

## Molecular Cloning and Identification of the Transcriptional Regulatory Domain of the Goat Neurokinin B Gene *TAC3*

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**Abstract.** Neurokinin B (NKB), encoded by *TAC3*, is thought to be an important accelerator of pulsatile gonadotropin-releasing hormone release. This study aimed to clarify the transcriptional regulatory mechanism of goat *TAC3*. First, we determined the full-length mRNA sequence of goat *TAC3* from the hypothalamus to be 820 b, including a 381 b coding region, with the putative transcription start site located 143-b upstream of the start codon. The deduced amino acid sequence of NKB, which is produced from preproNKB, was completely conserved among goat, cattle, and human. Next, we cloned 5'-upstream region of goat *TAC3* up to 3400 b from the translation initiation site, and this region was highly homologous with cattle *TAC3* (89%). We used this goat *TAC3* 5'-upstream region to perform luciferase assays. We created a luciferase reporter vector containing DNA constructs from -2706, -1837, -834, -335, or -197 to +166 bp (the putative transcription start site was designated as +1) of goat *TAC3* and these were transiently transfected into mouse hypothalamus-derived N7 cells and human neuroblastoma-derived SK-N-AS cells. The luciferase activity gradually increased with the deletion of the 5'-upstream region, suggesting that the transcriptional suppressive region is located between -2706 and -336 bp and that the core promoter exists downstream of -197 bp. Estradiol treatment did not lead to significant suppression of luciferase activity of any constructs, suggesting the existence of other factor(s) that regulate goat *TAC3* transcription.

**Key words:** Estrogen, GnRH, Neurokinin B, Promoter, Ruminant

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In mammalian females, two modes of gonadotropin-releasing hormone (GnRH) release regulate ovarian function. One is the pulsatile GnRH secretion that causes pulsatile luteinizing hormone (LH) and follicle-stimulating hormone (FSH) releases, which are responsible for follicular development and steroidogenesis [1–3]. The other is the surge mode of GnRH secretion followed by an LH surge, leading to ovulation. Both GnRH/LH pulse and GnRH/LH surge are controlled by estrogen feedback, but estrogen acts differently on these two secretory patterns: GnRH/LH pulses are negatively regulated by estrogen, while GnRH/LH surge is positively regulated by estrogen [4].

The question of which neuronal substrates are the target of estrogen feedback effects on GnRH secretion is still open to debate. Since GnRH neurons do not express estrogen receptor  $\alpha$  (ER $\alpha$ ) [5], other sites of estrogen action have been predicted. Currently, kisspeptin neurons, which express ER $\alpha$  [6, 7], are the major candidates to transfer estrogen signals to GnRH neurons and organize GnRH

secretion [8]. Kisspeptin neurons are located in two regions of the hypothalamus, the anterior hypothalamus, such as the medial preoptic area (mPOA) and anteroventral periventricular nucleus (AVPV), and the arcuate nucleus (ARC) [9–12]. Multiple lines of evidence have suggested that kisspeptin neurons in the ARC and the mPOA regulate GnRH/LH pulses and GnRH/LH surges, respectively [13]. Kisspeptin neurons in the ARC are called KNDy neurons since they co-express the neuropeptides neurokinin B (NKB) and dynorphin A (Dyn) in many species including goat [14–19], while kisspeptin neurons in the mPOA/AVPV do not express NKB or Dyn; thus, NKB and Dyn are possible key molecules that make KNDy neurons to be the generator of GnRH pulses.

NKB, a member of the tachykinin family, is produced from preproNKB which is encoded by *TAC3* gene in goat, cattle, and human [20]. NKB acts through the receptor NK3R, encoded by *TACR3* gene. The loss-of-function mutants for *TAC3* or *TACR3* in humans exhibit gonadal dysfunction and the disappearance of normal LH release [21, 22], indicating a key role of NKB in reproductive function. Recent studies reported that an intracerebroventricular administration of NKB induced GnRH pulses in goats [18], and LH pulses were suppressed by the intravenous and intracerebroventricular injection of NKB receptor antagonist in monkeys and rats, respectively [17, 23]. On the other hand, Dyn administration gradually decreased LH concentrations in goats [18]. These data suggest that NKB functions

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as an accelerator and that Dyn functions as a decelerator of pulsatile GnRH/LH secretion. Although the importance of NKB in controlling GnRH/LH pulses is well known, the regulatory mechanism of NKB expression is largely unknown, including extracellular factors that increase/decrease NKB expression and intracellular molecules that enhance/suppress *TAC3* transcription.

The present study aimed to clarify the transcriptional regulatory mechanism of goat *TAC3*. The mRNA sequence and the 5'-upstream genomic sequence of *TAC3* in other species including cattle and human have already been determined. However, it is worth determining those in goat because goat is a useful animal model for endocrinological study including the study of the GnRH/LH pulse generation system as described above and because we can obtain more precise experimental results if we apply the sequence information of goat when using goats as an animal model. Therefore, we first identified the full-length mRNA sequence of goat *TAC3* and the genomic sequence of the goat *TAC3* 5'-upstream region. Next, we evaluated the promoter activity of this region by luciferase assay using N7, immortalized mouse hypothalamic neural cells, and SK-N-AS cells, human neuroblastoma cells. Further, we examined the effect of estrogen on goat *TAC3* promoter activity.

## Materials and Methods

### *Animals and sample preparation*

Shiba goats were used for this experiment. They were maintained under natural conditions at the Field Science Center, Graduate School of Bioagricultural Sciences, Nagoya University, Japan. Goats were fed twice a day with free access to water and supplemental minerals.

To prepare total RNA for full-length mRNA analysis, the ARC tissue was obtained from the hypothalamus of a 3-week-old male goat after injection with an overdose of sodium pentobarbital. To prepare genomic DNA for determining goat *TAC3* 5'-upstream sequence, a 2 ml blood sample was collected from a 7-month-old female goat. All experiments were approved by the Committee on the Care and Use of Experimental Animals of the Graduate School of Bioagricultural Sciences, Nagoya University.

### *Determination of goat TAC3 full-length mRNA sequence*

The collected ARC tissue was homogenized and total RNA was extracted with TriPure Isolation Reagent (Roche Diagnostics, Basel, Switzerland) following the manufacturer's protocol, and then 5'-rapid amplification of cDNA ends (RACE) and 3'-RACE methods were performed using GeneRacer Kits (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The reverse gene-specific primer used for 5'-RACE was 5'-CTTGAAGGTG CCAAA CCTGG GGATG TT-3' and the forward gene-specific primer used for 3'-RACE was 5'-AACAT CCCC GGT TT GGCAC CTTC A AG-3'. These gene-specific primers were designed from a previously reported partial goat *TAC3* mRNA sequence (GenBank accession number AB499062). Obtained PCR products were sequenced to determine the full-length mRNA sequence of goat *TAC3*. The sequence of goat *TAC3* and the deduced amino acid sequence of goat preproNKB were compared with those of cattle and human using ClustalW (<http://clustalw.ddbj.nig.ac.jp/>).

### *Determination of goat TAC3 5'-upstream sequence*

Goat genomic DNA was extracted from blood using DNeasy Blood & Tissue Kits (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol, and then the 5'-upstream sequences of goat *TAC3* coding region were cloned using Genome Walker Universal Kits (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's instructions. Goat genomic DNA was digested with four restriction enzymes and the adaptors were ligated to both ends of the genomic DNA fragments. Subsequently, this adaptor-ligated DNA fragment was amplified by nested PCR using gene-specific primers recognizing goat *TAC3* sequence and primers recognizing the sequence of the adaptor. The gene-specific primers were 5'-CATGC TCCTG TGA CT CCTCA -3' for first PCR and 5'-AAAAC TCCGA GCCAA GCTG-3' for nested PCR. The obtained PCR product was sequenced. We repeated this experimental process twice more to determine further 5'-upstream of goat *TAC3*. The gene specific primers were as follows: 5'-ACCAC CAGGA TGAGC AAGAT CCTAT CCA-3' and 5'-ATGAT TGGGAGGTGAGTCTG GCACC TCT-3' for the second round and 5'-CTTGG GATTT GGAAG AGAAT CATAG CTCTG-3' and 5'-AATGG TTCCT GCTCA CCAGC ACCTT TCCCA-3' for the third round. The predicted transcription factor binding site in the obtained goat *TAC3* 5'-upstream region was analyzed using the Transcriptional Element Search System (TESS) program (<http://www.cbil.upenn.edu/tess>). The sequence of the goat *TAC3* 5'-upstream region was compared with those of cattle and human *TAC3* using ClustalW. The 5'-upstream sequences of cattle and human *TAC3* were obtained from the University of California, Santa Cruz (UCSC) genome bioinformatics website (<http://genome.ucsc.edu/>).

### *Construction of luciferase reporter vectors*

To construct the reporter vector for luciferase assay, the 5'-fragment of goat *TAC3* containing sequence -2706, -1837, -834, -335, or -197 to +166 were amplified by genomic PCR with primers including *XhoI* and *HindIII* sites at the 5' and 3' ends, respectively. Each amplified fragment was digested by *XhoI* and *HindIII*, and was inserted into a luciferase reporter vector, pGL4-basic (Promega, Madison, WI, USA). The constructed plasmids were designated as pGL-2706, pGL-1837, pGL-834, pGL-335, and pGL-197 according to the respective positions of the fragments.

### *Transient transfection and luciferase assay*

Luciferase reporter vectors were transiently transfected to N7 cells (Cellutions Biosystems, Burlington, ON, Canada) and SK-N-AS cells (American Type Culture Collection, Manassas, VA, USA). N7 cell line, derived from a mouse hypothalamic neural cell [24], is reported to express *Tac2* (NKB gene in mice) and *Esr1* (ER $\alpha$  gene) according to the manufacturer's information, and SK-N-AS cells, which originated from human neuroblastoma cells, have been used for the analysis of transcriptional regulation in human *TAC3* gene [25]. N7 cells and SK-N-AS cells were maintained in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), 1 mM sodium pyruvate (Gibco), 4 mM L-glutamine (Gibco), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco) at 37 C in 5% CO<sub>2</sub>. Non-essential amino acids (1%) were added to the medium for SK-N-AS cells.

N7 cells and SK-N-AS cells were seeded on 24-well tissue culture plates at  $2.5 \times 10^4$  cells per well and  $6.75 \times 10^4$  cells per well, respectively, and cultured for 12 h before transfection. pGL4 vector (900 ng/well) and pRL-TK vector (100 ng/well), an internal control, were mixed with Lipofectamine 2000 Reagent (Invitrogen), and added to the cells. Twenty-four hours later, cells were treated with the medium containing vehicle (100% ethanol) or 1 or 10 nM estradiol (E2) and were incubated for an additional 24 h. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Transfections were performed in duplicate and repeated at least 3 times.

#### Statistical analysis

For analysis of the effects of E2 on luciferase activity, Student's *t*-test was used. The luciferase activities of pGL-2706, pGL-1837, pGL-834, pGL-335, and pGL-197 in the non-E2-treated group were compared with that of pGL4-basic using Student's *t*-test. The luciferase activities are presented as activity relative to that of the pGL4-basic vector and as means  $\pm$  SEM.

## Results

#### Identification of goat *TAC3* full-length mRNA sequence

The full-length mRNA of goat *TAC3* was 820 b and was deposited in the DNA Data Bank of Japan (DDBJ) database (accession no. AB796345). It consisted of 143 b of 5'-untranslated region (5'-UTR), 381 b of open reading frame (ORF), and 296 b of 3'-UTR containing a 23 b poly(A) tail (Fig. 1A). The homologies of goat *TAC3* with cattle and human *TAC3* were 92% and 76%, respectively. The upper end point of the 5'-UTR was estimated to be the transcription start site; this is identical to the transcription start site of cattle *TAC3* and 33-b downstream of the human *TAC3* transcription initiation site.

The deduced amino acid sequence of goat preproNKB, consisting of 126 residues, was compared with those of cattle and human (Fig. 1B). Goat preproNKB had 94% and 65% homology with cattle and human preproNKB, respectively. The sequence of NKB was identical among the 3 species.

#### Cloning of goat *TAC3* 5'-upstream region

We obtained goat *TAC3* 5'-upstream region up to 3400 b from the start codon by genome walking method (Fig. 2), and the sequence was deposited in the DDBJ database (accession no. AB797216). The putative transcription start site of goat *TAC3*, determined by 5'-RACE, was located in 694-b upstream of the start codon. We defined the putative transcription start point as +1; that made the upper end point of the obtained 5'-upstream region -2706 b and the translation start site +695 b. Fifteen putative ER $\alpha$  binding sites (half-ERE) were found in the obtained region. A putative neuron restrictive silencing element (NRSE) was found within +37 to +52 b. A GC box and CAAT box were located within -104 to -94 b and -81 to -77 b, respectively. The entire 5'-flanking sequence from -2706 to +694 b was highly conserved between goat and cattle (89%). For goat and human, the region from the start codon to -197 b had 71% homology, but the region further upstream from -198 b was dissimilar between the two species.

#### Promoter activity in goat *TAC3* 5'-upstream region

All the luciferase reporter vectors containing the goat *TAC3* upstream region showed higher luciferase activity than the control vector, pGL4-basic, in N7 cells (Fig. 3B). The deletion of the goat *TAC3* 5'-flanking region resulted in an increase in luciferase activity and pGL-197, the shortest vector, exhibited the highest luciferase activity among the 5 vectors, showing a 55-fold increase in activity over pGL4-basic. The analysis using SK-N-AS cells also demonstrated the progressive increase in luciferase activity with the deletion of the 5'-flanking region (Fig. 3C). The luciferase reporter vectors in the non-E2-treated group had significantly higher luciferase activities than pGL4-basic, except in the case of pGL-834 in N7 and pGL-2706 in SK-N-AS. The addition of E2 decreased the luciferase activity in some constructs. E2 was most effective in 10 nM E2-treated pGL-2706, the luciferase activities of which decreased to 50% (N7) and 55% (SK-N-AS) of those in the non-E2-treated group. However, the suppressive effect of E2 was not significant in either construct, including the case of pGL-2706 treated with 10 nM E2 ( $P=0.30$  in N7 and 0.53 in SK-N-AS).

## Discussion

The present study identified the sequence of full-length mRNA and the 5'-upstream region of goat *TAC3*. In addition, we clarified the transcriptional regulatory region of *TAC3* for the first time in domestic animals using the obtained 5'-upstream region of goat *TAC3*.

The full-length mRNA sequence of *TAC3* and putative amino acids sequence of preproNKB were highly conserved between goat and cattle (92% and 94%, respectively). In contrast, the sequences of *TAC3* and preproNKB were less conserved between goat and human (76% and 65%, respectively). However, the amino acid sequence of NKB was identical between goat, cattle, and human, suggesting that the biological activity of NKB is common across these species.

The 5'-upstream sequences of goat and cattle *TAC3* also showed high homology (89%), suggesting that the transcription of goat and cattle *TAC3* is driven by similar mechanisms, and that the results obtained from the analysis of goat *TAC3* are applicable to cattle *TAC3*. The fact that the putative transcription start sites of goat and cattle *TAC3* were located the same distance from the start codon also supports this idea [26]. The region from the start codon to -197 bp is thought to be necessary for the basic transcription of *TAC3* across species, since this region was conserved among goat, cattle, and human. In contrast, the region above -197 bp is not similar between ruminants (goat and cattle) and human, suggesting a different mechanism for *TAC3* transcriptional regulation.

The luciferase assay using successive deletion of *TAC3* 5'-upstream revealed relatively higher luciferase activity for pGL-335 and pGL-197 than longer constructs, suggesting that the core promoter region resided in the region downstream of -197 bp. In addition, since the luciferase activity increased by the deletion of the 5'-upstream region, it is likely that some repressive elements are located in the region between -2706 and -336 bp of goat *TAC3*. These results were consistent with those of previous reports: Gillies *et al.* evaluated the promoter activity of the human *TAC3* 5'-upstream region by luciferase assay using SK-N-AS cells and showed that the -289 to +181 bp region had higher activity than the -757 to +181 bp region

**A**

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1                               acttccccagt  11
12  ccctccactccctgtttctccgttcacagcagcagcaccagcagcgtttgtggggagcgggcagtg  77
78  cttccggccagctccttgatcctgctagaccatctgtcccaggctccaagctgctccacaggcatc  143
144 atgCGGagcgcctgctgtttgagtcattctggcctcagcttggtcggagtttgggtgcggtc  209
    M R S A L L F A V I L A L S L A R S F G A V
210 tgtgaggagtcacaggagcatgtggggcccggtgggggtcacagcaagaaggactcaaattctctac  275
    C E E S Q E H V G P G G G H S K K D S N L Y
276 cagctgccccatcattgctccggagactctatgatagccgctgatctccctggatggattgctc  341
    Q L P P S L L R R L Y D S R V I S L D G L L
342 aagatgctgagcaaggccagcgttaggtcctaaggagtcgccacttcccagaaacgtgacatgcat  407
    K M L S K A S V G P K E S P L P Q K R D M H
408 gacttctttgtgggtctcatgggcaagaggaacctccagccagacactcctgttgatataaaccaa  473
    D F F V G L M G K R N L Q P D T P V D I N Q
474 gaaaacatccccaggtttggcaccttcaagtacccccagtggtgaatgagcactcctctcctgg  539
    E N I P R F G T F K Y P P S V E *
540 actcctgggctacatcgtgaagacacccaccctagatgcaaacgcttctatttcccttccttctc  605
606 cccaccctgtaacactccttgcttggactccttcttctctctgtcccactggctgcagaaactg  671
672 ccttgtagcatccccagtcctgtaacaattgactacagattgccctggaatttctgtagcatcc  737
738 taaaaatgatgtctctctctattcctcaacaataaaggatttttacatattatgacctaaaaaa  803
804 aaaaaaaaaaaaaaaaaa  820

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**B**

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Goat      1 MRSALLFAVILALSLARSFVAVCEESQEHVPGGGHSHKSDSNLYQLPPSLLRRLYDSRVISLDGL  65
Cattle   1 MRSTLLFAVILALSSARSLGAVCEESQEQVVPGGGHSKSDSNLYQLPPSLLRRLYDSRVVSLDGL  65
Human    1 MRIMLLFTAILAFSLAQSFVAVCKEPQEEVVPGGGRSKRDPDLYQL----LQRLFKS-HSSLEGL  60
          **   ***:.***:*  *:*:***:*.*.*.*  ***:***:*.:***   *:**.*  **:**
          66 LKMLSKASVGPKEsplPQKRDMHDFVGLMGKRNLPDTPVDINQENIPRFGTFKYPPSVE  126
          66 LKMLSKASVGPKEsplPQKRDMHDFVGLMGKRNLPDTPVDINQENIPSFYTFKYPPSVE  126
          61 LKALSQASTDPKESTSPEKRDHDFVGLMGKRSVQPDSPDQENVPFSGILKYPPRAE  121
          ** **:*.*.***.*  *:*:*****.*.:***:*.*:***:*  ** :***.*

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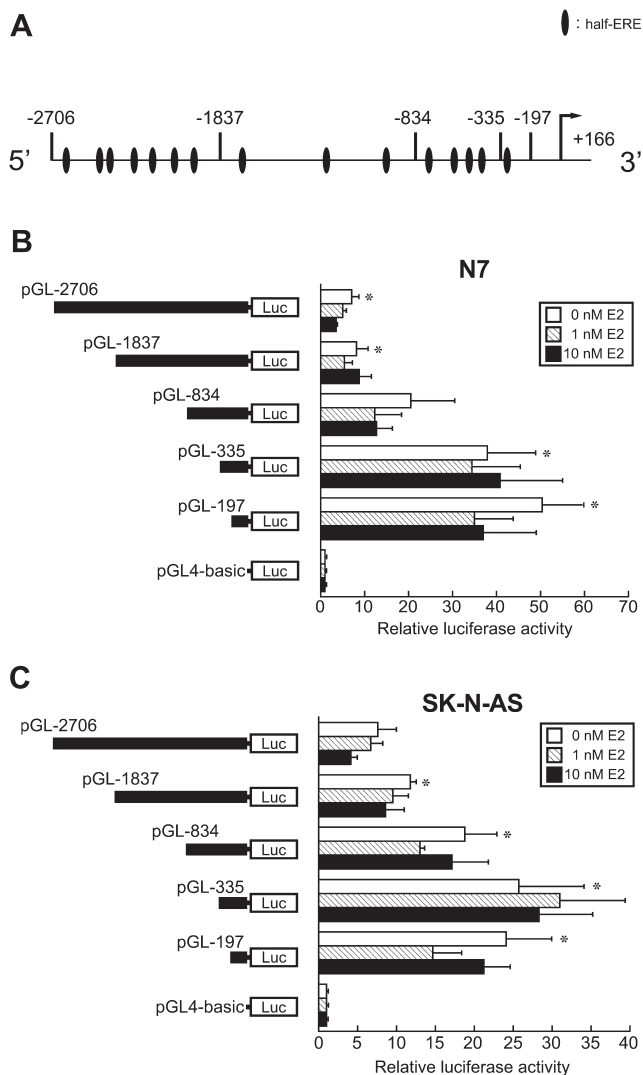
**Fig. 1.** Identification of goat *T4C3* gene. (A) Full-length mRNA and deduced amino acid sequences of goat *T4C3* gene. The deduced amino acids are shown under the nucleotide sequence in single-letter notation. Asterisk (\*) indicates stop codon. Numbers indicate nucleotide positions. (B) Alignment of amino acid sequences of goat, cattle, and human preproNKB. Identical (\*), conserved (:), and semi-conserved (.) amino acid residues are marked. The amino acid sequences of NKB are highlighted with grey shading.

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-2706                                                                                               CTGCAG
                                                                                               half-ERE
-2700 TACCTGCTTTACATGCTATTTGCATATGATCTGTGGGCAAGTGAAGCCAGCCTATCCTTGCCCTCCCTCCTTCATCACCTCTCTGACTT
-2610 AGCACCAGCTCTTTCAAGTCTTTGTTAATCCCAGAGATCAAGGGGTGATCAATTCTCCCTGCCATTCCCTTGCTCCCCCGATCCCTAGC
                                                                                               half-ERE
-2520 TCCTGCCATCCTGTGAGATGCCTTATCATCAAAGGACATTATTCATGTTGGACCTTTGCTGAAGCCTTGCTTCCCTGGTACCAGGGCAAG
half-ERE half-ERE
-2430 GGGCAAGGATGAATGGGGTGGTGGAGGCAAGGAGCTGACATAGAGATGGGACCCAGGAGACTTTAGTCTTGCCCTCATCTAATGTG
half-ERE
-2340 TTCTTCTCCTGCACTGCTAATACCTCCAGCCTGTATGGCTTGACCTCCTTTTCCAGGTTGGTGAGAGGAGGAGAGAGAGGCGCGGAGAT
half-ERE
-2250 GTCCAGGTCCAATTAATAATAGCACCTGAACCTTCTCAAAGGCATGAACCAGTCATATGTTTACATGTAAACAGCCTTCCCTTCTGCCC
half-ERE
-2160 TTTATCCCAACCCTGAGTCCGGGGCCATGGTTTCCCAGAGAGCCAAGTAATTAGCTGGGTAAATTACATGAGGCCCCAGTGGCCTCTCA
half-ERE
-2070 CGTAATTCATGGGGTCTCAAAGAGTGGGACACTGAGCAAATAAGCATGTAGGAACAAGACCCTAGCTGCTACCTCACTTGGGGATGAAG
half-ERE
-1980 CTGGAGTCCCGTCAGCCAGAACGACTTCTTAGTATGGCCCAAGACCGAGGGTGGGTGGGCTGGGGAAGGAAACATTTCCAGACTCTAGC
-1890 AATGCTTGAGAGGCTTTATGGAAGATCCACATGGTGGGCTGCAGTGAAGAAGCTAGACTCAGGCTACCTTTTTTGTGTTTATAAATAAAT
half-ERE
-1800 TTTTGTGAGCACCTATTATATGCCAACATTATAGAGCTAGGAATCCAATAGCAAACAGATGAAAAGTCTTGGCCCTCTAAAGATGAC
half-ERE
-1710 ATTCTGGTGGGAGGTGAGGTAGACAGACTTTATAAGTAAATTTGTAGTATGTTTGTATGTAATGTATGAAGAAAAGAAATGAGGGGATATG
-1620 GAGAATAAAGTGGTAGTAATTTTATTAATAAATTTTCTGGCCAAGCTGAGAGGGATGTGGGACCTTAGTTTCCAACCAGGAATTGAACCT
-1530 GTGCTTCTGCATTGGGAGCTCAGGGTCTTAACCACTGGATTGCCAAGGAAGTCCCTAGTGGTAGTAACTTAAATAGGGTGATTGGGAAA
-1440 GGTGCTGGTGAGCAGGAACCATTTATAATAAATATGCAACAGAGCTATGATTTCTTCCAAATCCCAAGATATAGAAGAGCACCCGATCCC
half-ERE
-1350 AATCTCTAAGTCTTTTCGGGAGGTGGACCCCCACCCTTCTGGGGACCTAAGTCTCCCTTACTGGTCAAGTATAGACCAGTGCAGTAC
half-ERE
-1260 AATGAAGGGGAGGTTGGGAGTGAGAAACACCTGGATTGAGGCTGTTCTCCAGGAGCTATATGTCTGAAGCATGTGACTGAACTTTTTG
-1170 AGCCTCAGTTCCCTCATCTGAAAATAGGAATGTTTCTACTCACACGGCGGAGGTGATGAAAATCAGTTGAATTGCTCTCTGCTGAAGCT
-1080 CCTTGCAAATGTTCAAATTTCTTCTCCATTATAAATAAACACTGAGAGTGCAGACTGCTCTGAAGCCGGCTTTCACCTCACCTCCCCTCA
half-ERE
-990 TCTCCTCTGGCGCATTCTTTTTCTCTGGGTCTGTCTCAGATCTGACTCTGACCCTCCCCCTGGGGCTCTTTACCTTTTGGGGCTGAA
half-ERE
-900 GTGAGCACAGTGAAGCCTCTCAACTGTGGGTATGAACTAGATGCTTGAAGTTTCAGGGGAGAAGATGCCTGAGTTGTTGGAGACAGAG
half-ERE
-810 TGGGGGATAAATGAAGCAAAGGAGAAAAAGAAGCAATTAAGAGATCGATGCTGGGCAAGGGCTGGGAGCAGCTTAGCCATGTCATCTC
half-ERE
-720 TCAGAGATGACAGCAGAAGGATGGAGGTGAGAACCCTGAAAGGATGCAACAACGTCAGGCTGGTTCATGGGTCAAGAAATGGGGTGGGAG
half-ERE
-630 GCCAGGGGAGGGCACTGGCCCTGGGAGCGATGGAGGGAGCAGACGCTGGAGGTTTGCACCTAGCAGCTGGGGGGAAGTGCAGACCCT
half-ERE
-540 AAGAGGTTGAAGGGCCAGTGCATGAGTCAGCTCCAGCTGGGAGGAGACAGAGGGACAACCTGAAGACTGGAGAGGTGCCAGACTCACCT
-450 CCCAATCATCTGGGATAGGATCTTGCTCATCTGTTGGTGGGGTGGCGGGGGGGGGTGGTGCAGAGAAGGACCTGCCAGAAATGGTTTT
half-ERE
-360 GGCTTGGCAGTTGAGGCTGGAAGTTTAAAGGGCAAGAGCTGGTGCATGGATATGGAGGAGAAAGAGCCAGGGACCTCAGCTATTGAGT
-270 TGCATAAGCAACATCATTGCTTAGTGCGCCAGCTCCAGGGCTGCTGGACTGTGAGTTGCTGGATAACCTCAGCTCACTGCTGGGTTTGT
GC box GC box
-180 GGTGTGTGTATGTATGTGTGTGTGTGTCTGTGCGTGTGTCTGCAGCCTATATCCCCCTTACCCCCACCAGCTCCCGCCCGCCAGC
CAAT box
-90 CACCCTGGGATTGGTGTGATTCTCAGCCCCCTCCCCGGCTCCCCAGACCTCCCAGAGCCTTTATCAGGGAGCTGGGCGGAGTTCTGCC
putative transcription start site putative NRSE
+1 ACTTCCCAGTCCCTCCACTCCCTGTTTCTCCGTTCCAGCAGCAGCACCAGCAGCGTTTGTGGGGAGCAGGCAGTCTCCGGCCAGCT
Translation start site
+91 CCTTGATCCTGCTAGACCATCTGTCCCAGGCTCCAAGCTGCTCCACAGGTAGGCACAAACCAGAGAATGTTGGATG

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**Fig. 2.** Sequence of the 5'-flanking region from -2706 to +166 b of goat *TAC3* gene. The numbering of nucleotide residues on the left is relative to a putative transcription start site designated as +1 (upstream, -; downstream, +). The start codon (ATG) is found 694 b-downstream of the putative transcription start site. The estimated binding sites of estrogen receptor  $\alpha$  (half-ERE), GC boxes, a CAAT box, and a putative neuron restrictive silencing element (NRSE) binding site are indicated.



**Fig. 3.** Luciferase (Luc) activity in the goat *TAC3* 5'-upstream region with estradiol (E2) treatment. Schematic representation of the goat *TAC3* 5'-upstream region used for the promoter assay, in which putative ER $\alpha$  binding sites (half-ERE) are indicated (A). Vectors containing different lengths of the *TAC3* 5'-flanking region or the empty vector (pGL4-basic) were transfected into mouse hypothalamic neuron-derived N7 cell line (B) and human neuroblastoma-derived SK-N-AS cell line (C). The luciferase activities are represented as activity relative to that of the pGL4-basic vector. Opened, striped, and closed bars indicate the 0, 1, and 10 nM E2 treatments, respectively. Values are means  $\pm$  SEM for four (N7) and three (SK-N-AS) independent experiments. \* $P < 0.05$  vs. pGL4-basic (Student's *t*-test).

[25]. The -289 to +181 bp region of human *TAC3* includes the region equivalent to the -196 to +166 bp of goat *TAC3*, indicating that the core promoter of the goat and human *TAC3* genes is regulated in a similar manner. Gillies *et al.* demonstrated that neuron restrictive silencing factor (NRSF) activates human *TAC3* transcription by binding to NRSE located within +50 to +70 b [25, 27]. NRSF has been known to recognize a 21-bp consensus sequence [28, 29],

NRSE, and the core sequence is included in this consensus sequence [29]. In the goat *TAC3* 5'-upstream region, an NRSE-like sequence existed 10-b downstream of human *TAC3*; however, one base of the goat *TAC3* NRSE-like sequence was different from the NRSE core sequence. Further research is needed to know whether NRSF acts as a transcriptional activator of goat *TAC3* similarly to human *TAC3*.

The complex of sex steroid and its receptor is known to act as a transcription factor. Generally, ER $\alpha$  regulates the transcription of genes in collaboration with another transcription factor, such as Sp-1 or AP-1 [30, 31]. A previous study in ovariectomized mice reported that E2 treatment decreased the number of *Tac2* mRNA-expressing cells in the ARC by 53% compared with the control [15]. Ovariectomized sheep treated with E2 also exhibited a significant decrease in *TAC3* mRNA-expressing cells in the hypothalamus [32]. Additionally, E2 treatment decreased the *Tac2* expression level in the ARC in wild-type mice but not in ER $\alpha$  knockout mice [33]. These previous results indicate that estrogen negatively regulates *TAC3* transcription via ER $\alpha$ . Analysis of the predicted transcription factor binding site found a number of binding sites for ER $\alpha$  (half-ERE), Sp-1, and AP-1 in the 5'-upstream region of goat *TAC3*. This may indicate the involvement of estrogen as a suppressive factor of *TAC3*; therefore, we examined the effect of E2 on the luciferase activity of each construct. The mean luciferase activities of pGL-2706, pGL-1837, and pGL-834 were lower in the 1 nM E2-treated group, which may be responsible for a number of the ER $\alpha$  binding sites included in those vectors: The regions from -2706 to -1838 bp, -1837 to -835 bp, and -834 to -336 bp contained 7, 3, and 4 putative ER $\alpha$  binding sites, respectively (Fig. 3A). The luciferase activity in pGL-197 also had a tendency to be suppressed by E2. We could not find an ER $\alpha$  binding site within the region from -197 to +166 bp, but this region contained several Sp-1 binding sites; therefore, it is possible that ER $\alpha$  acts indirectly on the region by binding and collaborating with Sp-1. Treatment with 10 nM E2 suppressed the luciferase activity of pGL-2706 by approximately half, which was the highest suppression ratio, indicating that multiple ER $\alpha$  binding sites located in the region from -2706 to +166 bp act as transcriptional suppressive elements. However, all the above-mentioned suppressive effects of E2 were not statistically significant. Estrogen is likely to have a certain effect on *TAC3* suppression but may not be a major factor that regulates *TAC3* transcription in goats. Thus, the effect of estrogen on goat *TAC3* transcription was not fully established in the current experiment, which was different from the results in previous reports using *in vivo* animal studies. The present results of our promoter assay suggested that estrogen does not directly act on *TAC3* transcription, although it may indirectly influence the decrease in goat *TAC3* expression through an unknown mechanism. It may also be possible that the putative ER $\alpha$  binding sites in the -2706 to +166 bp region are not the actual binding sites of ER $\alpha$  but the sequences outside the -2706 to +166 bp region are responsible for suppression of *TAC3* transcription by estrogen. One possible limiting factor of our study is that the N7 and SK-N-AS cells used in the luciferase assays may have been insufficient to fully detect the effects of estrogen on *TAC3* transcription. For example, a factor(s) that works with ER $\alpha$  may not have been enough in these cell lines to cause a significant reduction in luciferase activity by E2 treatment.

In conclusion, goat *TAC3*, encoding NKB, is highly homologous

to cattle *TAC3* in both the mRNA and 5'-upstream region sequences. The present study demonstrated the prerequisite regions for the gene expression of goat *TAC3*: the downstream region of -197 bp includes the core promoter of goat *TAC3*, and the region from -2706 to -336 bp includes transcriptional suppressor. The results of this study also suggested that estrogen does not impart a direct effect on goat *TAC3* transcription. Further studies on the identification of the major regulator(s) of *TAC3* transcription are thus warranted.

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