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Conserved motifs in the invertebrate iridescent virus 6 (IIV6) genome regulate virus transcription

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ABSTRACT

Invertebrate iridescent virus 6 (IIV6) is the type species of the *Iridovirus* genus in the *Betairidovirinae* subfamily of the *Iridoviridae* family. Transcription of the 215 predicted IIV6 genes is temporally regulated, dividing the genes into three kinetic classes: immediate-early (IE), delayed-early (DE), and late (L). So far, the transcriptional class has been determined for a selection of virion protein genes and only for three genes the potential promoter regions have been analyzed in detail. In this study, we investigated the transcriptional class of all IIV6 genes that had not been classified until now. RT-PCR analysis of total RNA isolated from virus-infected insect cells in the presence or absence of protein and DNA synthesis inhibitors, placed 113, 23 and 22 of the newly analyzed viral ORFs into the IE, DE and L gene classes, respectively. Afterwards, *in silico* analysis was performed to the upstream regions (200 bp) of all viral ORFs using the MEME Suite Software. The AA(A/T)(T/A)TG(A/G)A and (T/A/C)(T/G/C)T(T/A)ATGG motifs were identified in the upstream region of IE and DE genes, respectively. These motifs were validated by luciferase reporter assays as crucial sequences for promoter activity. For the L genes two conserved motifs were identified for all analyzed genes: (T/G)(C/T)(A/C)A(T/G/C)(T/C)T(T/C) and (C/G/T)(G/A/C)(T/A)(T/G) (G/T)(T/C). However, the presence of these two motifs did not influence promoter activity. Conversely, the presence of these two sequences upstream of the reporter decreased its expression. Single nucleotide mutations in the highly conserved nucleotides at the end of the second motif (TTGT) showed that this motif acted as a repressor sequence for late genes in the IIV6 genome. Next, upstream sequences of IIV6 L genes from which we removed this second motif *in silico,* were re-analyzed for the presence of potential conserved promoter sequences. Two additional motifs were identified in this way for L genes: (T/A)(A/T)(A/T/G)(A/T)(T/C)(A/G)(A/C)(A/C) and (C/G)(T/C)(T/A/C)C(A/T)(A/T)T(T/G) (T/G)(T/G/A). Independent mutations in either motif caused a severe decrease in luciferase expression. Information on temporal classes and upstream regulatory sequences will contribute to our understanding of the transcriptional mechanisms in IIV6.

1. Introduction

Invertebrate iridescent viruses (IIVs, family *Iridoviridae*, subfamily Betairidovirinae, genus Iridovirus) form icosahedral particles of 120–180 nm in diameter (Chinchar et al., 2017). Virions comprise a DNA/protein core surrounded by an internal lipid membrane, a protein capsid and in the case of those particles that bud out of cells, an outer viral envelope. IIVs have been reported to infect over 100 species of arthropods (Williams et al., 2017). *Invertebrate iridescent virus 6* (IIV-6), also known as Chilo iridescent virus (CIV), is the type species of the *Iridovirus* genus. The IIV6 genome consists of 212,482 bp of linear dsDNA (Jakob and Darai, 2002) with 215 non-overlapping and putative protein-encoding ORFs selected from the 468 computationally predicted ORFs (Eaton et al., 2007). Proteomic analysis has shown that IIV6 particles contain 54 structural, viral-encoded proteins (Ince et al., 2010). The replication of the IIV6 genome is presumed to be essentially similar to that of Frog virus 3 (FV3), the type species of the genus *Ranavirus,* in the subfamily *Alphairidovirinae* (Granoff, 1984; Williams and Ward, 2010). Viral genome replication starts in the nucleus and is followed by genome concatamerization and subsequent cleavage, particle assembly and maturation in the cytoplasm (Goorha, 1982). Since purified IIV6 DNA is not infectious, one or more virion-associated proteins are needed for the initiation of IIV gene transcription (Cerutti et al., 1989).

A previous study on IIV6 mRNAs detectable by northern blot analysis revealed three temporal transcript classes in IIV6 infections: immediate-early (IE), delayed-early (DE) and late (L) (D'Costa et al., 2001). Thirty

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eight of the detected transcripts were synthesized in the presence of protein synthesis inhibitors and were classified in the IE class; thirty four transcripts were produced in the presence of DNA synthesis inhibitors and were classified in the DE class, while 65 five transcripts were detected only in the absence of inhibitors and were classified in the L class. However, as the transcripts were classified prior to genome sequencing, the relationship between the ORFs and their temporal classification was not previously established. In a later study, the 54 IIV6 structural virion protein genes were analyzed for their temporal expression, showing that the majority of these were expressed as IE genes (Ince et al., 2013).

It is known that IIV6 transcripts possess generally short 5′ untranslated regions and lack poly A tails (Nalcacioglu et al., 2003). On the other hand, information regarding the promoter elements of IIV6 genes is rather limited. So far, potential promoter regions of only three IIV6 genes, *exonuclease* (012L, IE), *DNApol* (037L, DE) and *major capsid protein gene* (*mcp*) (274L, L), have been characterized in detail (Nalcacioglu et al., 2003; 2007; Dizman et al., 2012). These promoters have been identified by means of a luciferase reporter assay in conjunction with deletion mutagenesis of the sequences in the 5′upstream region of the respective ORFs. In the current study, we investigated the transcriptional class of all as of yet unclassified IIV6 ORFs (170 transcripts) to complete the temporal classification and to be able to search for essential, conserved promoter motifs in IIV6 genes. Therefore, the upstream regions of all genes in a particular class (classified in this paper and in previous studies) were compared and analyzed for conserved sequence motifs. The identified conserved sequences were examined for promoter activity in insect cells using the luciferase reporter assay.

2. Material and methods

2.1. Cell line, virus and virus infections

Spodoptera frugiperda 9 (Sf9) cells were maintained in Sf-900 II SFM (Gibco) supplemented with 5% fetal bovine serum (FBS, Sigma) at 28 °C as monolayer. Invertebrate iridescent virus 6 (IIV6) was propagated in these cells and the virus titer was determined in End Point Dilution Assays (EPDAs) (Cook et al., 1976). Virus infections were carried out with $2x10^6$ Sf9 cells in 6-well plates, infected at a multiplicity of infection (MOI) of 2. For the temporal classification of the genes, cultures were pre-treated 1 h before infection with cytosine-1-β-D-arabinofuranoside (Ara-C, 100 μg/ml) and cycloheximide (CHX, 150 μg/ml) to inhibit DNA and protein synthesis, respectively. The inhibitors remained present during the infection.

2.2. Reverse transcription PCR (RT-PCR)

To determine the temporal expression classes of IIV6 genes, RNA isolated as described below was subjected to RT–PCR. Forward and reverse gene specific primers were designed to amplify suitable regions from all viral genes (Table S1). Total RNA was isolated from infected and mock-infected Sf9 cells at 12 h post infection (p.i.) using Trizol Reagent (Sigma, T9424) following the manufacturer's instructions. Isolated RNA samples were treated with DNase I (Sigma, AMPD1-1KT) to remove any residual DNA and then extracted with phenol–chloroform. For cDNA synthesis, 1 μg of total RNA was mixed with 1 μl (10 μM) gene specific reverse primer and the total volume was adjusted to 12 μl with water. After incubation at 65 °C for 5 min, the samples were cooled on ice. Subsequently, 4 μl reaction buffer (5X), 1 μl RiboLock RNase inhibitor (20 μ/μl), 2 μl dNTP mix (10 mM) and 1 μl reverse transcriptase (Thermo Scientific, RevertAid M−MuLV RT, 200 u/μl) were gently mixed in and reactions were incubated at 42 °C for 60 min. The cDNA synthesis was terminated by heating at 70 °C for 5 min. and the resulting cDNA mixture was then used as template for gene specific PCR amplifications with forward and reverse primers. PCR products were analyzed in a 1% agarose gel stained with ethidium bromide. PCR performed with cDNA, obtained from infected cells in absence of inhibitors, was used as positive control (PC).

2.3. Conserved sequence analyses

MEME (multiple expectation maximization for motif elicitation) (Bailey et al., 2009) software was used to search for conserved sequences in IIV6 noncoding sequences in the 200 nt regions upstream of the translation initiation codons. To that aim, the upstream sequences were categorized based on experimental data (IE, DE or L) to be able to compare upstream sequences within each expression class. Parameters were set to zero or one occurrence per sequence and we searched only the provided (coding) strand.

2.4. Plasmid construction

Upstream sequences of selected genes from each temporal group were investigated to determine whether conserved motifs, found with the MEME software, are indeed important for promoter activity. One gene was selected from each of the three groups. 193R, 126R and 259R were selected as models for IE, DE and L class genes, respectively. Upstream regions of these genes were tested for promoter activity in combination with a luciferase reporter system. Subsequently, upstream sequences of two additional L genes, 061R and 084L were also investigated with this system. Two different regions were amplified from each upstream region; one is the long one containing the conserved motif (wt) and the other is the short one missing the motif (*del* for E and DE or *delL1 + L2* in case of L genes). These DNA fragments were amplified from the viral genome using two different forward primers and a common reverse primer, for each gene (Table 1). The resulting PCR products, containing *Kpn*I and *Hin*dIII sites at 5′ and 3′ ends, respectively, were ligated into the pJET1.2/blunt cloning vector (Thermo). Subsequently these fragments were cloned into upstream of the luciferase reporter ORF of the pSPLuc + vector (Promega), again using the restriction sites at the ends of the fragments.

2.5. Transfection and luciferase assay

Sf9 cells (2.5×10^6 cells/well) in 6-well plates were infected with IIV6 for 2 h and then transfected using Cellfectin (Invitrogen) with plasmid DNA (1 μg) harboring the upstream sequences. The various putative promoter constructs were tested in parallel. At 24 h after transfection, cells were collected by centrifugation at 1000*g* for 5 min. Firefly luciferase activities were measured in cell extracts using the single luciferase reporter assay system (Promega) following the manufacturer's instructions. Transfections were conducted in triplicate, and average values are reported.

2.6. Site directed mutagenesis

Highly conserved sequences, found in the upstream regions of the L gene 061R were mutated to understand the role of these sequences in determining promoter activity. Mutations were performed by PCR using primers specific for the upstream region of 061R, but carrying a number of mismatched nucleotides (Table 1) (Nalcacioglu et al., 2003). Amplified sequences were first cloned into the pJET1.2/blunt cloning vector and then transferred to the pSPLuc + vector, as described above.

3. Results

3.1. Transcriptional classification of all IIV6 transcripts

To be able to categorize the whole set of genes in the IIV6 genome according to their transcriptional classes, we examined the expression of 170 IIV6 genes at the transcriptional level by RT-PCR. The other 45 genes in the IIV6 genome have previously been classified

Table 1
Primers for the promotor analyses.

Primers	Tm (°C)	Primer sequences (5′- 3′)
193R-prom-Fw	46.4	*GGTACC*GAGGATTTAAAAAAGTTTTAATTTAAA
193R-del-Fw	46.4	*GGTACC*TTCAAAATTAATAATACATGATACAAT
193R-prom-Rv	48.1	*AAGCTT*ATTATAAATTCCACATGTATCCAT
126R-prom-Fw	50.1	*GGTACC*GGTTTTATAAAACAATTAGCACAATTT
126R-del-Fw	45.2	*GGTACC*GATAACCATTAAAAATTATAAATAATTG
126R- prom-Rv	47.4	*AAGCTT*TTCTAAATTTGAAAATAAACTTCTTAC
259R-prom-Fw	50.4	*GGTACC*GGTATTTTCGTAATTCATTTCTTGAT
259R-del-Fw	50.8	*GGTACC*GGATTGATGCTTTTAAATGAAAAATATG
259R- prom-Rv	51.7	*AAGCTT*TGTATTTATCACTAATTCGTGTTTTGT
084L- prom-Fw	50.5	*GGTACC*TAAAGTTTCAATTTTGGAAGTTCG
084L-del-Fw	50.5	*GGTACC*AACTAATGGAAGAAGACTTTCAG
084L- prom-Rv	49.5	*AAGCTT*AGGAGACATTCTTTTATTTACAATTAA
061R- prom-Fw	46.9	*GGTACC*CATCATTTTTTCACTTTCATTTAA
061R-del-Fw	45.9	*GGTACC*GTAATATTTCTTTAATACTGAAAAATC
061R- prom-Rv	46.9	*AAGCTT*AATTCCTACGCAAATAATTATAC
061R-mutL1-Fw	63.5	*GGTACC*CATCATTGGAGTGAGCGTCTTTAATAGTGGAGATTTATTTTTAGACATATCTTGTTTATTTTTA
061R-mutL2-Fw	62.4	*GGTACC*CATCATTTTTTCACTTTCATTTAATAGTGGAGATTTATTTTTAGACATATCGCAGTTATTTTTA
061R-mutL3-Fw	60.1	*GGTACC*ACTGTTCCGACGACGTTGATATTAAACACTACTAT
061R-mutL4-Fw	58.7	*GGTACC*ACTGAAAAATCAAAGTTGATATGCCTCTGTTGTAT

*Kpn*I (*GGTACC*) and *Hin*dIII (*AAGCTT*) are shown in italicized and underlined.

transcriptionally (Nalcacioglu et al., 2007; Ince et al., 2008; 2013; Dizman et al., 2012) and were not examined again, except for 012L (IE), 037L (DE) and 274L (L) that were used as positive controls in the current study. In order to classify the IIV6 genes, Sf9 cells were infected with IIV6 in the presence or absence of cycloheximide, which inhibits *de novo* polypeptide synthesis, and Ara-C, an inhibitor of DNA replication. Total cellular RNA was extracted from cells at 12 h p.i. and analyzed for the presence of IIV6 transcripts using gene specific primers. In infected cells, a total of 113 newly analyzed transcripts was detected in the presence of protein or DNA synthesis inhibitors, which means that viral protein synthesis and DNA replication are not necessary for these transcripts and therefore they are classified in the IE class (Fig. 1). The number of the additional transcripts detected in the presence of only the DNA synthesis inhibitor was 23 (Fig. 2A). Since these transcripts do not require viral DNA replication but require viral protein synthesis to be transcribed, they were classified in the DE class, and indeed the DE-positive control (037L) was also detected here. The other transcripts that were not detected in the presence of either inhibitors, were classified in the L class. The number of the late transcripts among the 170 newly tested ORFs was 22 (Fig. 2B). However, no RT-PCR products were obtained from the putative transcripts of 12 ORFs (069L, 121R, 146R, 148R, 201R, 212L, 236L, 238R, 315L, 414L, 426R, 463L). With the previously classified IIV6 genes, the total number of IE, DE and L genes became 138, 35 and 30, respectively.

Among the 138 IE genes identified in total, 61 have a known or a putative function according to gene ontology information obtained from the UniProt database. Eight of the 35 DE transcripts, have an identified or a predicted function. The remaining 27 transcripts of the DE class do not contain a known domain to predict their function (Table 2). The third set of transcripts, classified as L, includes 7 genes with a known or putative function and 23 genes of unknown function (Table 2).

4. Conserved motifs in the upstream region of IIV6 genes

After grouping the genes in the three temporal classes, sequences upstream of the translational start codon of each gene were investigated for the presence of conserved and potentially important motifs for promoter activity. For each classified group of genes, motifs were generated by the MEME Suite database (Fig. 3). The AA(A/T)(T/A)TG(A/G)A and (T/A/C)(T/G/C)T(T/A) ATGG sequences were identified with high probability as conserved motifs in the upstream regions of IE and DE genes, respectively (Fig. 3A-B). The program run for the upstream sequences of the 138 IE genes identified the conserved motif in all of these genes. However, for the 35 scanned DE genes, the motif obtained was only observed in 20 genes. For late genes the program detected two conserved motifs, (T/G)(C/T)(A/C)A(T/G/C)(T/C)T(T/C) (motif L1) and (C/G/T)(G/A/C)(T/A)(T/G)(G/T)(T/C) (motif L2), with a similar and high probability in the upstream regions of all scanned late genes (Fig. 3C-D). The locations of all these upstream motifs respective to the translation start site varies for each gene.

4.1. Investigating the motifs for promoter activity

To analyze the influence of the conserved motifs on promoter activity, deletion mutagenesis was performed on the upstream regions of 193R, 126R and 259R ORFs, belonging to the IE, DE and L classes, respectively. Two fragments, one containing the motif and the other not, were prepared for each ORF. Reporter plasmids harboring the wild type (wt) or mutant sequence upstream of a firefly luciferase reporter ORF, were transfected into *Sf9* cells. Cell lysates, obtained 24 h after transfection, were tested for luciferase activity.

The reporter plasmids that carried the wild type (wt) upstream regions for IE or DE genes (pSP193Rprom, pSP126Rprom), produced high luciferase activity, but the plasmids without the motif (pSP193Rdel, pSP126Rdel) produced a low activity (Fig. 4A-B). However, for L genes, the result was opposite. The plasmids containing both the L1 and L2 motifs produced a low luciferase activity (pSP259Rprom, pSP084Lprom, pSP061Rprom), but the plasmids without these two motifs (pSP259RdelL1 + L2, pSP084LdelL1 + L2, pSP061RdelL1 + L2) produced a high activity. This result was validated with two additional late genes (061L, 084L) by preparing similar deletion mutations and testing the luciferase activity as mentioned above (Fig. 4C).

4.2. Site-directed mutations in conserved late gene motifs

To analyze these L motifs in more detail, we modified the L1 and L2 motifs in the upstream region of 061R individually by PCR using primers with mismatches. Reporter plasmids were prepared carrying both L1 and L2 motifs, but one unmutated sequence and the other mutated, to determine the impact of such changes on promoter activity. Mutation of motif L1 (mutation 1) did not affect the promoter activity, however mutation at motif L2 (mutation 2) increased promoter activity. This result demonstrates that motif L2 acts as a repressor on L gene promoter activity by a factor of over 90% (Fig. 5). The fact that expression levels were not fully restored to high levels by deleting L2, suggest that L1 is insufficient for a fully-functional promoter sequence.

The fact that the detected L motifs do not act as promoters led us to search for other conserved sequences that might have promoter activity.

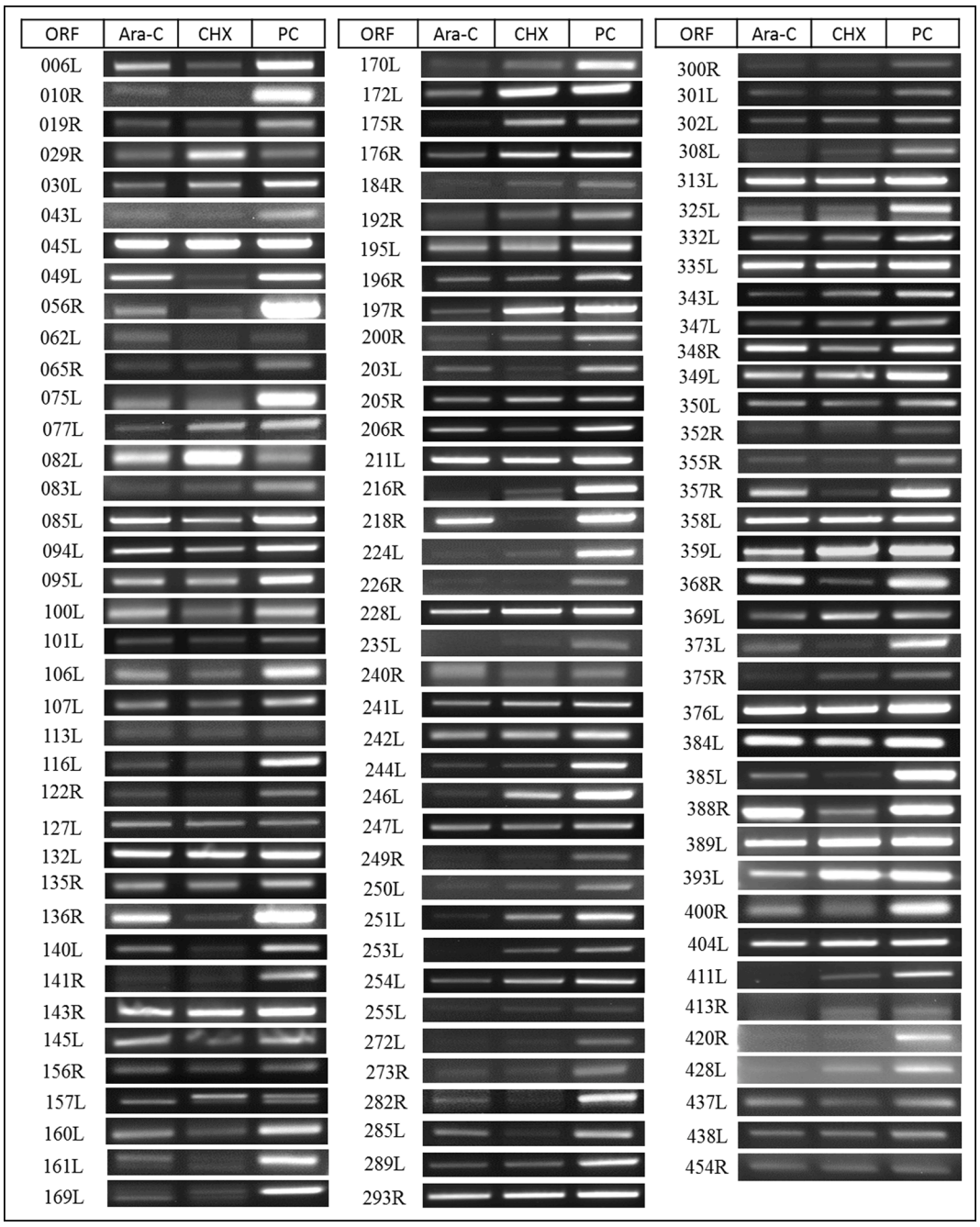

Fig. 1. Immediate-early (IE) gene transcripts of IIV6. Cells were infected with IIV6 in the presence of DNA (Ara-C) or protein synthesis (CHX) inhibitors. ORF-specific RT-PCR was carried out on total RNA isolated at 12 h post infection. Genes that give a positive RT-PCR signal in the presence of these inhibitors are categorized as IE genes. ORF: open reading frame; Ara-C: DNA synthesis inhibitor (cytosine arabinoside); CHX: protein synthesis inhibitor (cycloheximide). PC: Positive control, infection without addition of inhibitors.

Therefore, upstream sequences of all IIV6 L genes, from which the L1 and L2 motifs were *in silico* removed, were re-analyzed for the presence of potential promoter sequences using MEME Suite Software, resulting in two additional conserved sequences (motif L3 and motif L4) (Fig. 5). These motifs were individually mutated in PCR fragments that did not contain the L1 and L2 motifs and reporter analysis clearly showed that both motifs L3 and L4 contributed to L promoter activity (Fig. 5).

5. Discussion

This study presents extensive information on the transcriptional regulation of invertebrate iridescent virus 6 (IIV6) genes. Transcriptional studies on iridovirids (members of the family *Iridoviridae*) have been reported previously for Frog virus 3 (Majji et al., 2009), Singapore grouper iridovirus (Chen et al., 2006; Teng et al., 2008), Red sea bream iridovirus (Lua et al., 2005; Dang et al., 2007; 2008), IIV6 (D'Costa et al., 2001, 2004; Ince et al., 2008; 2013; Nalcacioglu et al., 2003; Dizman et al., 2012) and IIV9 (McMillan and Kalmakoff, 1994). The first transcriptional study on IIV6 genes identified 137 transcripts of which 38 corresponded to IE, 34 in DE and 65 in L temporal classes based on northern blot analysis. However, these authors did not clearly identify the ORFs in their study (D'Costa et al., 2004). Other transcriptional studies on IIV6 genes included temporal classification of a few genes: DNA polymerase (037L), major capsid protein (274L),

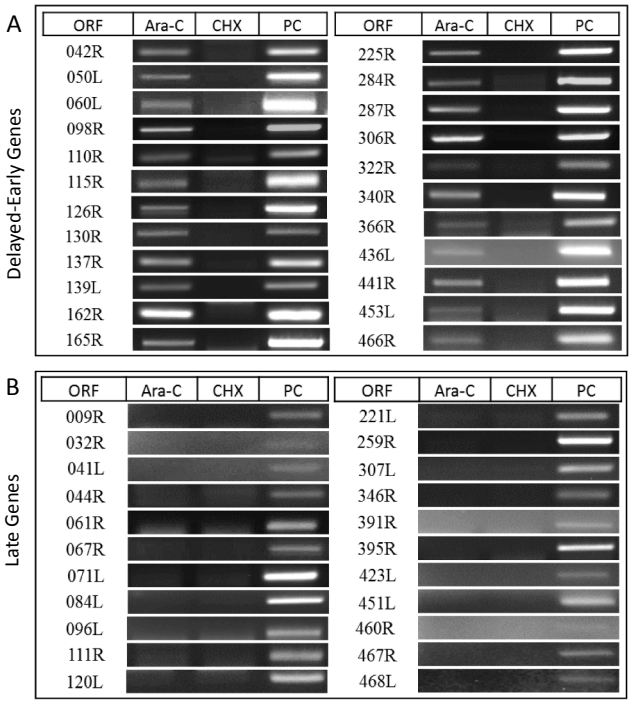

Fig. 2. Delayed-early (DE) and late (L) gene transcripts of IIV6. Cells were infected with IIV6 in the presence of DNA (Ara-C) or protein synthesis (CHX) inhibitors. ORF-specific RT-PCR was carried out on total RNA isolated at 12 h post infection. Transcripts that were not detected in the presence of the protein synthesis inhibitor (CHX) but were not affected by the DNA synthesis inhibitor are from DE genes (A). Genes that do not show transcripts in the presence of both inhibitors as categorized as L genes (B). ORF: open reading frame; Ara-C: DNA synthesis inhibitor (cytosine arabinoside); CHX: protein synthesis inhibitor (cycloheximide). PC: Positive control, infection without addition of inhibitors.

exonuclease (012L), inhibitor of apoptosis (193R), and putative myristoylated membrane protein (458R) (Nalcacioglu et al., 2003; Ince et al., 2008; Dizman et al., 2012; Aksu, 2017). A further study, performed on IIV6, classified 41 of the 54 structural protein coding genes into temporal classes using a ligation-based amplification of cDNA ends (LACE) technique (Ince et al., 2013). In the present study the remaining IIV6 genes that were not studied until now, have been classified according to their temporal expression class. Reverse transcription polymerase chain reaction (RT-PCR) has been successfully used to identify the gene transcripts using total RNA isolated from virus-infected cells in the presence of protein or viral DNA synthesis inhibitors. Since IIV6 transcripts do not contain a polyA tail (Nalcacioglu et al., 2003), specific primer sets designed for the 170 genes were used to detect the gene specific transcripts. Of these, 158 genes were placed into either of the three temporal classes. Transcripts of the remaining 12 genes (069L, 121R, 146R, 148R, 201R, 212L, 236L, 238R, 315L, 414L, 426R, 463L), could not be detected in the RNA isolated at 12 h.p.i., and also not in RNA samples isolated at other time points between 0 and 24 h p.i. from cells that were not treated with inhibitors (data not shown). The presence of the corresponding ORFs in the viral genome of our IIV6 isolate was confirmed by PCR (data not shown). The absence of these 12 transcripts may be due to the insect cells that we infected with IIV6 or a relatively low transcription level that prevented detection by RT-PCR. A similar problem was encountered by Ince, et al., (2013) while investigating the temporal classification of the IIV6 structural genes using the LACE technique. In that study, the transcripts of 13 genes (010R, 061R, 084L, 096L, 111R, 130R, 203L, 307L, 325L, 355R, 366R, 395R and 453L) could not be detected, although proteomics was used to identify the corresponding peptides. However, in our study, we have been able to detect and classify the transcripts of these 13 structural protein genes by

Table 2
Temporal classification of CIV gene transcripts based on inhibitor studies.

ORF	RNA Class	Molecular function	Biological process
006L	IE	Putative KilA-N domain-containing protein	
009R	L	Unknown	
010R	IE	Transmembrane protein	integral component of membrane
012L	IE	Exonuclease activity, nucleic acid binding	
019R	IE	Unknown	
022L*	IE	Helicase activity, ATP binding	
029R	IE	Unknown	
030L	IE	DNA helicase activity	DNA repair, telomere maintenance
032R	L	Unknown	
034R*	IE	Unknown	integral component of membrane
037L*	DE	DNA-directed DNA polymerase activity, DNA binding, nucleotide binding	DNA replication, viral DNA genome replication
041L	L	Unknown	
042R	DE	Unknown	
043L	IE	Unknown	
044R	L	Unknown	
045L	IE	DNA topoisomerase type II (ATP-hydrolyzing) activity, ATP binding, DNA binding, metal ion binding	DNA topological change
049L	IE	Nicotinamide riboside transmembrane transporter activity, Transmembrane protein	integral component of membrane
050L	DE	Unknown	
056R	IE	Unknown	integral component of membrane
060L	DE	Unknown	integral component of membrane
061R	L	Unknown	integral component of membrane
062L	IE	Unknown	
065R	IE	Unknown	integral component of membrane
067R	L	Helicase family protein	integral component of membrane
069L	ND	Putative Bro-N domain-containing protein	
071L	L	Unknown	
075L	IE	ATP binding, AAA-ATPase, similar to poxvirus A32, required for DNA packaging	
077L	IE	Metal ion binding, Putative zinc finger protein	
082L	IE	Unknown	
083L	IE	Unknown	integral component of membrane
084L	L	Putative myristoylated protein	integral component of membrane
085L	IE	Endonuclease activity, ribonucleoside-diphosphate reductase activity, thioredoxin disulfide as acceptor	DNA replication, intein-mediated protein splicing
094L	IE	Unknown	
095L	IE	Metal ion binding, Putative RING finger protein	
096L	L	Putative FAS1 domain-containing protein	
098R	DE	Serine/threonine protein kinase	
100L	IE	NAD + ADP-ribosyltransferase activity	
101L	IE	Unknown	
104L*	IE	Unknown	
106L	IE	Unknown	
107L	IE	DNA binding, DNA-directed 5′-3′ RNA polymerase activity	DNA-templated transcription

(*continued on next page*)

Table 2 (*continued*)

ORF	RNA Class	Molecular function	Biological process
110R	DE	Unknown	
111R	L	Unknown	
113L	IE	Unknown	integral component of membrane
115R	DE	Unknown	
116L	IE	Unknown	
117L*	DE	Unknown	
118L*	IE	Putative myristoylated protein	integral component of membrane
120L	L	Unknown	
121R	ND	Putative RING finger protein, metal ion binding	
122R	IE	Unknown	
123R*	IE	Protein tyrosine/serine/ threonine phosphatase activity, protein tyrosine phosphatase activity	
126R	DE	Unknown	
127L	IE	Unknown	
130R	DE	Unknown	
132L	IE	Metal ion binding, Putative zinc finger protein	
135R	IE	Unknown	integral component of membrane
136R	IE	Metallopeptidase	
137R	DE	Unknown	
138R*	IE	Unknown	integral component of membrane
139L	DE	Unknown	
140L	IE	Unknown	integral component of membrane
141R	IE	Unknown	integral component of membrane
142R*	IE	Ribonuclease III activity, RNA binding	RNA processing
143R	IE	Nucleoside kinase activity, ATP binding, Putative kinase protein	
145L	IE	Unknown	
146R	ND	Putative MSV199 domain-containing protein	
148R	ND	Putative MSV199 domain-containing protein	
149L*	DE	Unknown	
155L*	IE	Unknown	
156R	IE	Unknown	
157L	IE	Metal ion binding, Putative RING finger protein	
159L*	L	Unknown	
160L	IE	Unknown	
161L	IE	Helicase activity, DNA binding, ATP binding	
162R	DE	Unknown	
165R	DE	Metalloendopeptidase activity, zinc ion binding	
169L	IE	Unknown	integral component of membrane
170L	IE	Unknown	
172L	IE	Helicase activity, ATP binding	
175R	IE	Putative RING finger protein, metal ion binding	
176R	IE	DNA-directed 5′-3′ RNA polymerase activity, DNA binding, metal ion binding	DNA-templated transcription
179R*	DE	Protein kinase activity	
184R	IE	Helicase activity, DNA primase activity, ATP binding, D5 family NTPase	DNA replication
192R	IE	Unknown	
193R	IE	Apoptosis inhibitör, metal ion binding	suppression by virus of host cysteine-type endopeptidase activity involved in apoptotic process
195L	IE	Unknown	
196R	IE	Unknown	
197R	IE	Protein tyrosine/serine/ threonine phosphatase activity, protein tyrosine phosphatase activity	
198R	L	Unknown	
200R	IE	Putative MSV199 domain-containing protein	
201R	ND	Putative Bro-N domain-containing protein	
203L	IE	Unknown	integral component of membrane
205R	IE	DNA ligase (NAD +) activity	DNA repair, DNA replication
206R	IE	Unknown	
209R*	IE	Unknown	
211L	IE	Putative MSV199 domain-containing protein,	
212L	ND	Putative MSV199 domain-containing protein	
213R	IE	Transmembrane protein	integral component of membrane
216R#	IE	Unknown	
218R	IE	Unknown	
219L*	IE	Unknown	
221L	L	Unknown	
224L	IE	Cysteine-type peptidase activity	integral component of membrane
225R	DE	Thymidylate synthase activity	dTMP/dTTP biosynthetic process
226R	IE	Unknown	
227L*	IE	Unknown	integral component of membrane
228L	IE	Unknown	
229L*	DE	Unknown	
232R*	IE	Thiol-dependent ubiquitinyl hydrolase activity	DNA-templated transcription, termination
234R*	L	Unknown	integral component of membrane
235L#	IE	Methyltransferase activity	
236L	ND	Unknown	
238R	ND	Putative MSV199 domain-containing protein	
240R	IE	Unknown	
241L	IE	Cysteine-rich uncharacterized protein	
242L	IE	Putative GIY-YIG domain-containing protein, nuclease activity	DNA manipulation
244L	IE	Hydrolase activity, metal ion binding	
246L	IE	Unknown	
247L	IE	Unknown	
249R	IE	Unknown	
250L	IE	Unknown	
251L	IE	Thymidylate kinase activity, ATP binding	dTDP/dTTP biosynthetic process
253L#	IE	Unknown	
254L	IE	Unknown	
255L	IE	Unknown	
259R	L	Unknown	integral component of membrane
261R*	IE	Unknown	
268L*	IE	Unknown	
272L	IE	Unknown	
273R	IE	Unknown	
274L*	L	Structural molecule activity	Major capsid protein, viral capsid
282R	IE	Putative transcription factor, Putative replication factor and/or DNA binding/ packaging protein	regulation of viral transcription
284R	DE	Unknown	
285L	IE	Unknown	
287R	DE	Unknown	
289L	IE	Putative Bro-N domain-containing protein	

(*continued on next page*)

Table 2 (*continued*)

ORF	RNA Class	Molecular function	Biological process
293R	IE	Unknown	
295L*	IE	Vaccinia virus early transcription factor	integral component of membrane
300R	IE	Transmembrane protein	integral component of membrane
301L	IE	Unknown	
302L	IE	Putative zinc finger protein, metal ion binding, nucleic acid binding	
306R	DE	Putative SWIB domain-containing protein	
307L	L	Uvr/REP helicase	integral component of membrane
308L	IE	Unknown	
309L*	IE	Unknown	
312R*	IE	Unknown	
313L	IE	Putative KilA-N domain-containing protein	
315L	ND	Putative KilA-N domain-containing protein	
317L*	L	Unknown	
322R	DE	Unknown	
325L	IE	Unknown	integral component of membrane
329R*	DE	Unknown	
332L	IE	Uncharacterized RING finger protein, metal ion binding	
335L	IE	Unknown	
337L*	DE	Putative membrane protein, Myristylated membrane protein	virion membrane, integral component of membrane
340R	DE	DRBM domain-containing protein, RNA binding	
342R*	L	Unknown	
343L	IE	Probable DNA-directed RNA polymerase subunit, DNA-directed 5′-3′ RNA polymerase activity, DNA binding	DNA-templated transcription
346R	L	Unknown	
347L	IE	Flavin-linked sulfhydryl oxidase activity	
348R	IE	Unknown	
349L	IE	Putative transcription elongation factor S-II-like protein, nucleic acid binding, zinc ion binding	DNA-templated transcription
350L	IE	Unknown	
352L	IE	Unknown	
355R	IE	Putative CTD phosphatase-like protein, phosphoprotein phosphatase activity	
357R	IE	Unknown	
358L	IE	Unknown	
359L	IE	Unknown	
361L*	IE	Probable cysteine proteinase, cysteine-type peptidase activity	integral component of membrane
366R	DE	Unknown	
368R	IE	Unknown	
369L	IE	Probable RAD2-like endonuclease, endonuclease activity, metal ion binding	DNA repair, host cell nucleus
373L	IE	Unknown	
374R*	DE	Unknown	
375R	IE	Unknown	
376L	IE	Metal ion binding, ribonucleoside-diphosphate reductase activity, thioredoxin disulfide as acceptor	deoxyribonucleotide biosynthetic process, DNA replication
378R*	DE	Unknown	
380R*	IE	Protein serine/threonine kinase activity, ATP binding	
384L	IE	Unknown	
385L	IE	Unknown	
388R	IE	Putative MSV199 domain-containing protein	
389L	IE	Protein serine/threonine kinase activity, ATP binding	
391R	L	Chaperone binding	
393L	IE	Immediate-early protein ICP-46 homolog	
395R	L	Unknown	
396L*	DE	Unknown	
400R	IE	Unknown	
401R*	L	High mobility group protein homolog, HMG box DNA binding	host cell nucleus
404L	IE	Unknown	
411L#	IE	Unknown	
413R#	IE	Putative RING finger protein, metal ion binding	
414L	ND	Putative hydrolase, hydrolase activity, metal ion binding	
415R*	IE	Unknown	
420R	IE	Putative MSV199 domain-containing protein	
422L*	DE	Unknown	
423L	L	Unknown	
426R	ND	Unknown	
428L	IE	Probable DNA-directed RNA polymerase II subunit RPB2 homolog, DNA-directed 5′-3′ RNA polymerase activity, DNA binding, metal ion binding, ribonucleoside binding	DNA-templated transcription
436R	DE	Proliferating cell nuclear antigen (PCNA)	
437L	IE	Unknown	
438L	IE	dUTP diphosphatase activity	dUMP/dUTP biosynthetic process
439L*	IE	Probable kinase, protein kinase activity, ATP binding	
441R	DE	Unknown	
443R*	IE	Unknown	
451L	L	Unknown	
453L	DE	Putative thioredoxin-like protein	oxidation–reduction process
454R	IE	Putative DNA-directed RNA polymerase subunit, DNA-directed 5′-3′ RNA polymerase activity, DNA binding	DNA-templated transcription
457L	DE	Unknown	
458R	L	Putative myristoylated membrane protein	integral component of membrane
460R	L	Unknown	
463L	ND	Probable lipid hydrolase, hydrolase activity	lipid catabolic process
466R	DE	Unknown	integral component of membrane
467R	L	Unknown	
468L	L	Putative MSV199 domain-containing protein	

Stars (*) indicate that previously determined ORFs. Hash (#) symbols show the transcripts which are detected in the presence of CHX, but not determined in the presence of Ara-C, so those are classified as immediate early genes. IE, immediate early; DE, delayed early; L, late; ND, not determined. Molecular functions and biological process were obtained from UniProt database according to gene ontology. Grey shaded ORFs are core genes among all iridovirids.

RT-PCR. Bioinformatics analysis showed that most of the 12 remaining unclassified genes in the IIV6 genome contain domains associated with DNA binding; Bro-N (069L, 201R), RING finger (121R), MSV199 (146R, 148R, 212L, 238R) and KilA-N (315L). Also, two of these unclassified genes, 414L and 463L, have hydrolase activity.

Five ORFs (216R, 235L, 253L, 411L, 413R) were detected in the presence of the protein synthesis inhibitor (consistent with their

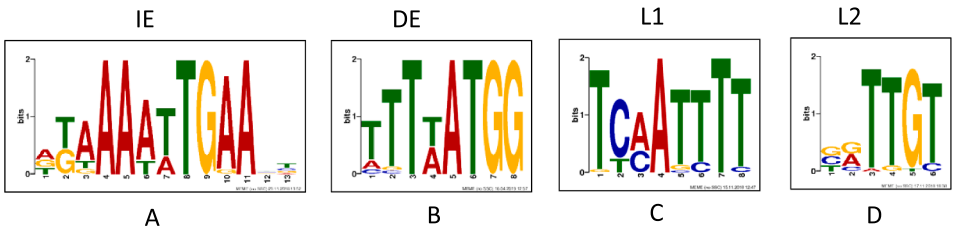

Fig. 3. Predicted motifs within the upstream sequences of IIV6 ORFs. Genes were categorized in temporal classes based on the experimental and literature data. Conserved motifs were searched for per temporal class by analysing the 200-nt sequences upstream of the annotated IIV6 translation start sites using MEME software. Parameters were set to zero or one occurrence per sequence and the program searched the coding strand only. Single motifs were obtained for (A) immediate early (IE) and (B) delayed early (DE) genes, while late (L) genes (C and D) contained two motifs (L1) and L2).

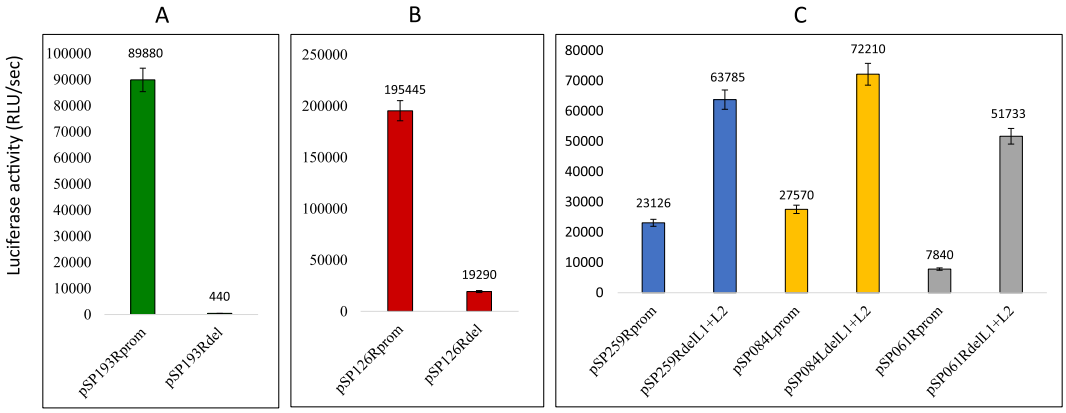

Fig. 4. Luciferase activities of promoter reporter plasmids of immediate-early (193R) (A), delayed-early (126R) (B) and late (259R, 084L, 061R) (C) ORFs with and without the motifs shown in Fig. 3. The prom and del extensions in the plasmid names refer to plasmids with and without the motifs, respectively. RLU/*sec*; relative light units emitted per second.

classification as IE genes), but not in the presence of the DNA synthesis inhibitor (consistent with their classification as L genes). This result was therefore not conforming the defined temporal groups. However, since these transcripts were detected in the presence of protein synthesis inhibitor like IE genes, they were also accepted as the products of IE genes. These genes may be transactivated early in infection by already existing proteins in the virion structure (IE), but at the same time be expressed in the late stage so that their gene products could be incorporated in the virion and may themselves assist in the transactivation process. A similar result was obtained for 219L and 295L by Ince et al., (2013).

So, more than half of the IIV6 genome can be immediately transcribed, most likely due to the action of transactivators incorporated in the viral particles. Compared to the other iridovirids (Majji et al., 2009; Chen et al., 2006; Teng et al., 2008; Dang et al., 2007), IIV6 has the highest number of ORFs in the IE class.

Among the 98 putative ORFs of Frog virus 3 (FV3), 31 have corresponding homologous in IIV6 (Tan et al., 2004). However, the temporal classes of these 31 IIV6 ORFs are mostly inconsistent with their counterparts in the FV3 transcriptome (Majji et al., 2009). For most of these homologues these inconsistencies differed by only one temporal class. Whether IIV6 has a closer resemblance in respect to transcriptional classes of its genes to invertebrate iridoviruses needs to be determined. Furthermore, the fact that genes are classified as IE genes, does not imply that a number of these genes may not continue to be expressed in the DE phase, or are expressed subsequently as late class transcripts, as we observed for a small number of IIV genes (D'Costa et al., 2001).

The obtained information on the temporal classes of almost all predicted IIV6 genes provided the basis for identifying whether ORFs in the same temporal class contained common upstream regulatory regions. The MEME Suite program identified the AA(A/T)(T/A)TG(A/G)A motif in the upstream sequence of all identified IE genes of IIV6. Previously, Dizman et al. (2012) mapped the limits of the putative promoter region of the IIV6 IE gene 012L using reporter gene assays. The *in silico* identified upstream motif is indeed present within the experimentally identified promoter region of 012L. The IIV6 *DNA polymerase* gene (037L) was reported previously as a DE class gene and it was shown experimentally that an AAAAT motif was essential for promoter activity (Nalcacioglu et al., 2007). The most prevalent motif, (T/A/C)(T/G/C)T (T/A)ATGG, identified for DE gene promoters is not present in the upstream sequence of 037L.

Two conserved motifs without promoter activity were detected in the upstream sequences of IIV6 L genes. Motif L2, ending with the sequence (T/A)(T/G)(G/T)(T/C) served as *cis*-acting repressor element for the ORF downstream, at least for the three ORFs (259R, 061L, 084L) tested in this study. The promoter region of the *mcp* has been analyzed previously (Nalcacioglu et al., 2003) and contains several repeats of motif L2. A *mcp* promoter construct starting at −53 relative to the ATG start codon, contained such a motif and had a high promoter activity, but the construct –23 that lacked this motif had reduced promoter activity in the luciferase reporter assay (Nalcacioglu et al., 2003). According to that result we may conclude that this motif does not function as a repressor for all IIV6 L genes, and it may be the context in which motif L2 is present is also important. Cis-acting repressor elements have been reported for a few viruses including Human cytomegalovirus (HCMV) (Biegalke, 1995; Lashmit et al., 1998), Equine herpesvirus 1 (EHV-1) (Ahn et al., 2010), Human Immunodeficiency Virus Type 1 (HIV-1) (Huffman and Arrigo, 1997) but to our knowledge this is the first identification of such an element in an insect virus.

Investigation of promoter motifs, using a computer program combined with biological assays, has been recently performed for Marseilleviruses (*Marseillevirus* genus, *Marseilleviridae* family) (Oliveira et al., 2017a). They detected an eight-nucleotide A/*T*-rich promoter sequence (AAATATTT) associated with 55% of the Marseillevirus genes. The IE motif, AA(A/T)(T/A)TG(A/G)A, we detected in the IIV6 genome was

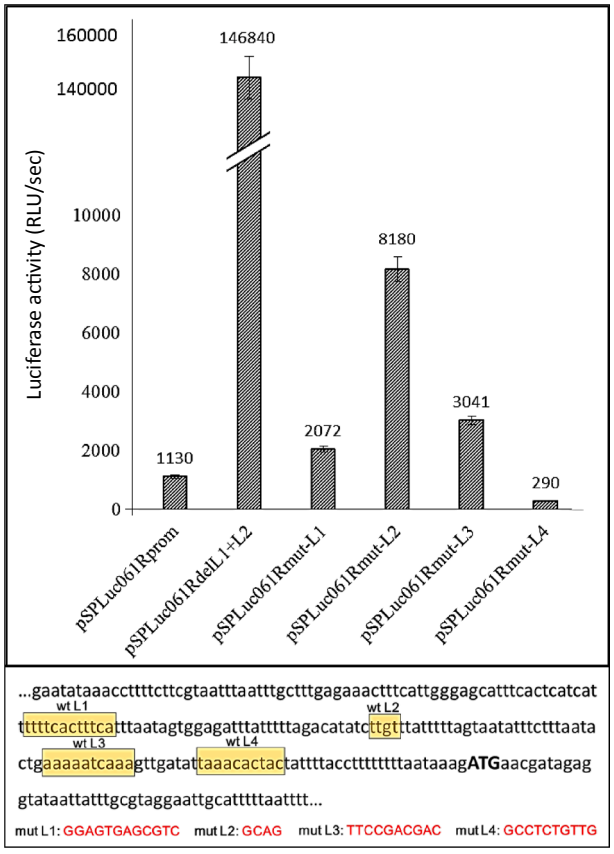

Fig. 5. Detection and analysis of additional upstream motifs for late genes. The position of the four identified motifs in the upstream region exemplified for ORF 061R. The mutated motifs are indicated with yellow boxes. The mutations made in these motifs are illustrated in red text. Luciferase activities of deletion and site directed-mutated promoter plasmids were compared to the wild type sequence for 061R. RLU/*sec*; relative light units emitted per second.

associated with the majority (68%) of viral genes. Furthermore, a specific 8 nt conserved motif, AAAATTGA, has been found in nearly 50% of IE (or DE) gene promoters in the Mimivirus genome (*Mimivirus* genus, *Mimiviridae* family) (Suhre et al., 2005). The similarity of this motif with the IIV6 IE motif is remarkable. In a review by Oliveira et al. (2017b), the currently available information about promoter regions in nucleocytoplasmic large DNA viruses (NCLDV), including iridovirids, was discussed. The fact that some promoter sequences found in one family are very similar to those found in their relatives suggest a common origin for NCLDVs genes and a promoter motif (TATATAAAATTGA) with the name "MEGA-box" was proposed for their common ancestor. The authors of the review suggested that the MEGA-box has been gradually evolved by nucleotide gain and loss into the functional promoters in the present-day NCLDVs. The resemblance of the IIV6 IE motif found in our study to the MEGA-box sequence reinforces this hypothesis.

Here, we have presented a study including both experimental and *in silico* analysis on transcriptional regulators of IIV6. Two hundred and three (158 in this study, 45 previously) of the 215 ORFs in the IIV6 genome have now been classified and include 138 IE genes, 35 DE genes and 30 L genes. Motifs detected in the upstream regions of the genes further delineate the temporal classes. The fact that all IE class genes contain the motif detected by the computer program increases the probability that this motif will essential for promoter activity for all IIV6 IE genes. This information increases our understanding the molecular mechanisms of IIV6 infection.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2020.107496.

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