Characterization of Korean Cattle Keratin IV Gene

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ABSTRACT : Keratins, the constituents of epithelial intermediate filaments, are precisely regulated in a tissue and development specific manner. There are two types of keratin in bovine. The type I is acidic keratin and the type II is neutral/basic keratin. 1.5 kb of 5' flanking sequence of Korean cattle Keratin IV gene, type II keratin (59 kDa), was cloned and sequenced. A symmetrical motif AApuCCAAA are located in a defined region upstream of the TATA box. Proximal SP1, AP1, E-box and CACC elements as the major determinants of transcription are identified. When it was compared to the bovine sequence from -600 bp to ATG upstream, the homology was 97% in nucleotide sequence. Several A and T sequences, located in the promoter region, are deleted in the Korean cattle. An expression vector consisted of Korean cattle Keratin IV gene promoter/SV40 large T antigen was transfected to HaCaT cell (Epithelial keratinocyte). The transformed HaCaT cells showed active proliferation when treated with PDGF (Platelet-derived growth factor) in 0.3% soft agar compared to control cells. These results indicate that Korean cattle Keratin IVgene promoter can be used as a promoter for transfection into epithelial cell. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 7 : 1055-1059)***

Key Words : Keratinocyte, PDGF, Keratin IV Gene, Korean Cattle

INTRODUCTION

The cytoplasmic intermediate filaments in vertebrate cells can be grouped into four classes (Keratin filaments, vimentin, neurofilament, unclear lamin). There are over 20 distinct keratins that based on their amino acid sequences (Jiang et al., 1989). The type I keratins in humans are found in a cluster on chromosome 17, and the type II keratins are on chromosome 12. A similar situation exists in mouse with the type I keratins on chromosome 11 and the type II on chromosome 15. Keratins function as heterodimers of type I molecule and type II molecule (Oliver, 1998). Keratin IV gene is found in epithelial cells of tail, skin, and esophagus of mice (Manfred et al., 1989; Tomohiko et al., 1997; Oliver et al., 1998). Keratin IV is type II keratin (59 kDa) and the partner of it is keratin 13 (Park et al., 2000). It has the highest expression in the esophagus and cornea, but to a lesser extent is found in the tongue, pharynx, larynx and the anus (Park et al., 2000). It also appears in the stage of embryo and early fetus (Tinsley et al., 1992; Park et al., 2000). The pattern, nine-exons and eight-introns, is characteristic of epidermal type II keratin genes. A symmetrical motif AApuCCAAA located in an upstream region of the TATA box of bovine (Manfred et al., 1987). Induction of AP1 transcriptional activity in response to UV irradiation contributes to remodeling of sun-exposed human skin (Antonello et al., 1998; Eitan et al., 2000).

In this paper, we cloned the 5' flanking sequence of Korean cattle keratin IV gene, and confirmed that the sequence could be used as an useful promoter for transfection into epithelial cells.

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MATERIALS AND METHODS

Screening

Bovine keratin IV PCR products (500 bp) that generated from keratin F primer (5'-CCAGAGTAGCCCC CAATTCC) and keratin R primer (5'-GGTTGAGAAGG GTGTGAGAGG) were P^{32}-labelled and used as a probe for screening keratin IV genomic DNA from Korean cattle genomic DNA library (λNLRIHW8535) (kindly provided by National Livestock Research Institute of Korea). PCR was conducted with PCR mixture (0.2 mM dNTP, 1.5 mM $MgCl_2$, 1×buffer, Taq polymerase (2.5 unit), 50 ng of template DNA, and 1 pmol primer for 1 min at 94°C, 1 min at 63°C, 10 min at 72°C by 35cycles.

Cloning and sequencing

Three positive clones were obtained from third screening and confirmed by southern blotting. Southern blot was done according to the protocol (Rediprime Ⅱ Random Prime Labeling System).

Cell culture and transfection

A 656 bp BamHI/KpnI DNA fragment containing a functional region of the keratin IV promoter was subcloned into the pBS KS vector containing SV40 T antigen gene. HaCaT Cell (utilized between passages 40 and 50) was cultured at 37°C and 5% CO_2 in DMEM, supplemented with 10% fetal bovine serum (Gibco BRL) and 50 mg/ml gentamycin (Gibco BRL). Cells were transfected with 10 μg of appropriate keratin vector and control plasmids

(Vector containing Albumin promoter and SV40 gene) using the transfection kit (lipofectamine, 2 mg/ml; Gibco BRL). Twenty-four hours prior to transfection, 5×10^5 cells were seeded on 60 mm dish yielding approximately 70% confluence. Forty-eight hours after transfection, keratin IV expression vector transfectants, control transfectants (albumin expression vector) and non-transfectants were collected using trypsin. 10 μg of keratin vector and 3 μg pMEM-Neo vector were prepared for Co-transfection. Selection media is 500 ml DMEM containing 200 mg/ml of G418 and 40 μl of gentamycin (Gibco BRL).

Reverse transcription (RT)-polymerase chain reaction (PCR)

RT-PCR was performed by the methods described by Federio et al. (1997) with reverse transcription system kit (Promega) to avoid false-positive results. First-strand cDNA was generated from 1 μg of total celluar RNA, which was derived from the HaCaT cells, transfected with expression vector DNA in a 50 μl reaction mixture containing reverse transcription 10×buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl and 1% Triton X-100), 10 mM dNTP Mixture, 25 mM $MgCl_2$, TaKaRa Ex Taq polymerase, 100 pmol oligo (dT)-48F primer (5'-GTTATGCCTACTT ATAAA GGTTAC-3'), 38R primer (5'-GCATTCCACCAC TGCTCCCA-3`). The size of expected PCR products were 314 bp and β-actin amplification was also performed as an internal control. The primer sequences for β-actin amplification were 5'- GTGGGCCGCCT AGGCACCAA-3' and 5'-CTCTTTGATGTCACGCACGATTTC-3'.

Growth in soft agar

Keratin, pMEM neo, and Non-transfectant cell line were suspended in 0.3% agar with PBS medium, plated at a density of 1×10^4 cells per 10 cm^2 dish, which was previously coated with 0.5% agar (sigma type Ⅰ) and maintained at 37°C. Colonies were scored using a microscope at 40×magnification.

-1490	gcccttgaag	aaaggtattt	ttttcctgc	ccagatgccg	aataagcact
-1440	ttctttaacg	tagggaaatg	ccctgctccc	cttggaaaac	tgcctcccat
-1390	aacactggag	agattgaaat	tgcttataaa	atagtcacaa	gagcttttct
-1340	caggttcatc	tccagtgtgg	gagtttgtct	ttcctcctct	ggctgtccat
-1290	ttaatcaaat	agtcttataa	agcaccctgt	ctacaattgc	cctcaatact
-1240	cctccatagt	gcttaattta	tggactaaaa	caatcaatct	ccttccccct
-1190	taaaccaagc	accatgatca	cttggcagcc	ttccctccct	ctaagaatgt
-1140	gtctggagat	ttaagatttc	aagcactgag	attaggaata	taaatttgaa
-1090	agttacttgt	gacatcatca	cacctgtaaa	gacacatttt	tatgattcac
-1040	tgtttctata	tgcccctcaa	ctttctgtac	acaccctatt	aaggtgtctg
-990	atcttgacgc	ctaagaaagt	tcagaatcta	tgagaatata	aattcagtga
-940	attcccttaa	tgatttttgt	cctactcatt	ttagattctc	aaaattaagt
-890	tctaagtgaa	cattccaaga	aactggagaa	aa**gggtggc**t	atgaattcca
-840	tttggtagga	aaaaaaaatg	tccacagtca	ccagctcaat	gggaaagcaa
-790	acactgggga	ggaaggtgca	ggcatggcta	gaaaccatga	gaagtcagct
-740	ttttgggcag	agtggacttg	ggtgatctct	tatcctatat	catgaaactg
-690	ccaattccac	acaagacaag	cttgttctta	tgctgtaaaa	actcatctcc
-640	tttgtcctct	tgcctttcaa	aggagtgtca	tgtccccaga	gtagccccca
-590	attcccaggc	caggccacca	ggaaggcagt	caggagatcc	agaaggacat
-540	gttcaaacat	ggcccaaaac	caccgcaagc	cactttcttg	ctcagaccac
-490	aggcaaatgc	accaaccctc	agagacagtt	aacctgaatg	ggaa**gggtgg**
-440	tgtgagtgga	gaagaaaact	tgtgtgggaa	gggggcaaga	gaagagtgtc
-390	tgagtaagca	gaaggagggg	acaattatca	cagtcagctc	cttgtctcct
-340	ttgtttgaga	gctgactaac	ccatgacttc	atgaatttac	atccagtggt
-290	ttgtgttggg	atcaagtcag	gctagaagcc	agaagaatct	ccatgactaa
-240	aggaaaccaa	agaagcaata	ttcatacttc	atacctttct	agaggca**ggg**
-190	**ggtg**atctca	ctatttgtaa	agcccgccct	ttctaatctg	caggctcacc
-140	ttccggactg	agcccggccc	attttttcc**t**	**atata**agctg	ctgccgggaa
-90	gctcctctca	tagatctgct	cctttcagct	ctgctttcca	cctctcacac
-40	ccttctcaac	ctattnnnnn	nnnnnnnnnn	nnnnnnnnnn	**ATG**GATAAAG

Figure 1. Nucleotide sequences of 5' upstream region of Korean cattle keratin Ⅳgene. AP-1; Square box, E-box; Lining, Sp1; Bold face letter, AAPuCCAAA; Black square box. Nnnnn: unknown nucleotide. Genebank accession number is AY242850.

RESULTS AND DISCUSSION

Cloning and sequencing of Korean cattle keratin IV gene

Korean cattle genomic DNA library (λNLRIHW8535) was utilized for screening of keratin IV gene. Three positive clones were selected through third screening and Southern blotting as described in material and methods. 1.5 kb DNA fragment corresponding to 5' upstream sequences of Korean cattle keratin IV gene was generated by long-range PCR and sequenced. Sequencing was performed by the method of dideoxy nucleotide chain termination (Sanger, 1977). The nucleotide sequence from -1,501 bp to ATG start codon was determined. Sp1 element (GGGGCGGC) exists at 3 regions (-202 or -445 and -858) and is indicated with bold face letters (Figure 1). E-box element (CATATG) detects at 4 regions between -482 and -1,033. AP-1 element locates at 2 places that mark as a square box in -241 to -247 and -1,042 to -1,048.

When Korean cattle keratin IV gene was compared with published bovine keratin IV promoter sequences, Korean cattle keratin IV exhibits 96.3% sequence homology from

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BOVINE    AAGCTTGTTCTTATGCTGTAAAAACTCATCTCCTTTGTCCCTCTTGCCTTTCAAAGGAGT
KOREAN    AAGCTTGTTCTTATGCTGTAAAAACTCATCTCCTTTGTCC-TCTTGCCTTTCAAAGGAGT
          ***************************************** ******************

BOVINE    GTCATGTCCCCAGAGTAGCCCCCAATTCCCAGGCCAGGCCACCAGGAAGGCAGTCAGGAG
KOREAN    GTCATGTCCCCAGAGTAGCCCCCAATTCCCAGGCCAGGCCACCAGGAAGGCAGTCAGGAG
          ************************************************************

BOVINE    ATCCAGAAGGACATGTTCAAACATGGCCCAAAACCACCGCAAGCCACTTTCTTGCTCAGA
KOREAN    ATCCAGAAGGACATGTTCAAACATGGCCCAAAACCACCGCAAGCCACTTTCTTGCTCAGA
          ************************************************************

BOVINE    CCACAGGCAAATGCCTATTAACCCTCAGAGACGTTCAACCTGAATGGGAAGGGTGGTGTG
KOREAN    CCACAGGCAAATGC--ACCAACCCTCAGAGACAGTTAACCTGAATGGGAAGGGTGGTGTG
           **************  *  *************  * ************************

BOVINE    AGTGGAGAAGAAAACTTGTGTGGGAAGGGGGCAAGAGAAGAGTGTCTGAGTAAGCAGAAG
KOREAN    AGTGGAGAAGAAAACTTGTGTGGGAAGGGGGCAAGAGAAGAGTGTCTGAGTAAGCAGAAG
          ************************************************************

BOVINE    GAGGGGACAATTATCACAGATCAGCTCCTTGTCTCCTTTGTTTGAGAGCATGACTAACCC
KOREAN    GAGGGGACAATTATCACAG-TCAGCTCCTTGTCTCCTTTGTTTGAGAGC-TGACTAACCC
          ******************* ****************************** **********

BOVINE    ATGACTTCAGTGAATTTACATCCAGTGGTATTGTGTTGGGATCAAGTCAAGGCTAGAAGC
KOREAN    ATGACTTCA-TGAATTTACATCCAGTGGT-TTGTGTTGGGATCAAGTCA-GGCTAGAAGC
          ********* ****************** ****************** **********

BOVINE    CAGAAGAATTTCTCCATGACTAAAGGAAACCAAAGAAGCAATATTCATACTTCATACCTT
KOREAN    CAGAAGAA--TCTCCATGACTAAAGGAAACCAAAGAAGCAATATTCATACTTCATACCTT
          ********  ************************************************

BOVINE    TCTAGAGGCAGGGGGTGATCTCACTATTTGTAAAGCCCAGCCCTTTCTAATCTGCAGGCT
KOREAN    TCTAGAGGCAGGGGGTGATCTCACTATTTGTAAAGCCC-GCCCTTTCTAATCTGCAGGCT
          ************************************ ********************

BOVINE    CTCT-ATAGATCTGTTCTTT--AGCTCTGCTTTCCACCTCTCACACCCTTCTCAACCTATT
KOREAN    CTCTCATAGATCTGCTCCTTTCAGCTCTGCTTTCCACCTCTCACACCCTTCTCAACCTATT
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Figure 2. Alignment of nucleotide sequences of 5'upstream region of Bovine keratin IV and Korean cattle keratin IV gene. Some of A, T is eliminated in K-cattle sequence.

* Indicates homology between both nucleotide sequences. Shaded square indicate TATA box and transcription initiation site.

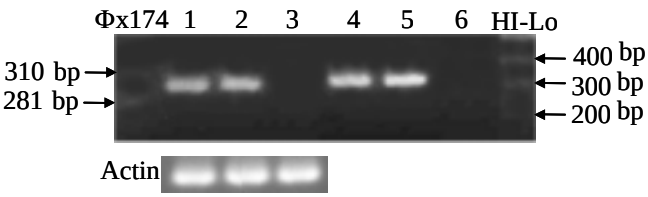

Figure 3. RT-PCR results showing SV40 transcript in K4SV transfected HaCaT cell. Lane 1, 2 and 3 indicates RT-PCR product derived from RNA from K4SV transfectant cells, RNA from albumin promoter/SV40 transfectant (positive control) and RNA from non-transfectant (negative control) respectively. Lane 4 and 5 indicates PCR product derived from K4SV vector DNA (PCR positive control) and Albumin promoter/SV40 vector DNA (PCR positive control). Lane 6 is PCR negative control. Actin was used as an internal control of RT-PCR. Φx174 and HI-LO indicates DNA size marker, respectively.

start codon to -600 bp of bovine keratin IV reported by Manfred et al., (1987) and Angel et al., (1995) as shown in Figure 2. Several nucleotides were deleted in Korean cattle keratin gene compared to those of the bovine gene. With further verification in Korean cattle keratin IV gene, these deletions might be used as Korean cattle specific markers as indicated by Yeo et al. (2002).

Expression of transfected gene into HaCaT cell

Expression vector (K4SV), as described in materials and methods, was co-transfected with selection vector (pMEM-Neo) into HaCaT cell line. RT-PCR was done to check expression of the transfected gene in the Keratin transfectant cells. As shown in Figure 3, the transfected gene was expressed like positive control.

Increased proliferation of transfected HaCaT cells

In order to know the phenotype of HaCaT cells expressing SV 40 by the regulation of bovine Keratin IV promoter, we characterized properties of cell proliferation by PDGF (platelet-derived growth factor) treatment to keratin transfectant cells. As shown in Figure 4-A and 4-B, keratin transfectant cells were significantly proliferated according to the treatment of PDGF. However mock transfectant cells treated with PDGF were not proliferated as shown in Figure 4-C. SV40 large T antigen expressed by the regulation of keratin gene with the treatment of PDGF affected HaCaT cells proliferation, even though we don`t know whether PDGF participate in the expression of keratin gene directly or not.

Keratin IV and other keratins are available to generate transgenic animals with specific tumors like skin, esophagus, stomach (Jone et al., 2000). Also expression vector (K4SV) could be available in generating transgenic animals as HaCaT cells transfected by it expressed reporter

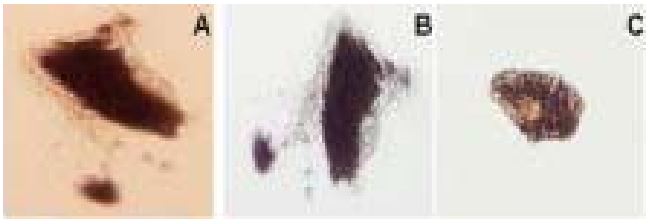

Figure 4. Morphology of K4SV transfected HaCaT cells treated with PDGF. Cells were maintained in 0.3% soft agar.
A. K4SV transfectant cell (2 days growth)
B. K4SV transfectant cell (4 days growth)
C. Mock transfectant cell (4 days growth)

gene and were significantly proliferated by the treatment of PDGF as shown in Figure 4.

Transcriptional regulation of keratin IV might be important in understanding the commitment to early differentiation in tissue. Keratin IV Expression was found in the periderm of 16-day-old mouse embryos. No data were available at other times in embryonic development. In humans, keratin IV presents in all layers of the epidermis at 10 weeks and becoming totally absent at around 20 weeks (Peter et al., 1994; Seth et al., 1998). On the basis of keratin IV absent at around 20 weeks, Keratin IV transfectant cell line (HaCaT, human origin cell line) may be identified to reduce a degree of Keratin IV expression day by day. Putative transcription start regions showed an asymmetrical sequence and several A and T sequences were absented irregularly. On the high degree of sequence homology and cell-type specific pattern of expression, two different bovine has a equivalent property. The pattern of expression of keratins may be altered due to various factors such as vitamins, hormones, enzymes, environmental stress conditions, tumor promoters, malignant transformation, and also by drugs affecting the protein synthetic machinery (Alpana et al., 1997).

In order to confirm if the 5' flanking sequence of Korean cattle keratin IV gene could be available as a promoter for transfection, we transfected K4SV vector into epithelial cells. An expression vector consisted of Korean cattle Keratin IV gene promoter/SV40 large T antigen was transfected to HaCaT cell (Epithelial keratinocyte). The transformed HaCaT cell showed active proliferation when treated with PDGF (Platelet-derived growth factor) in 0.3% soft agar when compared to control cells. The results indicate that Korean cattle Keratin IVgene promoter can be available as an useful promoter for transfection into epithelial cell.

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