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Lymphoid Tissue-Specific Transcriptional Activity of Interleukin-8 Homolog Promoter in Marek's Disease Virus

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Abstract: Interleukin-8 homolog (vIL8) is a viral chemokine encoded by Marek's Disease Virus (MDV) genome. To study transcriptional regulation of *vIL8* gene helps to investigate the role of its encoding protein in pathogenesis of MDV. In order to explore the transcriptional regulation mechanism of *vIL8* gene, its putative promoter (position-1,362 to +81 bp) was amplified from the genome of MDV GA strain. Then, the pGL3-Basic luciferase reporter vectors bearing 5' potential vIL8 promoter segments of different lengths were transfected into second-generation Chicken Embryo Fibroblasts (CEF) and primary Chicken Embryo Bursal (CEB) cells, respectively. The promoter activity was detected using luciferase assay system. The results show that the vIL8 promoter had activity in both CEF and CEB cells but the levels of activity were different between these two cell types. The vIL8 promoter region between -470 and +10 bp almost had no activity in CEF but had remarkably higher activity in CEB cells (0 vs. 23.2) suggesting some transcriptional regulatory element binding sites specific to lymphoid tissue may exist in this region. Therefore, the vIL8 promoter is very likely specific to lymphoid tissue. All the results may make an important basis for the further study on transcriptional regulation mechanisms of vIL8.

Key words: Marek's disease virus interleukin-8 homolog, promoter, transcriptional activity, regulation, China

INTRODUCTION

Marek's disease caused by oncogenic strains of Marek's Disease Virus (MDV), a member of the Herpesviridae family is an economically-important lymphoid neoplasm of chickens. Nevertheless, its pathogenesis and tumorigenicity are still unclear (Schat and Xing, 2000). Interleukin-8 homolog (vIL8) encoded by virulent oncogenic MDV strains belongs to CXC chemokines and MDV vIL8 expressed by baculovirus system has chemotactic activity against chicken peripheral blood mononuclear cells (Liu *et al*., 1999; Parcells *et al*., 2001). Some researches have confirmed that vIL8 is non-essential for virus replication but plays an important role in early cytolytic infections, presumably mainly through signaling susceptible lymphocytes to infection foci to aggravate viral infection (Cui *et al*., 2004, 2005). However, its transcription and regulation which are important for researches about its roles in pathogenesis of MDV are rarely reported at present. Jarosinski *et al*. (2003) sequenced approximately 950 bp upstream region of the presumed MDV vIL8 promoter and found four of six attenuated MDV strains have deletions in this region, compared with virulent strains. To perform further study on transcription and regulation of *vIL8* gene, researchers analyzed the vIL8 promoter sequence of virulent MDV GA strain using bioinformatics methods and detected the activities of 5' deleted vIL8 promoter segments of different lengths *in vitro*.

MATERIALS AND METHODS

Virulent MDV GA strain and *E. coli* DH5 alpha were preserved in Key Lab of Jiangsu Preventive Veterinary Medicine (Yangzhou, China). Chicken Embryo Fibroblasts (CEF) and Chicken Embryo Bursal (CEB) cells were prepared with 9 and 18 days old SPF chicken embryos (Merial Nanjing Animal Health Co., Ltd., Nanjing, China), respectively.

The cells were cultured in DMEM (GIBCO/BRL; Gaithersburg, USA) containing 5% (v/v) fetal calf serum. The primers were synthesized by Shanghai Shenergy Biocolor Bioscience and Technology Company (Shanghai, China). Restriction enzymes were purchased from TaKaRa (Dalian, China). T_4 DNA ligase was purchased from Promega (Madison, USA). Gel extraction kits were purchased from Omega Bio-Tek (Norcross, USA).

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Bioinformatics analysis: According to the genomic sequence of MDV GA strain published in GenBank (ID: AF147806), the sequence of the first exon and upstream sequence of *vIL8* gene about 2,000 bp in size were obtained. The transcription start site of *vIL8* gene was located by predicting with First EF software and Dragon Gene Start Finder (DGSF) software. Promoter prediction and element analysis were conducted using Promoter 2.0 software, MatInspector software and TESS promoter scan software.

Amplification and sequencing of *vIL8* gene promoter: The 2nd-generation CEF were inoculated with MDV GA strain and the cells were harvested 4 days post inoculation when a large number of plaques appeared. Viral genomic DNA was extracted routinely and dissolved in 50.0 μL double distilled water. According to the genomic sequence of MDV GA strain published in GenBank, a pair of primers was designed to amplify *vIL8* gene (position-1,362 to +81 bp) (Table 1). Using the viral genomic DNA as template and the designed primers, promoter DNA segments were amplified by PCR in a 50.0 μL reaction system through the following temperature profile: 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1.5 min and a final incubation at 72°C for 10 min. The PCR products were separated by 10 g L^{-1} agarose gel electrophoresis. The target fragment was then recovered and purified using the Gel Extraction kit. The purified fragment was ligated to pGEM-T easy vector (Promega; Madison, USA) followed by sequencing performed by Sangon Biotech (Shanghai, China). The positive recombinant plasmid was designated pT-PvIL8.

Construction of luciferase reporter vectors: Using a common downstream primer (PvIL8R) and six upstream primers (PvIL8F1-PvIL8F6) shown in Table 1, the 5' deleted promoter segments of different lengths were amplified from pT-PvIL8. CMV promoter segment used as an internal standard was amplified from pcDNA3.1/zeo (+) vector (Invitrogen; Carlsbad, USA) using PCMVF and PCMVR primers. Accordingly, two sites for digestion with Xho I and Hind III enzymes were inserted in the 5'-end of the primers. Following digestion with Xho I and Hind III enzymes and separation by electrophoresis, the target fragments were recovered and inserted into pGL3-Basic luciferase reporter vector (Promega; Madison, USA) that had been digested by the same two enzymes. Then, the ligation products were transformed into *E. coli* DH5 alpha competent cells and inoculated on LB plates containing ampicillin. After overnight incubation, plasmid was extracted from randomly selected colonies and identified through dual restriction enzyme digestion. The obtained positive clones were named pGL-PvIL8-1~6 and pGL-PCMV. They were used for analysis on promoter activity.

Transfection assay: Liposome-mediated transient transfection was conducted. The cells were seeded on 24 well plates at 5×10^5 cells/well, 1 day prior to transfection. When the cells grew to 80% confluence, the recombinant plasmid harboring the 5' deleted promoter segment was transfected into the cells at a plasmid/lipofectin ratio of 0.9 μg:2.0 μL. The cells transfected with pGL3-Basic (0.9 μg:2.0 μL) and those transfected with pGL-PCMV (0.1 μg:2.0 μL) were used as negative control and positive control, respectively. All transfections were done in duplicate with three batches of cells.

Determination of luciferase activities: After 72 h post transfection, culture medium was discarded. Then, the cells were washed with PBS 3 times and each well was added 100.0 μL lysis buffer provided by luciferase assay kit (Promega; Madison, USA). After complete lysis, cell lysate was centrifuged at 12,000 r min^{-1} for 30 min at 4°C. A total of 100.0 μL of luciferase assay reagent was added to 20.0 μL of the yielded supernatant and the luminometer was programmed to perform a 6-s measurement delay followed by a 6-s measurement read for luciferase activity. In order to subtract background luminescence caused by difference in transfection efficiency, Relative Light Unit (RLU) namely, the measured luciferase intensity relative to that of pGL-PCMV-transfected cells was used to indicate luciferase activity.

Table 1: Sequences and locations of primers

Primer	Sequences	Gene locations (bp)
P1	5'-CAG ACA TAC TCC TAT GCA CC-3'	-1,362 to -1,343
P2	5'-CCA TTT CCA GGC AAC AAA T-3'	+63 to +81
PvIL8F1	5'-CCG **CTC GAG** CAG GCA CAT AGG CAG ATG TC-3'	-1,336 to -1,317
PvIL8F2	5'-CCG **CTC GAG** CAA GAA TAG TTT GAA TTC TCG G-3'	-1,094 to -1,073
PvIL8F3	5'-CCG **CTC GAG** GTG AAA GAG TGA ACG GGA AG-3'	-882 to -863
PvIL8F4	5'-CCG **CTC GAG** GGT TCG GGT AAG GCG TTC-3'	-630 to -613
PvIL8F5	5'-CCG **CTC GAG** GTG TGT AGA GGG CGC ATG-3'	-470 to -453
PvIL8F6	5'-CCG **CTC GAG** ATG CCC CAT CGA ATG G-3'	-176 to -161
PvIL8R	5'-CCC **AAG CTT** CGC CCC AAC TGC TCA T-3'	-6 to +10
PCMVF	5'-CCG **CTC GAG** TTC GCG ATG TAC G-3'	-
PCMVR	5'-CCC **AAG CTT** AAT TTC GAT AAG CC-3'	-

The bold characters indicate sites of Xho I (**CTC GAG**) and Hind III (**AAG CTT**) enzymes

RESULTS AND DISCUSSION

Existence of regions with promoter activities upstream of *vIL8* gene: The transcription start site of *vIL8* gene was located at the same position as predicted by either First EF software or DGSF software. Many regions with promoter activities were found around 1,400 bp upstream of *vIL8* gene as analyzed by Promoter 2.0 software, MatInspector software and TESS promoter scan software. Several potential transcription factor binding elements including some immune regulator elements were also found in this region as analyzed by MatInspector software and TESS Promoter Scan software (Fig. 1).

Sequence analysis on vIL8 promoter region: As evidenced by 10 g L^{-1} agarose gel electrophoresis, a 1,443 bp band with theoretically expected size appeared in the PCR products (Fig. 2). The target band was recovered

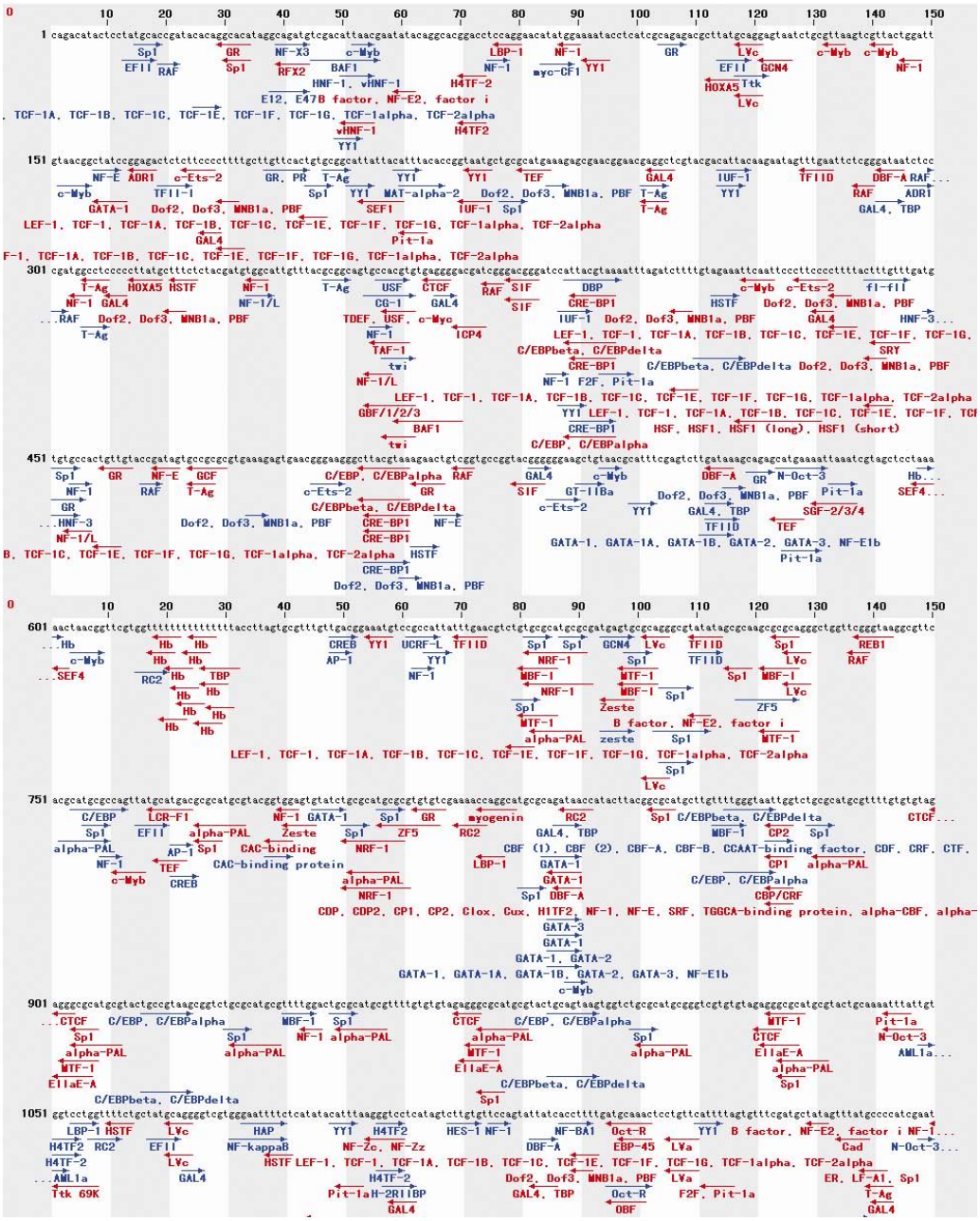

Fig. 1: Potential transcription factor binding elements predicted using TESS promoter scan software

and cloned into pGEM-T easy vector. The sequencing results show that the sequence of *vIL8* gene core promoter amplified from MDV genome had 100% similarity to 5' non-coding sequence of *vIL8* gene of MDV GA strain published in GenBank.

Obtainment of luciferase reporter vector controlled by 5' deleted vIL8 promoter: The 5' deleted vIL8 promoter segments were respectively inserted into the multiple cloning site of pGL3-Basic vector to construct a series of reporter vectors called pGL-PvIL8-1-pGL-PvIL8-6. The pGL-PCMV with *luciferase* gene controlled by the CMV promoter was used as internal control. The recombinant plasmids were identified by digestion with Xho I and Hind III. As evidenced by electrophoresis, bands of expected sizes appeared in the digestion products (Fig. 3) indicating successful construction.

Activities of 5' deleted vIL8 promoter segments of different lengths: After transfection, the second-generation CEF and primary CEB cells grew well with even confluence. After 72 h post transfection, luciferase activities were determined (Fig. 4). Compared with the pGL3-Basic vector, all recombinant vectors harboring the 5' deleted vIL8 promoter segments showed promoter activity.

The promoter region between -1,336 and +10 bp had the strongest activity and its RFU in CEF and CEB cells was 21.1 and 25.4, respectively. Deletion between 1,336 and -1,094 bp resulted in remarkable reduction of promoter activity and the RFU of promoter region between -1,094 to +10 bp in CEF and CEB cells was only 3.2 and 3.3, respectively. The promoter activities of regions from -1,094 to +10, -882 to +10 and -630 to +10 bp

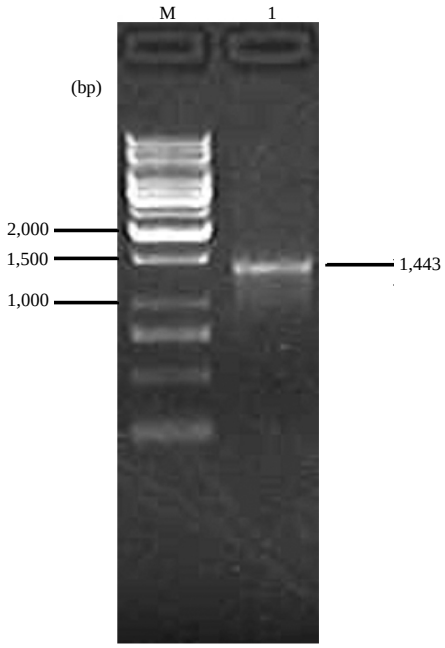

Fig. 2: PCR amplification of transcription regulatory region of MDV *vIL8* gene M: 1 kbp DNA ladder; 1: PCR products

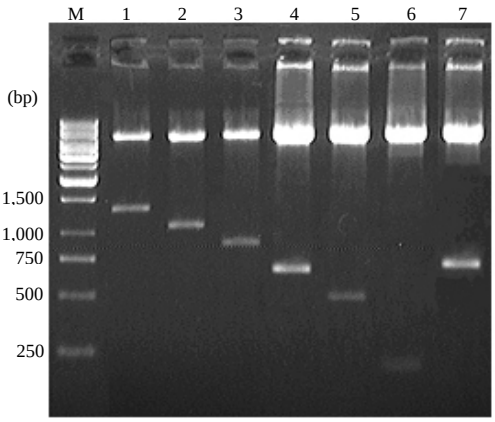

Fig. 3: Identification of luciferase reporter vectors M: 1 kbp DNA ladder; 1-6: pGL-PvIL8-1-pGL-PvIL8-6 digested with Xho I and Hind III; 7: pGL-PCMV digested with Xho I and Hind III

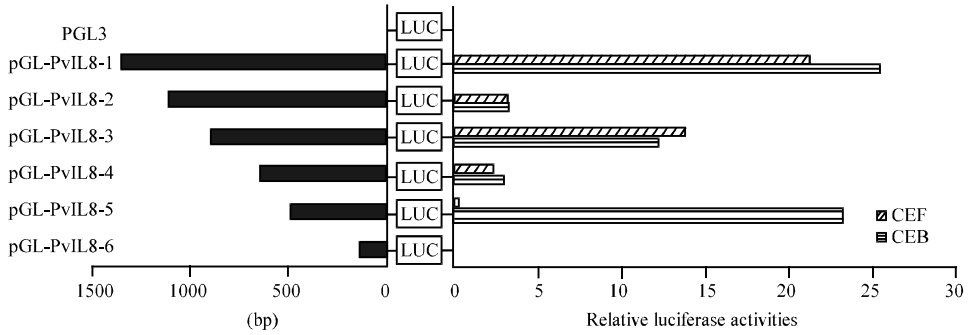

Fig. 4: Promoter activities of 5' deleted vIL8 promoter segments with different lengths in CEF and CEB cells

were not obviously different between CEF and CEB. The promoter region between -176 and +10 bp had very low activity which was close to that of pGL3-Basic. Interestingly, the activities of promoter region between -470 and +10 bp had the largest difference between CEF and CEB cells (0 vs. 23.2) showing tissue specificity of vIL8 promoter in lymphoid tissue.

In the early cytolytic phase of MDV infection, vIL8 attract and recruit lymphocytes to sites of infection which helps viral infection in lymphocytes and ultimately leads to tumor formation. However, the expression and regulation mechanism of *vIL8* gene is not clear. Thus, it is necessary to clarify promoter region, cis-acting elements and transcription factor binding sites involved in basal transcription of *vIL8* gene. In the study, many regions with promoter activities were found upstream of *vIL8* gene of MDV GA strain and some potential transcription factor binding elements including immune regulator elements were also found in the promoter region. On this basis, researchers cloned the region between -1,336 and +10 bp upstream of *vIL8* gene and analyzed its functional sequence. The sequencing results show the sequence of MDV *vIL8* gene core promoter was completely consistent with 5' non-coding sequence of *vIL8* gene of MDV GA strain published in GenBank.

In order to locate regulatory sites causing cell-specific expression differences and functional elements affecting regulation, researchers amplified the 5' deleted vIL8 promoter segments of different lengths from MDV GA strain according to software analysis. As evidenced by the results of luciferase activities, these segments induced different expression levels of luciferase. Almost all vIL8 promoter segments could induce luciferase expression both in the second-generation CEF and primary CEB cells which demonstrates the amplified sequence was the promoter of *vIL8* gene. In addition, the luciferase activity in the same cell type varied with promoter lengths and the expression levels of luciferase were similar in the CEF and CEB cells. When the region from -1,336-1,094 bp was deleted, the promoter activities decreased sharply inferring the existence of important cis-elements. However, the region between -1,094 and -882 bp may contain some elements inhibiting gene expression. The deletion of this region increased the expression level of luciferase. The absence of region from 882-630 bp reduced the expression level of luciferase indicating this region may have cis-elements. Nevertheless, their sites and corresponding trans-factors should be verified using further bioinformatics analysis and testing.

The vIL8 promoter segments of the same length had different transcriptional activities between the 2nd-generation CEF and primary CEB cells. The region between -470 and +10 bp and that between -1,336 and +10 bp had close transcriptional activity in the CEB cells but the former had remarkably weaker transcriptional activity than the latter in the CEF. This result is also supported by software analysis.

The region between -470 and -176 bp may contain many binding sites of lymphocyte-specific transcriptional regulatory elements including one Ikaros binding site, one NF-kappa B binding site and a number of cAMP-responsive element binding sites. The Ikaros binding sites play a key role in regulation of T- or B-cell-specific genes. The NF-kappa B binding sites are very important for immune response, lymphocyte proliferation and apoptosis. The cAMP-responsive element binding sites regulate expression of many genes. Presumably, these binding sites may contribute to lymphocyte-specific expression of *vIL8* gene. The existence of these elements also suggests that the expression of vIL8 mRNA may be regulated by immune factors.

CONCLUSION

This study shows that the lymphoid tissue-specific expression is important for MDV infection because MDV is classified as a lymphotropic herpesvirus. When foci of MDV infection are formed, vIL8 specifically expressed in lymphocytes can signal target peripheral lymphocytes to come into sites of infection. The vIL8-induced chemotaxis results in rapid expansion of infection foci and contributes to ultimate formation of lymphoma. Therefore, tissue-specificity of vIL8 promoter may be crucial for the roles of vIL8 which needs further studies.

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