Virus Research 189 (2014) 286–292

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Construction and characterization of a recombinant invertebrate iridovirus

Arzu Ozgen[a], Hacer Muratoglu[b], Zihni Demirbag[a], Just M. Vlak[c], Monique M. van Oers[c], Remziye Nalcacioglu[a,*]

[a] Karadeniz Technical University, Faculty of Science, Department of Biology, 61080 Trabzon, Turkey
[b] Karadeniz Technical University, Faculty of Science, Department of Molecular Biology and Genetics, 61080 Trabzon, Turkey
[c] Laboratory of Virology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

ARTICLE INFO

Article history:
Received 23 March 2014
Received in revised form 9 May 2014
Accepted 13 May 2014
Available online 12 June 2014

Keywords:
Chilo iridescent virus
Green fluorescent protein
Recombinant virus
157L
Apoptosis

ABSTRACT

Chilo iridescent virus (CIV), officially named *Insect iridescent virus 6* (IIV6), is the type species of the genus *Iridovirus* (family *Iridoviridae*). In this paper we constructed a recombinant CIV, encoding the green fluorescent protein (GFP). This recombinant can be used to investigate viral replication dynamics. We showed that homologous recombination is a valid method to make CIV gene knockouts and to insert foreign genes. The CIV 157L gene, putatively encoding a non-functional inhibitor of apoptosis (IAP), was chosen as target for foreign gene insertion. The *gfp* open reading frame preceded by the viral *mcp* promoter was inserted into the 157L locus by homologous recombination in *Anthonomus grandis* BRL-AG-3A cells. Recombinant virus (rCIV-Δ157L-*gfp*) was purified by successive rounds of plaque purification. All plaques produced by the purified recombinant virus emitted green fluorescence due to the presence of GFP. One-step growth curves for recombinant and wild-type CIV were similar and the recombinant was fully infectious *in vivo*. Hence, CIV157L can be inactivated without altering the replication kinetics of the virus. Consequently, the CIV 157L locus can be used as a site for insertion of foreign DNA, *e.g.* to modify viral properties for insect biocontrol.

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1. Introduction

Iridoviruses have large double-stranded DNA genomes and can infect invertebrates and poikilothermic vertebrates, including insects, fish, amphibians, and reptiles. The viral genomes are both circularly permuted and terminally redundant, which is a unique feature among eukaryotic viruses (Goorha et al., 1984; Williams et al., 2005). The family *Iridoviridae* is currently subdivided into five genera: *Chloriridovirus*, *Iridovirus*, *Lymphocystivirus*, *Megalocytivirus*, and *Ranavirus* (King et al., 2011). Twenty four iridovirus genomes have been sequenced, including seven genomes of insect infecting viruses (Lei et al., 2012; Mavian et al., 2012a,b; Piégu et al., 2013, 2014a,b,c; Shi et al., 2010; Williams and Ward, 2010; Wong et al., 2011; Zhang et al., 2013). *Chilo* iridescent virus (CIV), officially named *Insect iridescent virus 6* (IIV6), is the type species of this genus.

CIV is of particular ecological and economical importance, since it has been shown that this virus is lethal for a variety of insects. These include a number of herbivorous insects that cause huge damage in agriculture, for example, in rice farming and stone fruit culturing (Jakob and Darai, 2002). Consequently, this insect iridovirus can be considered as a potential agent for biological control (Hernandez et al., 2000; Jakob et al., 2002; Kleespies et al., 1999). However, CIV, as well as the other insect iridoviruses, show a low prevalence of patent infections and a low virulence (Williams et al., 2005), a disadvantage for their use in biocontrol.

Information on the molecular biology of CIV is limited and is based on a few genomic, transcriptomic and proteomic studies (Bigot et al., 2013; D'Costa et al., 2004, 2001; Dizman et al., 2012; Ince et al., 2013, 2010; Nalcacioglu et al., 2007, 2003), supplemented with several apoptosis related studies (Chitnis et al., 2011, 2008; Ince et al., 2008; Paul et al., 2007). Functional analysis has only been performed for the apoptosis inhibitor gene 193R, which was performed by silencing the 193R expression during infection of cultured insect cells and transfections with plasmids carrying the 193R gene (Ince et al., 2008). More information about the molecular biology of CIV may lead to a better understanding of the pathobiology of iridoviruses in insects.

To determine the contribution of a particular viral gene to replication and pathogenesis *in vivo*, an effective strategy is to generate

* Corresponding author. Phone: +90 462 377 35 54; Fax: +90 462 325 31 95.
E-mail address: remziye@ktu.edu.tr (R. Nalcacioglu).

http://dx.doi.org/10.1016/j.virusres.2014.05.012
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knockout viruses targeting this gene (Guangchun et al., 2011). Replacing the viral gene with a heterologous reporter gene is the first and easiest approach to generate such a knockout, recombinant virus. Gene targeting by homologous recombination is a powerful tool to precisely manipulate the genome for experimental uses and has been an efficient technique for studying DNA virus pathogenesis, including poxviruses, herpesviruses, baculoviruses, adenoviruses and African swine fever virus (Harrison et al., 2010; Lu et al., 2013; Miller, 2011; Rodríguez et al., 2009; Satheshkumar et al., 2009). The development of recombinant viruses expressing fluorescent proteins such as the green fluorescent protein (GFP) has greatly facilitated the understanding of virus infection and pathogenesis, including the dynamic motility of virus particles (Brandenburg and Zhuang, 2007; Greber and Way, 2006; Hernaez et al., 2006; Husain and Moss, 2003; Ohkawa et al., 2010; Ward and Moss, 2001), novel mechanisms for rapid virus spread (Doceul et al., 2010) and virus–host interactions (de Oliveira et al., 2008; Hollinshead et al., 2001).

Here we report the generation of a gene-knockout insect iridovirus by homologous recombination. The 157L gene in the CIV genome has been classified as encoding an apoptosis inhibitor protein (IAP) according to bioinformatic analysis (Jakob et al., 2001), but the encoded protein lacks the typical BIR domain and is probably not a functional IAP (Ince et al., 2008), in contrast to 193R. Therefore the 157L gene is thought to be a good locus for gene insertions. In the current study the CIV 157L gene was replaced by the green fluorescent protein gene (*gfp*). To that aim, the *gfp* open reading frame (ORF) was placed under the control of a copy of the promoter of the major capsid protein gene (*mcp;* 274L) and inserted into the CIV genome *via* homologous recombination. Recombinant, fluorescing CIV was selected, and its replication studied, in cotton boll weevil cells.

2. Materials and methods

2.1. Cell line and virus

The embryonic cell line derived from the cotton boll weevil *Anthonomus grandis*, BRL-AG-3A, was obtained from Prof. Dr. Guy Smagghe (Laboratory of Agrozoology, Ghent University, Belgium) and cultured in Hink's TNM-FH medium (Hink, 1970) supplemented with 10% fetal bovine serum (FBS, Sigma) at 28 °C. CIV was obtained from Dr. C. Joel Funk-USDA-ARS Western Cotton Research Laboratory, Phoenix, AZ, and was propagated on BRL-AG-3A cells grown in 75 cm^2 flasks at 80% confluency. Virus titers were determined by end point dilution assays and expressed as the tissue culture infectious dose 50 ($TCID_{50}$)/ml (Reed and Muench, 1938).

2.2. Construction of the transfer vector

The CIV ORF 157L was chosen as target gene for recombinant transfer vector construction. To generate recombinant CIV by homologous recombination, a transfer vector was constructed carrying the green fluorescent protein gene (*gfp*) under the control of the promoter of the CIV major capsid protein gene (*mcp*; 274L) between sequences, homologous to the left and right flanking regions of the 157L ORF (Fig. 1A). The *mcp* promoter (*mcp* pr) fragment starting at nucleotide (nt) −268 relative to the transcription initiation site and ending at the nt +18 of the *mcp* ORF was amplified from the CIV genome with primers P5/P6 (Table 1). The *gfp* sequence without ATG start codon was amplified from the plasmid pDU20*gfp* (Becker et al., 2004) with primer pair P7/P8 (Table 1). The amplified fragments were fused in frame by fusion PCR using the P5/P8 primer pair (Table 1). The 1032 bp PCR product containing *Eco*RI and *Hin*dIII sites up- and downstream of the *mcp* pr-*gfp* sequence was cloned into the pJET1.2/blunt cloning vector (Fermentas) generating the pJET1.2 *mcp* pr-*gfp* vector. The *mcp* pr-*gfp* cassette was introduced into the pBluescript SK$^-$ vector (Stratagene) using the same restriction sites.

Flanking sequences of the 157L gene, approximately 600 bp in length, were amplified by PCR from CIV genomic DNA using P1/P2 and P3/P4 primer pairs (Table 1). The amplified fragment from the upstream region, flanked by *Not*I and *Eco*RI sites, was cloned upstream of the *mcp* pr-*gfp* cassette in the pBluescript SK$^-$ vector. The fragment containing the downstream region with flanked by *Hin*dIII and *Xho*I sites was subsequently cloned downstream of the *mcp-gfp* cassette. The resulting transfer vector containing the upstream flanking sequence of ORF157L, *mcp* pr fused to *gfp*, followed by the downstream flanking sequence of 157L, was named in full as pBS-up157L-*mcp* pr/*gfp*-dw157L and will further be addressed as pBSΔ157L-*gfp*. The identity of this plasmid was confirmed by sequencing.

2.3. Generation of recombinant viruses

Recombinant CIV was produced by homologous recombination between the CIV genome and the transfer vector in BRL-AG-3A cells (Fig. 1A). To this aim, 1.5×10^6 cells/well were seeded in six-well plates and infected for 2 h with wild type (wt) CIV at an MOI of 1 $TCID_{50}$ unit/cell. After 18 h of infection, the CIV-infected cells were transfected with the plasmid pBSΔ157L-*gfp*, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Five days later, the cell supernatant containing progeny virus was collected and plaque assays were performed to purify the recombinant virus (rCIV-Δ157L-*gfp*). The plaque purification, based on green fluorescence of the plaques, was repeated seven times until all plaques were positive for GFP.

2.4. Diagnostic PCR

BRL-AG-3A cells (1.5×10^6 cells/well in six-well plates) were infected with three different rCIV-Δ157L-*gfp* clonal isolates at an MOI of 0.01 $TCID_{50}$ units/cell and incubated at 28 °C. After the cytopathic effects like hypertrophy accompanied by nuclear enlargement and cytoplasmic granularity were apparent, cells were harvested and centrifuged at 2500 × *g* for 15 min at 21 °C. The cell

Table 1
Primers used in this study.[a]

No	Primer name	Primer sequences (5′–3′)
P1	CIV 157L Ups Fw	ATGCGGCCGCGCTGGATTTGATTCATCTGG (*Not*I)
P2	CIV 157L Ups Rv	CGGAATTCTGTAGATACAATGATTAAATTTG (*Eco*RI)
P3	CIV 157L Dwn Fw	CCCAAGCTTATATTCCTATTAAAAAGTTGTG (*Hin*dIII)
P4	CIV 157L Dwn Rv	CCGCTCGAGGGAGAGATACATAATGGAAC (XhoI)
P5	CIV MCP Prom Fw	CGGAATTCCAATACATAACAATCTTTCATTAT (*Eco*RI)
P6	CIV MCP-GFP Rv	CAGCCCGGGGGATCCGTTGACGAAGAAATAGACATCTTTG
P7	CIV MCP-GFP Fw	CAAAGATGTCTATTTCTTCGTCAACGGATCCCCCGGGCTG
P8	GFP Rv	CCCAAGCTTTCACTTGTACAGCTCGTCC (*Hin*dIII)

[a] Enzyme cleavage sites are underlined and written in parentheses at the ends.

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