Virology 499 (2016) 1–8

Contents lists available at ScienceDirect

Virology

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

MacoNPV baculovirus midgut-specific gene expression during infection of the bertha armyworm, *Mamestra configurata*

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ARTICLE INFO

Article history:
Received 22 July 2016
Returned to author for revisions
2 September 2016
Accepted 3 September 2016

Keywords:
Baculovirus
Mamestra configurata
MacoNPV
RNA sequencing
Digital PCR
Transcriptomics
Occlusion derived virus

ABSTRACT

Baculoviruses have two forms, occlusion derived virus (ODV) which is responsible for primary infection in host midgut tissue and budded virus (BV), which infects all other host tissues during secondary infection. This study examined the primary infection by ODV of midgut cells of bertha armyworm *Mamestra configurata* fourth instar larvae and measured the expression of viral genes over a time course of infection. Both digital PCR and RNA sequencing methods showed the profile of transcription to be different from those produced by AcMNPV BV infection of *in vitro* cell cultures. This included having unique collections of genes expressed early, as well as much greater late gene expression of *p6.9* and much reduced expression of *polh* and *p10*. These differences likely reflect characteristics unique to the critical step of *in vivo* midgut cell infection, and provide insights into the processes that regulate viral gene expression in different host tissues.

1. Introduction

Baculoviruses are insect-specific DNA viruses that have found use both in agriculture as biological agents for pest control, as well as in biotechnology as platforms for protein expression. They are capable of producing high levels of specific viral proteins late in the infection cycle which makes them uniquely suited for use as vectors for protein production in cultured insect cell systems (Murhammer, 2016; O'Reilly et al., 1992). They are also highly specific pathogens of arthropods, including insects of the Lepidoptera, and useful as biological control tools in agricultural settings (Bonning, 2005; Clem and Passarelli, 2013; Rohrmann, 2013). Thus, their basic biology and mechanisms of interaction with their hosts are of great interest.

The baculovirus infection process is complex, involving two distinct types of infectious virions; occlusion-derived virus (ODV), which leads to infection of new hosts in the environment and is required for primary infection of lepidopteran midgut cells, and budded virus (BV), which is required for escape from the midgut and systemic, or secondary, infection of all other tissues. The vast majority of studies on baculovirus molecular biology have been performed using *in vitro* cell culture systems infected with BV. These two types of virion are genetically identical, but have envelopes derived from different sources and contain distinct complements of virus proteins (Blissard, 1996; Hou et al., 2013, 2016). Little is known about how the switch between producing these two virion types is accomplished. In studies of infection by *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) of larval *Trichoplusia ni* midgut, Granados and Lawler (Granados and Lawler, 1981) observed two alternative routes for nucleocapsids entering columnar cells of the gut; entry into the nucleus with subsequent nuclear infection, or direct migration to the basal plasma membrane. It was concluded that the nucleocapsids at the basal membrane of the cell budded into the insect hemocoel leading to secondary infection, although visual verification of the budding event could not be obtained. Initial cells infected in the midgut include both columnar epithelial and regenerative cells and escape from this tissue appears to be via midgut-associated tracheal cells (Engelhard et al., 1994; Keddie et al., 1989). Recently, a mechanism for BV midgut escape enabling secondary infections has been proposed (Means and Passarelli, 2010; Passarelli, 2011). In this study, investigation of the role of viral Fibroblast Growth Factor (vFGF) in secondary infection revealed that the virus appears to use vFGF to attract tracheoblasts that impinge upon the midgut basal lamina from the hemocoel side. Once escape from the midgut has been accomplished, cell-to-cell and tissue-to-

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http://dx.doi.org/10.1016/j.virol.2016.09.001
0042-6822/

tissue transmission of virus throughout the host is accomplished by BV.

Infection by BV has been extensively characterized using cells grown in culture, where a very high degree of synchronization of infection can be achieved. Infection proceeds in a temporal progression, with transcription of immediate early genes, followed by early genes that promote and facilitate DNA replication, late genes that give rise to proteins responsible for packaging daughter genomes in capsids, and finally very late genes enabling assembly, maturation and dissemination of the virus (Rohrmann, 2013). During the virus life cycle, BV is produced during the late expression phase and ODV is produced during the very late phase of virus gene expression.

In practice, the main difference between the primary and secondary infection processes is the type of cell playing host to the virus. Primary infection of midgut cells is a transitory infection event, with production of BV as the main product, after which the tissue generally recovers as the infected cells are sloughed over time (Keddie et al., 1989). Secondary infection by BV of other tissues is a process that leads to the production of ODV in the nucleus and the structural dissolution of the insect and its tissues. In this way, primary gut infection, which is the more ancestral process used by gammabaculoviruses and deltabaculoviruses, is separate from the more derived process of secondary infection, which was potentially acquired by alphabaculoviruses and betabaculoviruses during co-evolution with their lepidopteran hosts (Passarelli, 2011).

At the genetic level, RNA sequencing (RNA-Seq) analysis of the progression of gene expression during infection has primarily been carried out in cultured insect cells (Chen et al., 2013; Katsuma et al., 2011; Xue et al., 2012). However, as indicated above, fundamental differences exist between the primary and secondary infection phases and our goal is to characterize the interaction *in vivo* between the virus and its host. Previous *in vivo* studies with baculovirus include our preliminary analysis of *Mamestra configurata* NPV-A (MacoNPV-A) gene expression in the *M. configurata* larval midgut (Donly et al., 2014) and gene expression studies with *Helicoverpa zea* nucleopolyhedrovirus (HzSNPV) in *Heliothis virescens* larval hemocytes (Breitenbach et al., 2011). A recent study used granulovirus infection of *Plodia interpunctella* to investigate the insect response to infection and yielded some viral reads, but coverage of the PiGV genome was only ~44% (McTaggart et al., 2015). Here we again use the MacoNPV-A - bertha armyworm model to investigate the alphabaculovirus transcriptome during midgut infection. MacoNPV-A is a group II alphabaculovirus having a multiple nucleocapsids per envelope phenotype and its genome contains 169 potential ORFs encoding proteins of at least 50 amino acids (Li et al., 2002). In our previous study of the MacoNPV-A *in vivo* infection process, we used 454 pyrosequencing and conventional qPCR (Donly et al., 2014). In the current study, we have increased the resolution of detection by employing RNA-Seq based on Illumina chemistry to increase the number of reads obtained by an order of magnitude over the previous pyrosequencing results. We have also employed digital PCR to acquire fully quantitative results instead of conventional relative qPCR. The resulting quantitative data allows for direct comparison of expression between genes and is not limited to comparison of expression patterns as relative qPCR data is. Utilizing this dual approach has revealed that gene expression models produced using *in vitro* infection with BV inoculum are different in several ways from midgut primary infections. This study provides the most detailed analysis yet of gene expression by a baculovirus during *in vivo* midgut infection.

2. Materials and methods

2.1. Preparation of viral stocks

The MacoNPV-A strain 90/2 (Li et al., 2002) was produced by oral infection of 4th instar *M. configurata* (bertha armyworm) larvae (Erlandson, 1990). Occlusion bodies (OB) were harvested from larval cadavers, purified by centrifugation on sucrose gradients and quantified by using a hemocytometer.

2.2. Insect culture and infection protocols

A laboratory culture of *M. configurata* was maintained on a semi-synthetic diet (Bucher and Bracken, 1976) and larvae were reared at 21 °C, 60% relative humidity and a 18:6 light/dark photoperiod.

Newly molted *M. configurata* 4th instar larvae were held without food for 4 h and then infected with MacoNPV-A OB using the canola leaf disc method. Briefly, 2 μl of inoculum, a 5.0×10^7 OB/ml suspension containing 0.5% Fluorescent Brightener 28 (Sigma), or a control containing no OBs, was pipetted onto 3.1 mm^2 canola leaf discs placed in each well of a 24-well NuTrend plate containing 1.0 ml of solidified 1.0% agar per well. Larvae were starved for 4 h prior to being placed individually on leaf discs; larvae that consumed the entire disc within 90 min were deemed to have consumed a 1.0×10^5 OB/larva dose. This time point was set at 0 h post-inoculation (hpi) because most of the treated larvae required the entire 90 min to completely consume the leaf disc and acquire the full dose of virus. In addition, at 6, 12, 18, 24 and 48 h afterward, 5–6 larvae were randomly selected and their midguts dissected as previously described (Braun, 1996; Erlandson et al., 2002) with minor modifications including the omission of the dispase treatment. The peritrophic matrix and food bolus were removed from the midgut through a small incision, the midgut excised, and the connective tissue and associated fat body and trachea were teased away using fine forceps. The freed midgut was then directly immersed in 0.5 ml of TRIzol (Invitrogen). The midgut samples in TRIzol were stored at −70 °C until tissue collection was complete. Independent bioassay infections were carried out to provide two biological replicates for cDNA library synthesis and RNA-Seq Illumina sequencing and three biological replicates for digital PCR.

2.3. RNA extraction and analysis

Total RNA was extracted from each pool of 5–6 midguts by homogenizing in 1.0 ml of TRIzol reagent according to the manufacturer's protocol (Invitrogen). Total RNA was resuspended in 100 μl of sterile DEPC-treated H_2O and quantified by measuring absorbance at 260 and 280 nm using a Nanodrop spectrophotometer (Thermo Fisher Scientific). The quality of the RNA was assessed using an RNA 6000 Nano Kit and a Bioanalyzer 2100 instrument (Agilent).

2.4. Digital PCR template preparation

DNA was eliminated from the extracted RNA for qPCR experiments using Turbo DNA-free DNAase (Ambion) to eliminate all viral DNA produced during infection and any genomic DNA carryover from the inoculum. Successful elimination of viral DNA contamination was confirmed by testing of no-reverse transcriptase (NRT) controls. The DNA-free total cellular RNA was then quantified using an RNA 6000 Nano Kit and a Bioanalyzer 2100 instrument (Agilent). A synthetic RNA was added to each sample at a uniform rate which could be used as an internal control to normalize for any variation in cDNA synthesis efficiency resulting