of CREM transcript in the 20 weeks old goat testes. The section a) was reacted with normal serum and section b) was reacted with anti-CREM antibody. The control a) exhibited no positive signals. Signals in the round Spermatid (ST) were indicated by arrows

(Weinbauer *et al*., 1998; Blocher *et al*., 2003) and mouse (Nantel *et al*., 1996; Blendy *et al*., 1996). It could be suggested CREM in adult goat testis may also involve in regulating the post-meiotic expression of related gene. In addition, it is worth mentioning again that the amino acid sequence of ICER is identical to the sequence of CREM activators except for the first few amino acids and ICER can be activated by CREM. However, researchers still can not distinguish between CREM activators and the ICER repressor due to lack of specific antibodies. Therefore, we can not determine that whether ICER protein was expressed in round spermatids in goat testis.

CONCLUSION

In this study, researchers obtained the full-length of goat CREMτa cDNA for the first time and determined the expression patterns of CREM mRNA in different tissues and different ages of testis in goat. CREM was ubiquitously detected in all tissues, suggesting CREM play important biological roles in goat. The high expression level of CREM mRNA and immunohistochemical localization of CREM in goat testis suggested that CREM may play important roles in male fertility. Further studies about regulation mechanisms of CREM in goat testis need to be conducted.

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