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Transcriptomic analysis of *Chilo* iridescent virus immediate early promoter

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ABSTRACT

Chilo iridescent virus (CIV) is an insect virus belonging to the *Iridoviridae*. The DNA genome (212,482 base pairs) is entirely sequenced, however very little is known about viral gene regulation, expression and function. The structure and transcriptional regulation of the CIV 012L gene is investigated in this study. Infection of *Bombyx mori* SPC-BM-36 cells in the presence of Ara-C (inhibits DNA replication) or cycloheximide (inhibits protein synthesis), followed by RT-PCR on isolated total RNA, showed that CIV 012L is transcribed as an immediate-early gene. Detecting the RNA transcript of the CIV 012L early in infection confirmed the data about the temporal class of the gene obtained with the inhibitors. Time course transcription of the gene showed that the transcription starts immediately after infection and reach up to maximum level at 4 h p.i. 5′ RACE analysis on RNA isolated from CIV-infected BM cells showed that the transcription initiation site is located 30 nucleotides upstream of the translational start codon. To map the limits of the putative promoter of this gene, upstream sequences of various lengths were cloned in front of a firefly luciferase reporter gene. The resulting plasmid constructs were tested in a transfection assay, in which the baculovirus IE-l promoter fused to Renilla luciferase was used as an internal control for transfection efficiency. A gradual reduction in luciferase expression occurred as the deletions extended from −200 to −10, relative to the transcription start site. It is clearly shown that sequences between −20 and −10 relative to the transcription start site have key promoter activity for CIV 012L gene. However this key sequence could not be found at the upstream region of CIV's other potential immediate early genes.

1. Introduction

The family *Iridoviridae* comprises five genera: *Iridovirus*, *Chloriridovirus*, *Lymphocystivirus*, *Ranavirus* and *Megalocytivirus* (Jancovich et al., 2011). The type species for the genus *Iridovirus* is *Chilo* iridescent virus (CIV), an alternative name is insect iridescent virus type 6. The genome of CIV has been entirely sequenced (Jakob et al., 2001). CIV has a broad host spectrum and has, in general, a limited mortality effect on its hosts (Williams et al., 2005; Williams, 2008). Up to now there have been several studies about CIV describing its structure, ecology and molecular biology.

Studies on infected cell-specific poly-peptides and transcripts have provided evidence for a temporal cascade subdividing the CIV mRNAs into three temporal classes: immediate-early (IE or α), delayed-early (DE, β) and late (L, γ) genes (Barray and Devauchelle, 1987). Immediate-early genes of CIV transcribes from parental genome in the early stage of infection. Generally, the transcripts of viral IE genes may function as regulatory trans-acting factors and manipulate important host cellular functions, including transcription, apoptosis, cell cycle control and immune responses (Hobbs and DeLuca, 1999; Moss and Shisler, 2001; Kinchington et al., 2001). DE genes are normally expressed later and include enzymes associated with DNA replication. L genes are expressed after the onset of viral DNA replication and encode mainly structural proteins of viral particles (Chambers et al., 1999; Ebrahimi et al., 2003). IE gene expression does, by definition, not require *de novo* protein synthesis. Thus, inhibition of *de novo* protein synthesis allows expression of only IE genes. DE and L genes on the other hand do require some IE or DE gene products, respectively, to be transcribed. Therefore, transcripts which are seen in the presence of DNA replication inhibitor but not in the presence of protein synthesis inhibitor can be classified as DE. However transcripts which have been seen in the absence of both inhibitors can be classified as L. Studies of the CIV gene expression patterns are necessary and useful for fundamental characterization of the viral replication cycle that will provide clues for a better understanding of viral replication and gene expression

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Table 1
Oligonucleotides used for RT-PCR and 5′-RACE.

Primer name	Sequences (5′–3′)
012L-F	5′-CTGCTCAGGAGGTATATGGT-3′
012L-R	5′-GAGAGGCTTCTGATGAAG-3′
SP1	5′-CCTATCATGCGCGGCCA-3′
SP2	5′-GAGCTACTCCGTCGATAC-3′
SP3	5′-CTGTCTACACACCAATGA-3′

strategies and give additional insights on the pathogenic mechanism of the virus.

In the scope of gene expression, promoter studies in the *Iridoviridae* family have been performed for only limited genes, including two early genes of frog virus 3 (FV3) (ICR-169 and ICR-489; Willis, 1987; Beckman et al., 1988) and two early genes and a major capsid protein gene of Bohle iridovirus (BIV) (Pallister et al., 2005). Both of them belong to the genus *Ranavirus*. Promoter regions were also studied for two CIV genes, one from delayed early class (ORF 037L; DNA pol-DNApolymerase) and the other from late class (ORF 274L; MCP) (Nalcacioglu et al., 2003). The sequence until −53 relative to the transcription start site was able to drive the expression of luciferase when a plasmid containing this gene was transfected into cells for late gene MCP. For DNApol, an exact sequence defined as AAAAT was found as the key promoter region. Both of these promoter activities are seen only if transfected cells were subsequently infected with CIV.

In this study we selected a gene from CIV genome (012L; exonuclease) that is reported as an immediate early gene according to the transcriptional analysis of CIV's restriction fragments from a genomic library (D'Costa et al., 2003). ORF 012L contains a 5′–3′ exonuclease N-terminus domain and is homologous to exonuclease II of *Saccharomyces pombe* (21.4% identity/37% homology) (Jakob et al., 2001). We studied transcription of CIV 012L gene followed by RT-PCR on isolated total RNA and identified the potential promoter region of it by generating further series of deletion mutants.

2. Materials and methods

2.1. Cells and virus

Bombyx mori SPC-BM-36 cells (DSMZ) were cultured at 27 °C in Grace's insect medium containing 10% fetal bovine serum (FBS) (Invitrogen). *Chilo* iridescent virus type-6 (CIV) was provided from C. Joel Funk (USDA-ARS Western Cotton Research Laboratory). SPC-BM-36 cells were infected with 5 μg/ml of CIV particles as described by D'Costa et al. (2001).

2.2. Time course analysis of CIV 012L specific transcripts

SPC-BM-36 cells were infected with CIV as described above. At 1 h p.i., the inoculum was removed and replaced with fresh medium. Cells were harvested at various times after infection (0, 1, 2, 4, 6, 8, 10 and 12) and pelleted at 300 × *g* for 5 min. Total RNA was isolated from each cell pellet using Trizol (Invitrogen) according to the manufacturer's instructions. An aliquot of 10 μg total RNA was treated with 2 U of DNase I (Ambion) at 37 °C for 30 min to remove any residual DNA and then extracted with phenol–chloroform and quantified after precipitation. The RNA preparations were screened for the presence of CIV 012L specific transcripts by RT-PCR. Briefly, 2 μg of RNA and 250 nM of reverse primer (012L-R; see Table 1) specific for CIV 012L mRNA were denatured at 70 °C for 5 min and then placed on ice. First-strand cDNA was synthesized in M-MuLV buffer (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) with 600 nM dNTPs and 100 U M-MuLV reverse transcriptase (New England BioLabs) in a total volume of 25 μl. The reverse transcription was performed at 37 °C for 1 h, followed by heating at 70 °C for 15 min. One fifth of the completed RT reaction mixture was used as template for PCR amplification with specific forward and reverse primers (012L-F, 012L-R). PCR was performed in a final volume of 50 μl containing 400 nM of each primer, 0.2 mM of each dNTP in 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9.0 at 25 °C), 50 mM KCl, 0.1% Triton X-100 and 0.5 units of *Taq* DNA polymerase (Promega). PCR products were analyzed in a 1% agarose gel stained with ethidium bromide. PCR performed with RNA as template, omitting the RT step, was performed to verify the absence of DNA contamination.

2.3. The temporal class of CIV 012L specific transcripts

SPC-BM-36 cells were infected with CIV as described above. Appropriate cultures were pretreated 1 h before infection with cycloheximide or Ara-C (both from Sigma) at final concentrations of 200 and 100 μg/ml, respectively, to inhibit either protein or DNA synthesis. These inhibitors were maintained at the above levels throughout the infection. Un-adsorbed virus was removed by washing with fresh medium containing no FBS and the infected cells were overlaid with Grace's insect medium containing 10% FBS. Total RNA were isolated from cells at 12 h p.i. as described above, treated with DNAse I and subjected to RT-PCR using the same primers as above. PCR products were analyzed in a 1% agarose gel stained with ethidium bromide. As a control cellular RNA was checked for the presence of CIV 012L gene transcript. Also all RNA samples of cellular, Ara-C and cycloheximide treated ones and mock infected one were subjected to direct PCR omitting to RT step in order to see if there is any DNA contamination or not.

2.4. 5′ untranslated region (5′-UTR) analysis

Transcriptional start point of CIV 012L mRNA was determined using 5′ RACE kit (Roche) according to the manufacturer's instructions with a set of three specific primers (SP1, SP2 and SP3; see Table 1). First strand cDNA was synthesized from 2 μg total RNA isolated at 12 h p.i. using the first gene-specific primer (SP1). The first-strand cDNA was then isolated and dA tailed. The cDNA was then subjected to two consecutive nested PCRs with specific primers. SP2 was used for the first PCR with an oligo dT anchor primer (Roche). SP3 primer was used for the second PCR in combination with a PCR anchor primer (Roche). The amplified fragment was cloned into pGEMT-Easy vector (Promega) and analyzed by automated sequencing (Macrogen, Korea).

2.5. Preparation of promoter containing plasmids

Viral DNA was isolated from CIV infected cells according to the Summers and Smith (1987). A luciferase reporter gene system was used to assay different lengths of CIV 012L upstream region for promoter activity. Recombinant plasmids were constructed by generating deletion mutants starting at positions −200, −130 or −80. These fragments were amplified by PCR from CIV DNA. The primers used at the 5′ end of the sequences to be cloned introduced KpnI restriction site. The primer at the 3′ end (Luc Rev) annealed from position +42 for CIV 012L relative to the transcriptional start and introduced a BglII restriction site. The amplified DNA fragment was digested with KpnI and BglII and cloned in-frame with a luciferase reporter gene in the vector $pSPLuc^+$ (Promega). In this way the promoter plasmids P-200, P-130 and P-80 were generated. The other promoter plasmids for CIV 012L (−40, −20, −10 and +11) were amplified by PCR from the P-200 promoter plasmid. The primers directed against the 5′ end had KpnI restriction sites as described above. At the 3′ end a primer (Luc NarI R) was used that annealed downstream of the NarI site in the luciferase ORF. In this way the sizes of the generated fragments were increased to simplify their