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Transcriptional Analysis of the Gene Encoding the Putative Myristoylated Membrane Protein (ORF458R) of Invertebrate Iridescent Virus 6 (IIV6)

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Abstract—Invertebrate iridescent virus 6 (IIV6) is a member of the genus *Iridovirus* and belongs to the Iridoviridae family. The entirely sequenced dsDNA genome, composed of 212.482 bp, encodes 215 putative open reading frames (ORFs). *ORF458R* encodes a putative myristoylated membrane protein. RT-PCR analysis of *ORF458R* expression in the presence of DNA replication and protein synthesis inhibitors showed that this gene is transcribed in the late phase of the virus infection. Time course analysis showed that transcription of *ORF458R* initiates between 12 and 24 h p.i. and starts to decrease after this point. Transcription of *ORF458R* initiated 53 nucleotides upstream of the translation start site and ended 40 nucleotides after the stop codon. Dual luciferase reporter gene assay showed that sequences between −61st and +18th nucleotides are essential for promoter activity. Interestingly, a remarkable decrease in promoter activity, in the presence of sequences between −299th and −143rd nucleotides, suggested a repressor activity between these regions. Our results showed that *ORF458R* is transcriptionally active, and separately located sequences at its upstream region with promoter and repressor activities regulating its expression. This information on the transcriptional analysis of *ORF458R* will contribute to our understanding of the molecular mechanisms of IIV6 replication.

Keywords: invertebrate iridescent virus 6, *ORF458R*, transcriptional analysis, insect virus

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INTRODUCTION

The family *Iridoviridae* has two subfamilies *Alphairidovirinae* and *Betairidovirinae*. Invertebrate iridescent virus 6 (IIV6), also called Chilo iridescent virus (CIV), is not only a member of the genus *Iridovirus* of *Betairidovirinae* but also the type species of the genus [1]. Although IIV6 can infect a broad range of insects, its virulence is quite low [2]. IIV6 is considered a non-replicative virus in mammalian cells and also an avenue for vaccine and therapeutic research by inducing a mammalian immune response since the immune response against IIV6 reduces the DNA replication of Vesicular stomatitis virus and Kunjin virus [3].

The ~212 kb viral genome of IIV6 contains 468 ORFs predicted from the computer-based analysis [4] and 215 ORFs of them encode putative proteins [5]. Transcripts of IIV6 genes divide into three temporal classes: immediate early (IE), delayed early (DE), and late (L) genes [6]. According to the first study on the classification of IIV6 transcripts based on the Northern blot hybridization method [7], DNA synthesis is not necessary for the transcription of 38 genes that are classified as immediate early genes (IE or α). While 34 genes are classified as delayed early genes (DE or β) which require de novo protein synthesis of early genes for their transcriptions, 65 genes that require the transcription of both DNA synthesis and protein synthesis are classified as late genes (L or γ).

There is limited information about the molecular mechanism of IIV6 infection. Although IIV6 is the most studied virus among the iridoviruses, replication and infection knowledge of IIV6 mostly come from studies on Frog virus-3. We have previously identified the promoter activities and transcription classes of *DNApol* and *MCP* genes of IIV6 [8, 9]. A few IIV6 genes had been functionally studied including, *193R* [10], *012L* [11], *136R*, and *165R* [12]. IIV6 virion proteins were identified using a proteomic approach [13]. The first recombinant insect iridovirus was constructed and characterized using homolog recombination by replacing *ORF157L* with a green fluorescent protein gene (*gfp*) [14]. Furthermore, the insecticidal activity of IIV6 was enhanced by introducing an insect-specific neurotoxin into the genome [15]. A recent study was carried out to determine interactions among the structural proteins of IIV6 by using the yeast two-hybrid system [16].

Table 1. Oligonucleotides used for RT-PCR, LACE and RACE analysis

Primer	Genome location	Sequence (5' → 3')
458R FW1	207044–207065	GAAGGCCTGGAATTCGGTGCCAGTGTAAGTTCAG
458R FW2*	207589–207610 and 207737–207756	TACAGAAAGTAAAGTTGAAGGTTACTTTAACTGGACCGATCG
458R RV1*	207589–207610 and 207737–207756	CGATCGGTCCAGTTAAAGTAACCTTCAACTTTACTTTCTGTA
458R RV2	208434–208456	GCTCTAGACGGGATCCAAGCCATAATTTGTTTTGTTCAA
SP1	207365–207384	ATTGATGTGTTTACAATATC
SP2	207308–207327	TTAACTCCTTTAATAGCAGC
SP3	207218–207237	GCATTTATATCAATACTTGC
RACE oligo d(C)	–	GACCACGCGTATCGATGTCGACCCCCCCCCCCCCCCCCV
RACE anchor	–	GACCACGCGTATCGATGTCGAC
LACE P1	–	PO_4-GACCACGCGTATCGATGTCGAC$(T)_{15}$VN
LACE P2	–	ATCGATACGCGTGGTC

*Primers for phusion PCR to remove the transmembrane domains from the gene.

Myristoylated viral proteins have a role in viral assembly, structure, budding, and virus-host interaction [17–22]. In this study, we focused on transcriptional and structural analysis of *ORF458R*, a putative myristoylated membrane protein.

EXPERIMENTAL

Cells and viruses. BRL-AG-3A embryonic cell line originated from *Anthonomus grandis* was provided by Prof. Dr. Guy Smagghe (Laboratory of Agrozoology, Ghent University, Belgium) and maintained at 28°C in Hink's TNM-FH medium [23] supplemented with 10% fetal bovine serum (FBS, Gibco). Invertebrate iridescent virus type 6 was provided by Dr. C. Joel Funk (USDA-ARS Western Cotton Research Laboratory, Phoenix, AZ). IIV6 was propagated in BRL-AG-3A cells, in 25 cm^2 flasks at the proper confluence. Endpoint dilution assay (with tissue culture infectious dose 50 ($TCID_{50}$)/mL) was used to determine the virus titer [24].

Temporal class and time course analysis of *458R* transcripts. To determine the transcription class, BRL-AG-3A cells were allowed to be confluent at a density of 1×10^6 cells/well in a 6-well plate at 28°C. After two hours of attachment, cells were treated with either cytosine β-*D*-arabinofuranoside (Ara-C, Sigma; 200 μg/mL) or cycloheximide (CHX, Sigma; 100 μg/mL) for 1 h to inhibit DNA and protein synthesis, respectively. Treated cells were infected at a multiplicity of infection (MOI) of 2 and maintained in the fresh medium in the presence of inhibitors. The cells were harvested with 1 mL Trizol Reagent (Invitrogen) at 24 h post-infection (h p.i.).

For the time course analysis, 1×10^6 BRL-AG-3A cells/well in a 6-well plate were infected as described above without using inhibitors. Cells were harvested at 0, 2, 4, 8, 12, 24, 36, 48 h p.i.

Total RNA samples were isolated using Trizol Reagent according to the manufacturer's instructions. RNA samples were treated with DNAse I (Sigma) to eliminate possible DNA residuals. The purity of RNA samples was tested by NanoDrop spectrophotometer (Thermo Scientific) and the integrity of RNA was confirmed by 1% agarose gel electrophoresis.

Since mRNAs of Iridoviridae family members do not have a poly-A tail downstream of transcripts (8) except two for TIV (*Tipula iridescent* virus) mRNAs [25], it is not possible to use the oligo-dT primer for cDNA synthesis from IIV6 transcripts. Therefore, total RNA samples were subjected to reverse transcription using specific primers (Table 1). The reaction was prepared in a final volume of 20 μL including 1 μg total RNA as template, 2 μL M-MuLV buffer (10×), 1 μL dNTP (10 mM), 1 μL M-MuLV Reverse Transcriptase (200 U/μL; New England BioLabs), 1 μL *458R* Rv2 primer (10 mM) and 1 μL RNasin (40 U/μL). The synthesis was initially performed at 42°C for 1 h and then heated to 65°C for 20 minutes. The double-stranded cDNAs were amplified by polymerase chain reaction (PCR) using *458R*-specific Fw1 and Rv1 primers for transcription class and *458R*-specific Fw2 and Rv2 primers for time course analysis (Table 1). PCR products were analyzed on a 1% agarose gel.

Determining the untranslated regions (UTRs) of *458R* cDNA. RACE (Rapid Amplification of cDNA Ends; Roche) method was followed for determining the 5'-UTR of *458R* by following the manufacturer's instructions except for the step for the use of poly-G tail instead of poly-T tail since the IIV6 genome has large AT-rich regions. Total RNA, harvested at 36 h post-infection, was reverse transcribed using M-MuLV

Table 2. Oligonucleotides used in promoter analysis

Primer	Position	Sequence (5' → 3')
458R-Luc Fw1	−299/−281	GG**AGATCT**GTGTGGGAGCTTCAGGGCT
458R-LucFw2	−238/−219	GG**AGATCT**GTCGCTTGTTCTCACGTGTG
458R-LucFw1	−195/−176	GG**AGATCT**GCGTTCCGACACACGTCTGT
458R-Luc Fw2	−143/−127	GG**AGATCT**CTCCAATTGGGGCAAAGC
458R-Luc Fw3	−100/−81	GG**AGATCT**GCCGACGCATTAATCTTATT
458R-Luc Fw4	−61/−39	GG**AGATCT**GGTTTTATAAATATAACTACAA
458R-Luc Rv	+18/−5	**AAGCTT**ACTTACACTGGCACCCATGTTAG
Luc NarI Rv	from pSPLuc	GAAT**GGCGCC**GGGCCTTTCTTTAT

The sequences AGATCT, AAGCTT, and GGCGCC, refer to BglII, HindIII and NarI restriction enzymes, respectively, and are printed in bold.

Reverse Transcriptase (200 U/μL; New England BioLabs) with *458R*-specific SP1 primer as described above. cDNAs were purified from the mixture with NucleoSpin Gel (Macherey-Nagel), and PCR Clean-up kit (Macherey-Nagel), and 3'-end of cDNAs were tailed with poly-G. cDNA included poly-G tail was subjected to nested PCR (Phusion Taq DNA Polymerase; New England BioLabs) using RACE Oligo-d(C) and *458R*-specific SP2 primers (98°C-30″, 98°C-10″, 56°C-20″, 72°C-12″, 72°C-10′) at 50 μL final concentration. The second step of nested PCR was carried out with RACE Anchor and *458R*-specific SP3 primers following the same procedure mentioned above. PCR products were analyzed on a 1% agarose gel, and desired DNA bands were cloned into pJET1.2 Vector (Thermo Fisher). Restriction analysis and sequencing (Macrogen) were performed for the verification of insertion and determination of 5'-UTR sequence, respectively.

The cDNA sample obtained from RNA harvested at 36 h p.i. was processed by the LACE (Ligation-based amplification of cDNA ends) method [26] to determine the 3'-UTR of the gene. LACE P1 oligonucleotide (100 μM) was ligated to each of the total RNA's 3'-end by using RNA ligase. The reaction mixture, containing 2 μg RNA, 3 μL ligase buffer (10X), 1 μL ATP (10 mM), 1 μL BSA (1 mg/mL), 1 μL T4 RNA Ligase (10U/μL; New England BioLabs) in a total volume of 30 μL, incubated at 4–8°C for 16 h. Subsequently, RNA's were precipitated using two volumes of isopropanol and 0.1 volume 3M sodium acetate (pH 5.2) at −20°C for 15 min and centrifuged at 16000 *g* for 7 min. The pellet was washed with ethanol (75%), air-dried and suspended with nuclease-free ddH_2O. 5 μL LACE P2 primer (10 μM) and 4 μL dNTPs (10 mM) were added to oligonucleotide ligated RNA and heated at 65°C for 5 min. The reverse transcription was carried out as described above after adding 5 μL DTT (Dithiothreitol; 5 mM), 1 μL Rnasin (2 U/μL), and 1 μL M-MuLV Reverse Transcriptase (200 U/μL; New England BioLabs) on reaction mixture. The cDNA strand was amplified with standard PCR (*Taq* DNA Polymerase/BioBasic, 95°C-1′, 95°C-20″, 58°C-30″, 72°C-90″, 72°C-10′) using *458R* specific Fw2 and LACE P2 primers (Table 1). PCR products were analyzed on a 1% agarose gel electrophoresis. Desired DNA band was purified and cloned into pJET1.2 Vector (Thermo Fisher). Restriction analysis and sequencing (Macrogen) were performed to verify insertion and determine the 3' UTR sequence, respectively.

Preparing promoter constructs. The promoter analysis of *458R* was carried out as described by Nalcacioglu et al. [8]. Six different forward and one reverse primers (Table 2) were designed based on the upstream region of *458R* to detect promoter activity of the gene. First, three promoter fragments (P1, P2, P3) were amplified using *458R*-Luc Fw1 and *458R*-Luc Rv primers from the IIV6 DNA template. These fragments were cloned upstream of the luciferase reporter gene of the pSPLuc+ vector (Promega). The recombinant pSPLuc+ plasmid containing P1 fragment was used as a template to amplify shorter fragments with *458R*-Luc Fw4, *458R*-Luc Fw5, *458R*-Luc Fw6, and Luc NarI Rv primers. The generated PCR products (P4, P5, P6) were also inserted into the pSPLuc+ vector.

Transfections and luciferase assays. BRL-AG-3A cells were seeded at a density of 9.5×10^5 cells/mL into 35 cm^2 wells on the day of transfection and were infected at a MOI of 1. Cells were subsequently transfected with pSPLuc+ plasmids (2 μg for each of them) including P1, P2, P3, P4, P5, and P6 fragments using Cellfectin (Invitrogen) at the time of two h p.i. The pIC-IE-1 (M.C.W. van Hulten, Wageningen University), containing the *Renilla* luciferase reporter gene, was co-transfected to normalize transfection efficiency. Cells were harvested and analyzed at 18 h p.i. Dual-luciferase reporter assay system (Promega) was used to measure Firefly and *Renilla* luciferase activities in collected cell extracts according to the manufacturer's instructions.

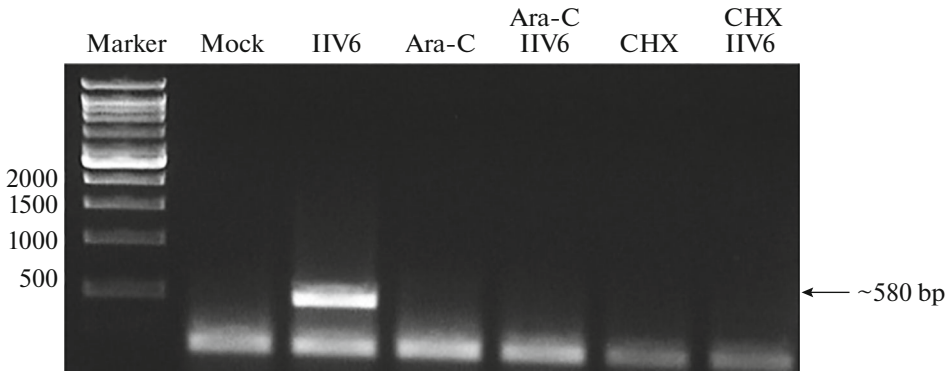

Fig. 1. Temporal class analysis of IIV6 *458R* transcripts detected by RT-PCR. Marker: 1 kb DNA Ladder (Vivantis). Lanes Mock, IIV6, Ara-C, Ara-C/IIV6, CHX and CHX/IIV6 indicates RT-PCR reactions with RNA's isolated from uninfected, virus infected, uninfected but Ara-C treated, virus infected plus Ara-C treated, uninfected but CHX treated, virus infected plus CHX treated BRL-AG-3A cells.

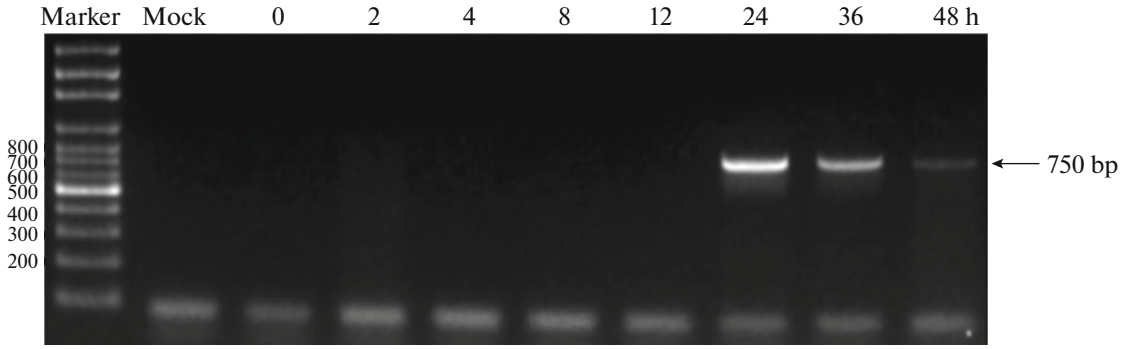

Fig. 2. Transcription time analysis of IIV6 *458R* transcripts detected by RT-PCR. Marker: 100 bp DNA Ladder (Solis Biodyne). The time points post-infection, when RNA samples were extracted from IIV6 infected BRL-AG-3A cells, are indicated at top of the gel in hours.

RESULTS

Temporal Class and Time Course Analysis of 458R

RNA isolation was performed from uninfected and infected BRL-AG-3A cells in the presence of Ara-C or CHX at 24 h p.i. cDNA synthesis was carried out using the RNA samples. Results clearly showed that *458R* is not transcribed in the presence of Ara-C and CHX which suggests *458R* is transcribed as a late gene (Fig. 1).

To determine the transcription time of *458R*, RNA isolation was performed from the infected cells which were harvested at 0, 2, 4, 8, 12, 24, 36, and 48 h p.i. cDNA synthesis was performed by using the RNA samples. RT-PCR products were analyzed on 1% agarose gel. The results showed that the transcription of the gene starts between 12 and 24 h p.i. (Fig. 2) and reaches the maximum level at 24 h p.i., and gradually decreases until 48 h p.i.

Untranslated Regions of 458R cDNA

RNA sample, harvested at 36 h p.i., was used as a template in the RACE analysis to identify the 5'-UTR of the *458R* cDNA. The cDNA was synthesized using SP1 primer and subsequently tailed with poly-G. polyG-tailed cDNA was amplified by using oligo d(C) and *458R*-specific SP2 primers. This product was used as a template to perform another PCR by using the primers RACE Anchor and *458R*-specific SP3. Resulted product, from four different clones, was sequenced to analyze the 5'UTR of *458R*. Sequence results showed that the gene transcription initiates at 53 nucleotides relative to the upstream of the translation start codon.

3'-UTR of *458R* cDNA was determined using the LACE method. cDNAs were synthesized from RNA extracted at 36 h p.i. to determine the 3' untranslated region of the gene. A standard PCR reaction was set up to produce double-stranded cDNAs which were subsequently amplified using *458R*-specific Fw2 and LACE P2 primers. PCR products, analyzed on agarose gel (1.5%) (Fig. 3), were purified and cloned into the pJET1.2 vector. Insertion was verified by restriction enzyme digestion. According to the sequences of four clones, the size of 3'-UTR of the *458R* cDNA composes of 40 nucleotides after the translation stop codon.

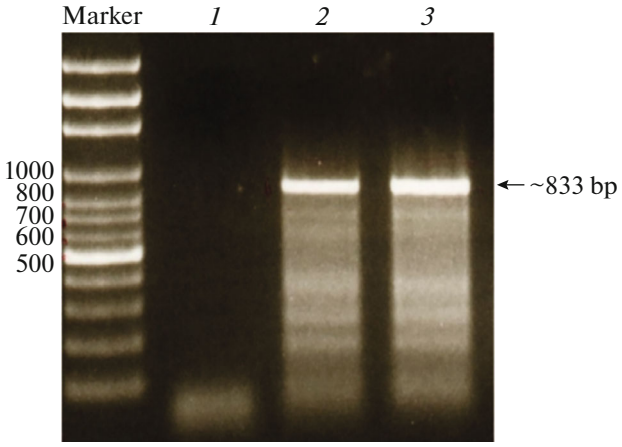

Fig. 3. Agarose gel analysis of the PCR product for 3'-UTR of *458R*. Marker: 100 bp DNA ladder (Solis Biodyne), lane *1*: negative PCR control, lane *2* and *3*: RT-PCR products generated by *458R* specific Fw2 and LACE P2 primers, respectively.

Promoter Analysis

Several sequences from the regions located upstream of the *458R* gene were tested for the ability to activate the luciferase gene. Promoter activity started decreasing when the region between −100th and −61st nucleotides, relative to the translation start site, was deleted. However, deletion of the region between −61st and +18th nucleotides, decreased the luciferase activity dramatically which means the key promoter region of the *458R* might be located between −61st and +18th nucleotides of the upstream region. However, the region between −299 and −143 nucleotides also decreased the promoter activity. Repression on promoter activity suggested the presence of a potential repressor sequence between these nucleotides (Fig. 4).

DISCUSSION

IIV6 infects a broad range of insects and has structural proteins with insecticidal activity but is not considered as a bio-pesticide material due to the often-low prevalence of patent infections [27, 28]. Additionally, although IIV6 can enter a mammalian cell but not replicate, IIV6 stimulated immune response causes a reduction in viral replication of arboviruses, VSV, and KUNV [3]. This gives some clues for the potential of IIV6 to be utilized in gene therapy studies as a material for vaccine or therapeutic development. Therefore, it is necessary that IIV6 should be analyzed for identifying molecular features and also replication mechanisms of the virus for widening usage of IIV6. In this respect, we determined to elucidate the temporal expression pattern, time course, and gene anatomy of the ORF *458R* which encodes a putative myristoylated membrane protein and has probably a role in one or more stages of viral infection from attaching to egress.

The computer-based analysis shows that *458R* has an identity (30%) with *118L*, a structural membrane protein of IIV6. Also, *458R* represents identities to *53R* from Frog Virus-3 (FV3) and *088L* ORFs from Singapor Grouper Iridoviruses [29, 30]. Like *53R* of FV-3 both *458R* and *118L* of IIV6 encode putative myristoylated proteins [5]. *53R* is needed for virion assembly and causes a reduction in mature virion formation in FV3 [31]. FV3 *53R* protein has homologs in all sequenced iridoviruses [32]. *53R* of *Rana grylio* virus, probably a strain of FV3 encodes a membrane protein [33], and has a role in viral replication, infection, and assembly [34]. SGIV *088L* is a core gene of the family and encodes a late membrane protein [35]. This gene is not only crucial for SGIV infection but also has a potential target for SGIV intervention [36]. *458R* may have a significant role in virus replication due to their homologs having crucial functions for the virus life cycle as mentioned above.

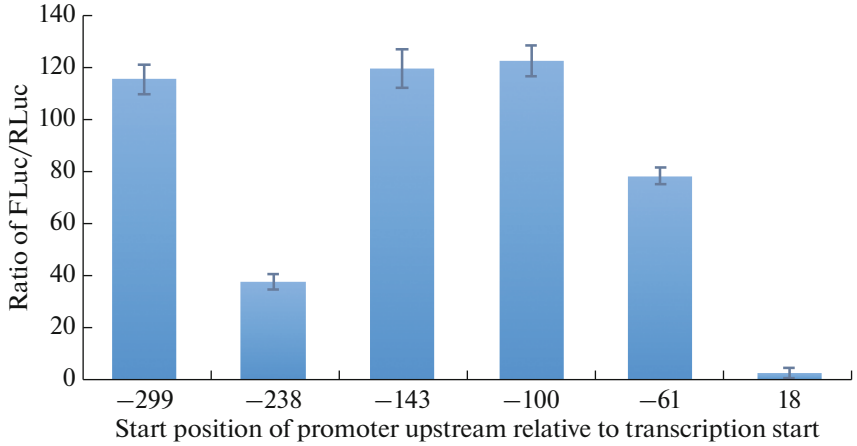

Fig. 4. Luciferase activities of the sequences at the upstream region of *458R*. Results are normalized by dividing the Firefly luciferase activity by *Renilla* luciferase activity.

It has already been shown that *458R* is a transcriptionally active gene [37]. In the current study, it is observed that transcription of *458R* is blocked by the inhibitors of DNA and protein synthesis indicating that it is a late gene. The protein of *458R* is considered as one of the structural proteins of IIV6 since late genes generally encode enzymes or viral structural proteins [38–40]. However, it does not mean that all late genes encode viral structural proteins. There are immediate early and delayed early genes of IIV6 that encode structural proteins [26, 41].

Generally, the transcription period of virus genes varies among the genes of the same temporal class. Time course analysis showed that transcription of the *458R* gene starts between 12 and 24 h after infection, and continues until 48 h and decreases gradually. These results also confirm earlier conclusions indicating that IIV6 late genes transcription occurs at 6, 12, and 24 h p.i. [37].

Transcription of *458R* is initiated at the 53rd nucleotide upstream of the translation start site. There are three other IIV6 genes of which their 5'-UTR has been identified [8, 42]. These include *mcp*, *dnapol,* and *exonuclease* genes which have 35, 14, and 30 nucleotide lengths as 5'-UTR, respectively. Compared to these three genes, *458R* has longer 5'-UTR. To draw the gene anatomy of *458R* clearly, we also defined the transcription stop site of the gene. Transcription terminates 40 nucleotides after the translation stop codon. 3'-UTR sequence showed that the *458R* gene does not contain a poly-A tail. TAATG/CATTA complementary motif, stated as a hairpin structure of IIV6 previously [43], is found at 3'-UTR of *458R*. There are 8 nucleotides between TAATG and CATTA sequences.

The potential promoter sequence of *458R* was also investigated and found that the key promoter sequence of the gene is located between –61st and +18th nucleotides, relative to the translation start codon. Deletion of the nucleotides between –61 and +18 decreased the transcription activity sharply by 96%. However, sequences between –100 and –61 have also decreased the promoter activity slightly. Additionally, sequences between –299 and –143 also showed a negative effect on promoter activity. This may be the result of a repressor sequence activity present between these sequences. Further mutations are needed to explain the role of these sequences on promoter activity. The potential promoter sequence of a late gene (*274L*) of IIV6 had been previously studied [8]. No similarity has been detected between the potential promoter regions of *458R* and *274L* genes. In conclusion, *458R*, a putative myristoylated transmembrane protein, starts transcription between 12 and 24 hours post-infection as a late gene and includes 53 and 40 nucleotides long 5'- and 3'-UTR sequences, respectively. Furthermore, the gene is controlled by both promoter and potential repressor sequences.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving humans or animals performed by any of the authors.

ADDITIONAL INFORMATION

The text was submitted by the author(s) in English.

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