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Identification of the porcine sialoadhesin gene promoter region and its cell-specific expression in porcine alveolar macrophage cells

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ABSTRACT. Porcine reproductive and respiratory syndrome (PRRS), which is caused by the PRRS virus (PRRSV), is a communicable disease. PRRS caused huge economic losses to swine breeding. The porcine alveolar macrophage (PAM) cell is the main target cell of PRRSV; therefore, it is very important to identify the specific gene promoter that controls expression in PAM cells so that the anti-PRRSV exogenous gene can be efficiently and specifically expressed in PAM cells to improve porcine resistance to PRRSV. In this study, the transcription initiation site for sialoadhesin (*Siglec-1*), which is a porcine alveolar macrophage-specific gene, was determined by 5' rapid amplification of cDNA end, and 88 bp of the 5'-untranslated region was cloned. *Siglec-1* promoter activity was detected by a dual-luciferase reporter assay, which showed that the fragment from -173 to +81 bp had the strongest promoter activity. Additionally, the cell-specific expression of the promoter fragments was tested in a PAM cell line (CRL-2844 cells), porcine

kidney 15 cell line (PK-15 cells), porcine fetal fibroblast (PEF) cells, and porcine preadipocytes. These results also showed that the fragment from -173 to +81 bp had the strongest cell-specific expression in PAM cells.

Key words: Porcine reproductive and respiratory syndrome; Porcine reproductive and respiratory syndrome virus; Promoter activity; Cell-specific expression; *Siglec-1*

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a viral infectious disease, which is accompanied by the clinical manifestations of respiratory problems in pigs of all ages and severe reproductive failure in sows that is characterized as the leading cause of late-term abortions and an increasing number of stillborn and weak pigs (Vanderheijden et al., 2003). This disease first appeared in the United States in 1987, and it broke out in Europe in 1989; then, it gradually spread to other regions around the world (Wensvoort et al., 1991; Collins et al., 1992). PRRS has caused huge economic losses for the world pig industry, it has become one of the major epidemics of large-scale pig farms, and it is also a major problem for disease control in the global swine industry (Chen et al., 2006).

PRRS virus (PRRSV) is a single-stranded RNA virus that belongs to the Arteriviridae family (Dea et al., 2000). *In vivo*, PRRSV infects the monocyte/macrophage lineage, primarily alveolar macrophages. It also infects macrophages in tonsils, spleen, liver, thymus, lymph nodes, and Peyer's patches (Duan et al., 1997a,b). Additional research showed that the virus also infects porcine testicular germ cells, whereas freshly isolated blood monocytes are hardly infected (Sur et al., 1996,1997). Porcine alveolar macrophages (PAMs) are the main target cells of PRRSV in the acute phase of infection (Teifke et al., 2001). The restricted cell tropism of PRRSV is determined by cellular membrane factors of the target cells (Kreutz, 1998).

PAMs are the target cells that are specifically infected by PRRSV in porcine. Thus, it is important to identify the promoters that are involved in PAM-specific gene expression, which could initiate the efficient and specific expression of anti-PRRSV exogenous genes in PAM cells. The *Siglec-1* gene, which belongs to the lectin family (Varki and Angata, 2006), encodes an essential PRRSV receptor that mediates attachment and internalization on macrophages; it is highly expressed in PAM cells. In humans and mice, Siglec-1 is only expressed on discrete subsets of tissue macrophages, including the spleen, bone marrow, lymph nodes, colon, liver, and lung (Crocker and Gordon, 1989; Hartnell et al., 2001). In porcine, Siglec-1 is critical for the entry of PRRSV into PAMs, and it mediates the internalization of PRRSV into porcine kidney 15 (PK-15) cells, which are resistant to PRRSV entry and contain an exogenous *Siglec-1* gene (Vanderheijden et al., 2003).

In this study, *Siglec-1* promoters of different lengths were cloned, and their transcriptional activity was analyzed in a PAM cell line, CRL-2844. The cell-specific expression of the *Siglec-1* promoter was verified in porcine preadipocyte, PK-15, porcine fetal fibroblast (PEF) and CRL-2844 cells by a dual-luciferase reporter assay system.

MATERIAL AND METHODS

Cell culture

CRL-2844, PK-15, PEF, and porcine preadipocyte cells were used in this experiment. CRL-2844 cells were cultured in 10% fetal bovine serum (FBS) and Roswell Park Memorial Institute 1640 (RPMI 1640) medium. PK-15 and PEF cells were cultured in 10% FBS and Dulbecco's modified Eagle medium nutrient mixture F-12 (DMEM/F12). Porcine preadipocyte cells were cultured in 10% FBS and DMEM. The culture medium was changed every 3 days.

Determination of the transcription initiation site of *Siglec-1*

The transcription initiation site of *Siglec-1* was determined by 5' rapid amplification of cDNA end (5'-RACE). RNA was extracted from CRL-2844 cells using Trizol (Takara, Dalian, China). 5'-RACE was performed according to the SMART™ RACE cDNA amplification kit instructions (Takara, Dalian, China). The 5' sequence of *Siglec-1* was determined using the universal primer A mix (UPM) supplied by the kit (5'-CTAATACGACTCACTATAGGGCAA GCAGTGGTATCAACGCAGT-3') and a gene-specific primer (GSP) (5'-CACGTTTCTGG TCTTTGAGCTTCGTCC-3'). The 5'-RACE product was characterized by nested polymerase chain reaction (PCR) using the nested gene-specific primer (NGSP) (5'-GCTGATCTCA AAGCGGAAGTTATAGGA-3') and UPM. RACE products and nested PCR products were cloned into pMD-19T. The sequences were verified by sequencing.

Analysis of promoter activity

DNA was extracted from CRL-2844 cells. In order to test the promoter activity, 8 fragments (Table 1) of various lengths were amplified from the 5'-flanking region of the *Siglec-1* gene with the introduction of *Xho*I and *Hin*dIII sites. The amplified products were digested and cloned into the multiple cloning site of the pGL3-basic vector, which contains the firefly luciferase gene as a reporter. The vectors were verified by restriction enzyme digestion and sequencing.

Eight constructed vectors and the invariant control vector pRL-TK, which contains the *Renilla* luciferase gene as a reporter, were used to co-transfect CRL-2844 cells using Lipofectamine™ 2000. The pGL3-basic and pRL-TK vectors were co-transfected as a control. The culture medium was replaced with fresh RPMI 1640 medium after 6 h, and the cell extracts were prepared 24 h after transfection by passive lysis buffer (PLB). Then, the promoter activity was determined using a dual-luciferase reporter assay kit (Promega, Madison, WI, USA) according to manufacturer instructions. Briefly, 20 mL PLB cell lysate was transferred to a tube containing 100 mL luciferase assay buffer II (LAB II) and mixed for 2-3 min. Then, the tube was placed in the luminometer, and the firefly luciferase activity measurement M1 was recorded. Next, 100 mL Stop & Glo® substrate was added to the same tube and vortexed briefly. The tube was placed in the luminometer again, and the *Renilla* luciferase activity value M2 was recorded. The promoter activity was denoted by the value M1/M2.

Table 1. Primer sequences for *Siglec-1* vector construction.

Vector	Primer sequence (5'-3')
pLUC173	GCGCTCGAGAGAGGACTTTCTGGGGCATG
pLUC574	GCGCTCGAGAGGACTGGCCCCAGAGTGA
pLUC691	GCGCTCGAGGACCCCAAAAGGGACGTT
pLUC740	GCGCTCGAGTGCTCACCAGGGACAAGG
pLUC901	GCGCTCGAGTTGGGCAGGGTCCTTGTG
pLUC1444	GCGCTCGAGTTTTCTAGGGCCACTCCTGTG
pLUC2188	GCGCTCGAGTCGTCTGTGGATGGACACTCGGAAAC
pLUC3412	GCGCTCGAGTGCAGCTGCGGCTCAGATTCA
Common downstream primer	GCGAAGCTTTCTGGCGCTGCTTCTGG

Cell-specific analysis of the *Siglec-1* promoter

To test cell-specific expression of the *Siglec-1* promoter, the promoter and pRL-TK vectors were co-transfected into CRL-2844, PK-15, PEF, and porcine preadipocyte cells. The relative promoter activity was measured by a dual-luciferase reporter assay kit.

Statistical analysis

All data are reported as means ± SE. All experiments were performed 6 times. Tissue-specific activity of the promoter was analyzed with the independent *t*-test procedure (SPSS Inc., Chicago, IL, USA). The transcriptional activities of deletion promoters were compared with one-way analysis of variance (SPSS Inc.). $P < 0.05$ was considered to be statistically significant.

RESULTS

5'-untranslated region (UTR) of the porcine *Siglec-1* gene

The gene-specific PCR and nested PCR products of 5'-RACE were about 1000 bp (Figure 1A) and 500 bp (Figure 1B), respectively. The product of the 5'-RACE was sequenced and the result is shown in Figure 1C. Sequence alignment revealed 88 bp before the start codon (Figure 1D).

Promoter activity and cell-specific analysis

The upstream 3500 bp before the start codon was used in the promoter activity analysis. Eight fragments, which contained the sequences from -173 to +81, -574 to +81, -691 to +81, -740 to +81, -901 to +81, -1444 to +81, -2188 to +81, and -3412 to +81 bp, were cloned into pGL3-basic vectors, and the vectors were named pLUC173, pLUC574, pLUC691, pLUC740, pLUC901, pLUC1444, pLUC2188, and pLUC3412, respectively. The vectors were verified by restriction enzyme digestion. All products were consistent with the expected lengths of 254, 655, 772, 950, 982, 1525, 2268, and 3493 bp.

The transcription activities of the deletion promoters are shown in Figure 2. All vectors except pLUC3412 showed significantly higher activity than the pGL3-basic vector. The pLUC173, pLUC574, and pLUC691 vectors had the strongest transcriptional activity of all vectors. However, the pLUC173 vector had the shortest promoter fragment.

Because the pLUC173 vector had the highest activity for gene expression, we tested this promoter fragment for cell-specific expression. The results are shown in Figure 3. Among CRL-2844, PK-15, PEF, and porcine preadipocyte cells, the pLUC173 vector had the highest promoter activity in CRL-2844 cells.

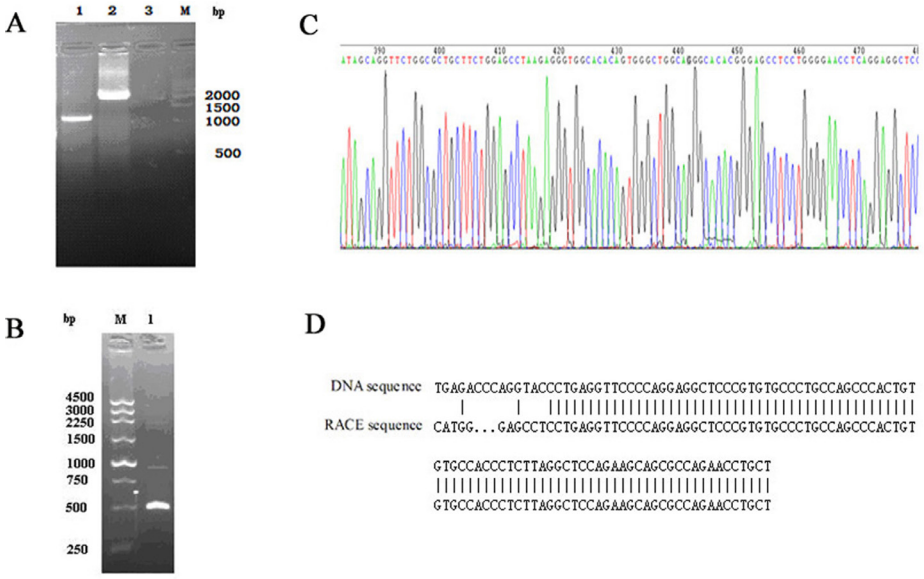

Figure 1. Results of 5'-RACE. **A.** Agarose gel (1%) image of 5'-RACE products. **B.** Agarose gel (1%) image of nest PCR products. **C.** Map analysis of 5'-RACE sequencing production. **D.** Sequencing production of 5'-RACE.

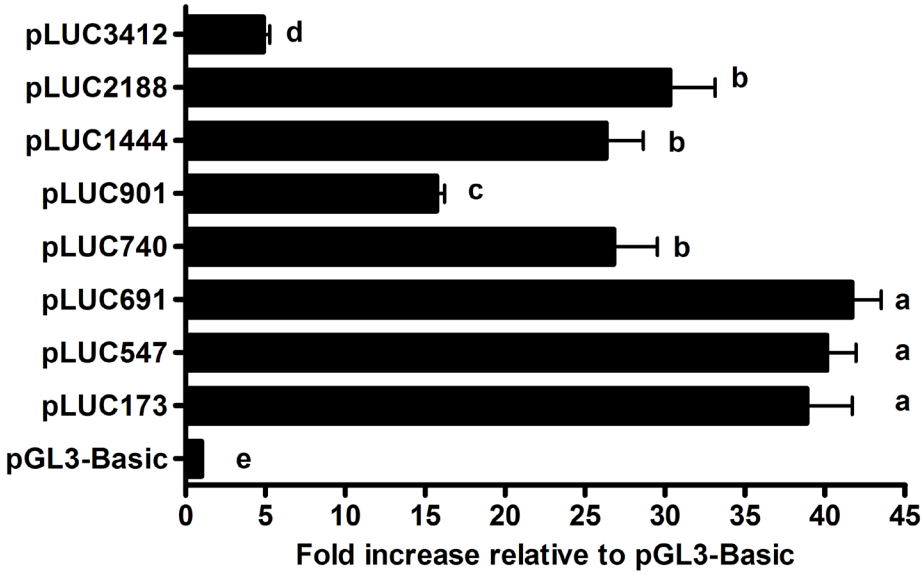

Figure 2. Promoter activity analysis. Means with different letters denotes significant difference ($P < 0.05$).

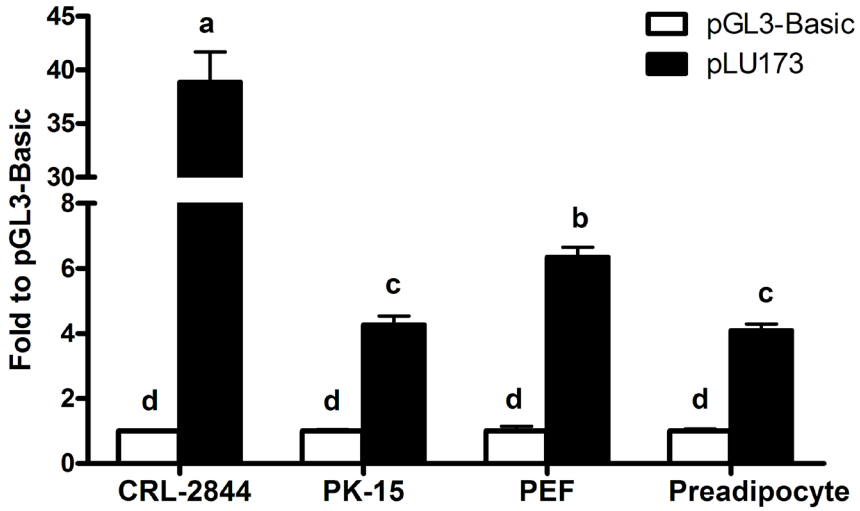

Figure 3. Cell-specific analysis of promoter fragments. Means with different letters denotes significant difference ($P < 0.05$).

DISCUSSION

The complexity of the mammalian genome is the major obstacle to identifying *cis*-regulatory elements. The development of transcription start site identification technology provides a reliable endorsement for gene promoter positions. 5'-RACE can rapidly amplify the 5' end of mRNA using cDNA as a template. The products of this method have limited errors. Moreover, this method is highly efficient, fast, and sensitive. In this study, we obtained the *Siglec-1* 5'-UTR using 5'-RACE. The GSP and NGSP mapped to +865 and +360 bp after the start codon; therefore, the PCR products should be greater than 865 and 360 bp, respectively. The lengths of the 5'-RACE products, which were approximately 1000 and 500 bp, were consistent with the expected results. Thus, we successfully obtained the 5'-UTR of *Siglec-1*.

If a positive regulatory factor binds the promoter *cis*-regulatory element, the activity of the promoter is enhanced. In the opposite situation, if the promoter *cis*-regulatory element is bound by negative regulatory factors, the promoter activity is reduced. From the restriction enzyme digestion and sequencing, we successfully constructed deletion promoter expression vectors. The pLU740, pLU901, and pLU3412 vectors had lower promoter activity than the pLU691 and pLU2188 vectors. This result may indicate that there were negative regulatory factors in the regions from -691 to -901 and -2188 to -3412 bp. The pLU173 promoter activity was 40-fold higher than that of the control group, and this vector had the shortest promoter fragment. Hence, the core promoter was located in the region from -173 to -1 bp. The pLU173 vector had the highest promoter activity in PAM cells; hence, *Siglec-1* had a cell-specific expression pattern in PAM cells. We hypothesize that PAM cell-specific transcription elements exist in the promoter region from -173 to -1 bp of *Siglec-1*. Additional research needs to be done to identify the transcription elements. In this study, we successfully identified the *Siglec-1* cell-specific promoter region. This is helpful for improving porcine disease resistance and breeding.

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