FUNCTIONAL GENOMICS OF CHILO IRIDESCENT VIRUS:

A transcriptoproteomic approach

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It is almost better to tell your own lies than someone else's truth. In the first case you are a man, in the second, you are no better than a parrot.

Who are you? A man or a parrot…

Kendi uydurduğun bir yalanı söylemek, başka bir ağızdan işitilip tekrarlanmış bir gerçeği söylemekten hemen hemen daha iyidir. Birinci ihtimalde sen bir insansın. İkincisindeyse, bir papağandan hiç farkın yoktur.

Sen Kimsin? İnsan mı? Papağan mı? (Dostoyevski)

Contents

Abstract

Iridoviruses are disease causing agents in (pest) insects, fishes and amphibians with serious ecological and economic impacts. Insight in the composition of the virions and the transcriptional regulation of the virion protein genes is crucial to unravel the biology of this lesser known family of viruses. In this thesis, the virions of *Chilo iridescent virus* (CIV) (genus *Iridovirus*) were analyzed by mass spectrometry, revealing 54 virion proteins. A novel transcriptomic approach for non-polyadenylated RNA transcripts, called LACE, was developed and applied to unravel the temporal class of the virion protein genes. This showed that many virion protein genes were expressed as early genes. Another intriguing finding is that an infected cell-specific 100 kDa protein interacted with a crucial delayed-early promoter motif in the DNA polymerase gene and it turned out that this motif was conserved in other (putative) delayed early genes in CIV and other iridoviruses. The hypothesis is that this 100 kDa protein is responsible for transcriptional activation of delayed-early genes. CIV is an example of an invertebrate iridoviruses that deals with induction and inhibition of apoptosis during infection. In this study, a gene for a functional inhibitor of apoptosis (193R), unique for an iridovirus, was identified. In addition, several candidates for pro-apoptotic proteins were found in the virion. In this dissertation fundamental knowledge was obtained on the proteome of CIV virions and the regulation of CIV gene expression. Due to the development and application of novel technics, this thesis provides new venues to answer remaining questions concerning the infection cycle of this interesting iridovirus.

Key words

Iridovirus, transcriptomics, proteomics, virus-host interaction

Chapter 1

General introduction

Introduction

Chilo iridescent virus (CIV) is the type species of the genus *Iridovirus* and belongs to the family *Iridoviridae*. CIV, officially named *Invertebrate iridescent virus 6* (IIV-6), was originally isolated from diseased larvae of the rice stem borer, *Chilo suppressalis* (Lepidoptera; Pyralidae) in Japan (Fukaya & Nasu, 1966). The term iridescent in the virus name comes from the iridescent appearance of infected insects with colors that typically range from violet to green. This phenomenon is due to the massive proliferation of CIV particles and their paracrystalline arrangement in the cytoplasm of cells of patently infected host insects. CIV may cause patent, fatal infections in insects especially in the larval or pupal stages, or covert, unapparent infections, which are not lethal and in which insects appear healthy and may develop to the adult stage and reproduce. Insect iridoviruses principally infect agriculturally and medically important insect species, especially mosquitoes (Diptera) and soil-related Lepidopteran and Coleopteran species, in moist or aquatic habitats.

Iridoviruses also occur in poikilothermic vertebrates, such as amphibians and fish. These viruses are implicated in the catastrophic decline of frogs around the world. An iridovirus outbreak in 2008 in the United Kingdom decimated the frog population in certain areas. Recently, in the Netherlands an iridovirus outbreak occurred in Dwingelderveld taking the life of thousands of frogs. In fish iridoviruses can cause serious disease in salmon (erythrocytic necrosis) and other fish (lymphocystis).[1]

Classification

Currently the family *Iridoviridae* is organized into five genera: *Ranavirus, Lymphocystivirus, Megalocytivirus*, *Iridovirus*, and *Chloriridovirus* (Chinchar *et al.*, 2005, King *et al.*, 2011). This division is based on virion particle size, host range and the disease caused presence or absence of a DNA methyltransferase, the GC content of the genome and phylogenetic analysis based on the major capsid protein (MCP). The genera *Iridovirus* and *Chloriridovirus* contain invertebrate infecting viruses and CIV has been used as the standard model for studies on invertebrate iridoviruses (IIVs). The members of the other genera infect cold-blooded vertebrates such as amphibians, fish and reptiles. Apart from CIV, the genus *Iridovirus* has one other member with an official species state: *Invertebrate iridescent virus 1* (IIV-1), which was the first IIV species isolated and which infects the soil-dwelling European crane fly *Tipula paludosa* (Diptera) (Xeros, 1954).

Additionally, this genus harbors eleven tentative species. Currently, there is not enough information available to confirm that all these viruses are different enough to be regarded as individual species in this genus (Chinchar *et al.*, 2005, Williams *et al.*, 2005).

[1]This chapter was updated from review on "The biology of *Chilo iridescent virus*" published in *Virologica Sinica* 24, 285-294 (2009).

CIV infections have been reported from all continents except Antarctica and at least two strains of CIV are being studied in laboratories in different parts of the world (Williams & Cory, 1994). The isolate that has been completely sequenced by Jakob *et al.* (2001) in Germany most likely differs from the isolates used in New Zealand, Australia, and the USA (Williams *et al.*, 2005). The CIV isolate used for the studies in this thesis was a gift from C. Joel Funk (USDA-ARS Western Cotton Research Laboratory, USA), which originally comes from James Kalmakoff (University of Otago, Dunedin, New Zealand).

Relatedness of CIV to other iridoviruses

Phylogenetic analysis using the 26 core iridovirus genes from the 15 completely sequenced iridoviruses (Eaton *et al.*, 2010) showed a clear division of the family *Iridoviridae* into the five genera (Fig. 1). Core genes are defined as genes that occur in all sequenced iridoviruses. Members of the genera *Ranavirus, Lymphocystivirus* and *Megalocytivirus* form three distinct clades respectively with a common ancestor, while members of the genera *Iridovirus* and *Chloriridovirus* form two clades that are more distantly related to each other and to members of the vertebrate clade.

Despite the limitation that only two invertebrate iridoviruses have been sequenced, the phylogeny shows a distinct lineage for each genus within the family *Iridoviridae,* with the vertebrate genera being more closely related to each other as compared to the invertebrate genera (Eaton *et al.*, 2010).

The iridovirus proteins D5 ATPase, A32 ATPase, A1L/VLTF2 transcription factor, MCP and viral DNA polymerase B, belonging to the iridovirus core genes, share amino acid sequence similarities to the corresponding proteins in other nucleocytoplasmic large DNA viruses (NCLDV) in the families, notably the *Asfarviridae*, *Ascoviridae*, *Phycodnaviridae*, *Mimiviridea,* and *Poxviridae* (Fig. 2) (Jancovich *et al.*, 2011). These studies provide support to earlier studies based on comparative analyses of the capsid protein, DNA polymerase, thymidine kinase, and ATPase III, that led to the hypothesis that ascoviruses may have evolved from invertebrate iridoviruses (Stasiak *et al.*, 2000, Stasiak *et al.*, 2003). Obviously, iridoviruses are more distantly related to other large DNA viruses, such as herpesviruses, adenoviruses, and baculoviruses, which will not be discussed here.

Morphology and composition

CIV is a large virus with icosahedral symmetry (see Fig. 3). The virus particle is composed of three concentric domains: an outer proteinaceous capsid, an intermediate lipid membrane with associated polypeptides, and a central DNA-protein complex containing the genome (Chinchar *et al.*, 2005, Williams, 1998, Williams *et al.*, 2005). The virions are usually 120-200 nm in diameter. In the genus *Lymphocystivirus,* virions may be up to 350 nm in size. Virions can be enveloped, when released by budding (termed extracellular virus), but the majority is non-enveloped (naked virus) and originates from the accumulation of newly matured progeny virions in large cytoplasmic, paracrystalline arrays (termed intracellular virus), which are released upon cell lysis.

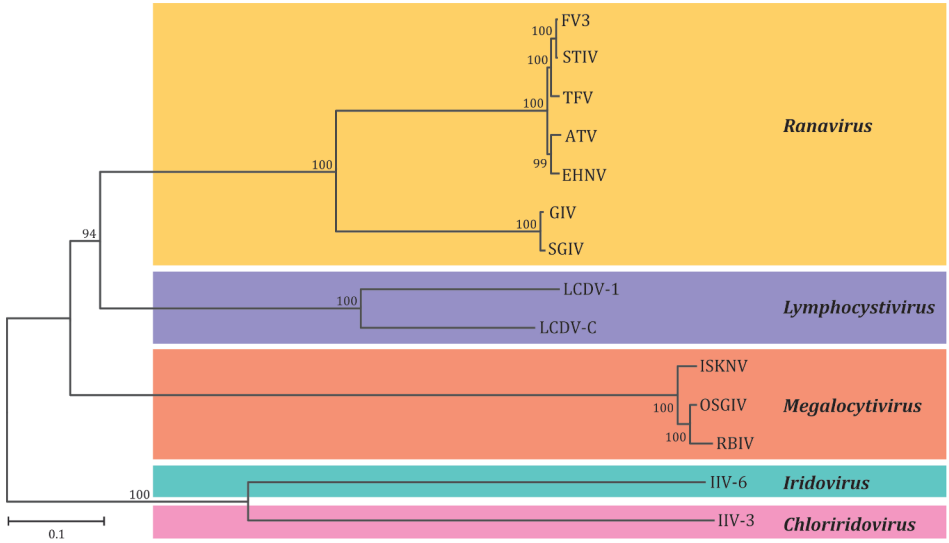

Figure 1. Phylogenetic relationships within the family *Iridoviridae* based on 26 core genes. The names of the other viruses are abbreviated as follows; CIV, *Chilo iridescent virus* (Jakob *et al.*, 2001); IIV-3, *Aedes taeniorhynchus iridescent virus* (Delhon *et al.*, 2006); ATV, *Ambystoma tigrinum stebbensi virus* (Jancovich *et al.*, 2003); TFV, *Tiger frog virus* (He *et al.*, 2002); FV3, *Frog virus 3* (Tan *et al.*, 2004); SGIV, *Singapore grouper iridovirus* (Song *et al.*, 2004); GIV, *Grouper iridovirus* (Tsai *et al.*, 2005); STIV, *Soft-shelled turtle iridovirus* (Huang *et al.*, 2009); LCDV-C, *Lymphocystis disease virus* - isolate China (Zhang et al., 2004); LCDV-1, *Lymphocystis disease virus* – 1 (Tidona & Darai, 1997); ISKNV, *Infectious spleen and kidney necrosis virus* (He *et al.*, 2001); RBIV, *Rock bream iridovirus* (Do *et al.*, 2004); OSGIV, *Orange-spotted grouper iridovirus* (Lü *et al.*, 2005); EHNV, *Epizootic hematopoietic necrosis virus* (Jancovich *et al.*, 2010). This figure was obtained from the 9th report of the International Committee on Taxonomy of Viruses (ICTV) with permission (King *et al.*, 2011).

A lipid bilayer covers the inner side of the capsid (Constantino *et al.*, 2001). Recent cryo-electron microscopy and three-dimensional image reconstruction studies on the capsid (naked virion) of CIV revealed a maximum diameter of 185 nm and showed fibrils rising out from the surface of the virion (Yan *et al.*, 2009). This study also showed, in addition to MCP, a group of less abundant proteins in the capsid structure. The capsid proteins form a complex, which contains a “finger” protein, a “zip” protein, a pentameric complex and an anchor protein. The molecular mass estimations for the finger and zip proteins, the anchor protein and the monomer of the pentameric complex were estimated to be 19.7, 11.9, 32.4 and 39.3 kDa, respectively. Showing that there are different types of minor capsid proteins associated with the capsomers outside the inner lipid membrane of CIV (Yan *et al.*, 2009).

The lipid composition of the internal viral membrane is the same whether the virus is propagated *in vivo* in larvae or *in vitro* in invertebrate cell cultures and is clearly different from that of the hosts (Balange-Orange & Devauchelle, 1982). Compositional analyses of CIV virions revealed a relatively high abundance of glycerophosphatidylinositol (PI), which indicates that CIV preferentially incorporates PI lipids into its internal membrane. Other invertebrate iridescent viruses analyzed by gas-liquid chromatography showed similar lipid compositions (Williams & Thompson, 1995).

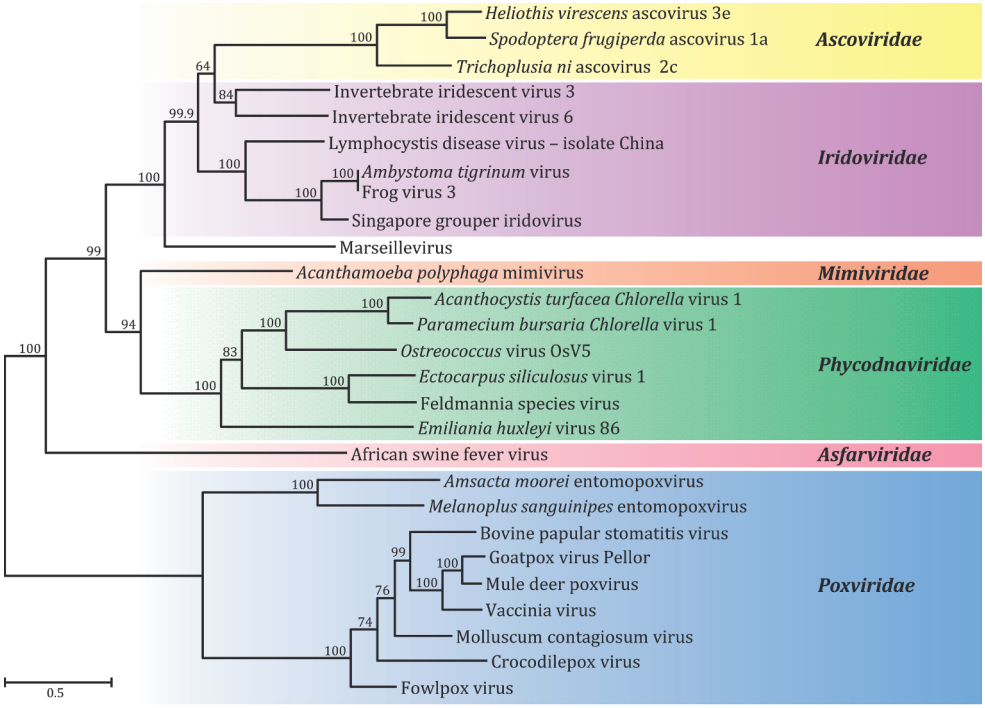

Figure 2. Phylogenetic relationships of iridoviruses with viruses in other NCLDV (This figure was obtained from the 9th report of ICTV with permission) (King *et al.*, 2011).

An in depth lipidomic study of *Singapore grouper iridovirus* (SGIV) also showed a high abundance of PI (Wu et al., 2010), suggesting that this might be a common feature of iridoviruses. The internal lipid membrane has been reported to be synthesized *de novo* in the cytoplasm of infected cells without any visible continuity with the cellular membrane (Marina et al., 2000, Stoltz, 1973). PI and other lipids are mainly synthesized in the endoplasmic reticulum (ER). The major structural lipids in eukaryotic membranes are glycerophospholipids. PI has a relatively higher abundance in the ER membrane than in other cellular compartments in mammalian and yeast cells (van Meer et al., 2008). Analysis of the lipid composition of the ER of iridovirus-infected cells would improve our understanding of how iridoviruses acquire the lipid components present in the internal membrane in virion structure.

Virion proteins

Different research groups have studied polypeptides present in the CIV particle. After solubilization of CIV particles with SDS-β-mercaptoethanol, 16 polypeptides were resolved with molecular masses ranging from 7 to 120 kDa, with a major polypeptide of 51 kDa. After solubilization with SDS-urea, 26 polypeptides were resolved with molecular masses ranging from 10 to 230 kDa (Barray & Devauchelle, 1979, Day & Mercer, 1964, Reyes *et al.*, 2004). How these proteins are related to the ORFs in the CIV genome is largely unknown.

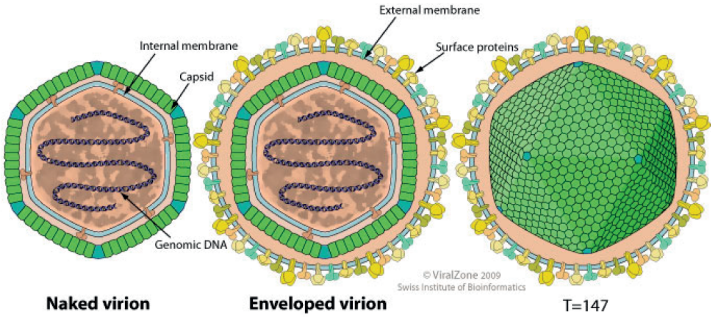

Figure 3. Graphical representation of the iridovirus structure (ViralZone, 2009).

Host range and pathology

The host range of CIV within the class Insecta has been investigated by intrahemocoelic or *per os* inoculation and was found to include more than 100 insect species belonging to six orders (Lepidoptera, Coleoptera, Diptera, Hymenoptera, Hemiptera and Orthoptera) (Hama, 1968, Henderson *et al.*, 2001, McLaughlin *et al.*, 1972, Mitsuhashi, 1967, Ohba, 1975, Ohba & Aizawa, 1979, Williams & Thompson, 1995). CIV can infect 13 species of mosquitoes *per os* (Williams & Thompson, 1995). In mosquitoes, CIV usually causes covert infections that reduce insect fitness (Marina *et al.*, 2003b). CIV also infects a number of non-insect arthropods (Ohba & Aizawa, 1979).

CIV replicates *in vitro* in a large number of insect cell lines derived from insects belonging to the different orders mainly Lepidoptera, Coleoptera, Hymenoptera and Hemiptera (Constantino *et al.*, 2001, Monnier & Devauchelle, 1976, Monnier & Devauchelle, 1980, Ohba, 1975), these including cells from the boll weevil *Anthonomus grandis* (D'Souza *et al.*, 1997), the root weevil *Diaprepes abbreviatus* (Hunter & Lapointe, 2003), and the hemipteran whitefly *Bemisia tabaci* (Funk *et al.*, 2001). CIV can even infect reptile cells (McIntosh & Kimura, 1974). In a recent study, the cell line AFKM-On-H, derived from hemocytes of the European corn borer *Ostrinia nubilalis* (Lepidoptera) did not productively support CIV infection (Belloncik *et al.*, 2007). However, CIV readily infects *Ostrinia nubilalis* larvae and replicates abundantly in different tissues and cells, including the fat body and hemocytes (Belloncik *et al.*, 1988). In addition to that, it was reported that disruption of the lipid bilayer in IIVs had a profound effect on their ability to infect cultured cells, but not to infect whole insects when the virus was injected into the haemocoel (Martinez *et al.*, 2003), suggesting that viruses may take different routes to establish an infection *in vivo* and *in vitro*.

In spite of its wide host range, CIV has attracted little attention as a potential biopesticide, because of the limited host mortality (Williams *et al.*, 2005) and very low prevalence of lethal infections, although epizootics have been reported occasionally (Hernandez *et al.*, 2000, Ricou, 1975). Non-lethal infections with CIV are common in insect populations (Ohba, 1975, Tonka & Weiser, 2000) and such infections may seriously reduce the reproductive capacity, body size and longevity of infected individuals (Marina *et al.*, 1999, Marina *et al.*, 2000, Marina *et al.*, 2003a, Marina *et al.*, 2003b). Little is known about the factors that determine the virulence of iridoviruses, but it is assumed that covert infections open the way to vertical transmission of the virus from parent to offspring.

CIV persistence and sensitivity to external factors

IIVs infect hosts that live in moist or aquatic habitats. Soil is believed to be an important environmental reservoir for IIVs. The effects of soil moisture and microorganisms on the persistence of CIV have been studied (Reyes *et al.*, 2004). The loss of infectivity of CIV was very rapid in dry soil. However, the soil moisture level did not further affect the rate of inactivation of virus in moist or wet soil. Soil sterilization prior to adding CIV significantly improved the persistence of CIV infectivity, both in damp and wet soil, indicating that other microorganisms affected the infectivity of CIV. A half-life of 4.9 days was recorded for CIV in non-sterile soil, 6.3 days in sterilized soil, and 12.9 days in the absence of soil (Reyes *et al.*, 2004). This study also concluded that persistence outside of the host in soil habitats is an important aspect of the ecology of IIVs.

Temperature also affects the rate of inactivation as IIVs are thermolabile and are inactivated within minutes at temperatures above 55°C (Day & Mercer, 1964, Stoltz, 1971). Aqueous suspensions of CIV showed a 10 fold reduction in titer after 50 days either at 4°C or at 25°C (Marina *et al.*, 2000). Ultraviolet radiation has been used to inactivate CIV in laboratory studies (Belloncik *et al.*, 1988) and exposure to solar UV light also resulted in a very rapid inactivation of CIV in water, with infectivity dropping by approximately nine logs after 24 hours exposure to sunlight (Williams *et al.*, 2005). The loss of activity of CIV due to UV light was also confirmed for aquatic habitats with water temperatures fluctuating between 24°C and 41°C, showing that direct sunlight causes a loss of infectivity of 99.99% in 36 hours (Hernandez *et al.*, 2005).

The sensitivity of CIV to a selection of organic solvents, detergents, enzymes and heat treatment was assayed in *Spodoptera frugiperda* (Sf9) cells and by injection into *Galleria mellonella* larvae. Sensitivity (defined as a reduction of at least 1 log in activity) was detected following treatment with the detergents and various solvents. No sensitivity was detected when CIV was exposed to Tween-80, lipases and proteinases. Viral activity was reduced by heating (Martinez *et al.*, 2003).

Genomic organization and codon usage

CIV virions contain a single, linear, double stranded DNA molecule of 212,482 bp (Jakob *et al.*, 2001). Iridoviruses are unique among animal viruses in that the viral DNA in the virus particle is linear with terminal redundancies, but that the genomic map is circular due to the circular permutation of the viral DNA (Delius *et al.*, 1984, Fischer *et al.*, 1990) meaning that, as a consequence of the DNA replication and packaging strategy, the direct terminal repeats vary per DNA molecule. For example, if we represent the viral genomic sequences with letters, the sequence analysis of linear genomes from individual virus particles for instance results in **ABCDE**.....UVWXYZ **ABCDE**, **CDEFG**.....WXYZAB **CDEFG**, **FGHIJ**....ZABCED **FGHIJ** (Chinchar *et al.*, 2009). The viral genomic DNA of CIV was found to be AT rich, with 71.37% A+T and 28.63% G+C (Jakob *et al.*, 2001). The original description of the CIV genome predicted 468 open reading frames (ORFs) for polypeptides ranging from 40 to 2432 amino acid residues. However, this value is likely to translate into functional genes since it includes both overlapping and non-overlapping ORFs. If only non-overlapping ORFs are considered, then CIV likely encodes 211 proteins instead of the predicted 468 (Eaton *et al.*, 2007). The CIV genome contains extensive

regions of short direct, inverted, and palindromic repetitive DNA sequences (Bilimoria, 2001, Jakob *et al.*, 2001). The function of these regions is unknown, but some they may have coding functions as well, as late transcripts were detected in a IIV-9 repeat region (Kelly & Tinsley, 1974). Usually, these regions are involved in DNA replication or function as enhancers of transcription. ORFs 261R, 396L and 443R (R and L refer to the direction of transcription of the ORF on the genome) represent large repetitive DNA elements in the CIV genome. IIVs (genera *Iridovirus* and *Chloriridovirus*) do not have a highly methylated genome in contrast to vertebrate infecting iridoviruses (genera *Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus*), which possess genomes methylated by a virus encoded DNA methyltransferase (Willis & Granoff, 1980) except for SGIV (genus *Ranavirus*), which lacks a DNA methyltransferase gene (Song *et al.*, 2004).

Conserved genes

The CIV genome contains 26 ORFs scattered over the genome that are conserved across viruses from all the genera of the family *Iridoviridae* (Eaton *et al.*, 2007). These, so called core genes, were used in the phylogeny presented in Fig. 1. These core genes encode proteins for crucial processes, including DNA replication, gene transcription, nucleocapsid assembly and virion architecture. The encoded proteins may also be involved in essential interactions with the host, for instance to enter host cells, abrogate the host metabolism and establish infection. The conserved genes that have been identified as essential players in viral transcription/DNA replication are 022L, 037L, 142R, 143R, 176R/343L, 184R, 282R, 349L, 355R, 369L, 376L, 428L, and 436L, and in protein processing and modifications 098R, 179R/439L and 380R (Nalçacıoğlu *et al.*, 2009). The other core genes encode MCP (ORF 274R), homologues of the FV3 immediate early infected cell protein ICP46-like protein (ORF 393L), ATPase (075L), myristylated membrane proteins (118L and 458R), a hypothetical protein of *Clostridium tetani* (337L), Erv1/Alr-like protein (307L), and proteins, for which we have no clue for their function yet (ORFs 067R, 295L, 287R, and 347L) (Nalçacıoğlu *et al.*, 2009).

Viral entry and replication strategy

The information that we have about the replication mechanism of iridoviruses comes mainly from studies on FV3 and this has become the model for all iridoviruses (Granoff, 1984). CIV and other iridoviruses are known as nucleocytoplasmic viruses. Their replication start in the nucleus and is completed in the cytoplasm. The first step in the infection is the binding of the virus particle to the host cell surface in a receptor-mediated way. Following binding to the host cell, the enveloped viral particles are internalized by receptor-mediated endocytosis, whereas intracellular virion particles enter the host cells by direct “fusion” between the virus shell and the cellular membranes (Braunwald *et al.*, 1985). The protein encoded by CIV ORF 096L is a good candidate for binding to the unknown cell surface receptor, since it has a fasciclin domain. Fasciclin has been shown to play cell adhesion functions in other systems (Clout *et al.*, 2003). After entry, the viral DNA is transported to the cell nucleus and early viral transcripts belonging to the immediate early (IE) and delayed early (DE) classes are synthesized in the nucleus using the input virion DNA as template (Williams *et al.*, 2005). These transcripts encode proteins needed for DNA replication and expression of late genes. This replication starts in the nucleus and results in DNA molecules up to twice the viral genome size. Late in infection,

the newly made DNA is transported into the cytoplasm and is transformed into larger concatemers, which may be up to ten-fold the genomic size (Willis *et al.*, 1984b). The late viral mRNA synthesis occurs after the onset of DNA replication and takes place in the cytoplasm of infected cells. It maybe catalyzed by a virus modified cellular polymerase or a novel, viral encoded RNA polymerase. The concatemeric form of the DNA is found within cytoplasmic viral assembly sites. Concatemers are split into fragments slightly larger than the length of the circular genome sequence and virion assembly takes place. Finally, virions exit the cell either by budding or cell lysis as described above (Fig. 4).

Transcriptional regulation

Previous studies on infected cell-specific polypeptides and on CIV transcripts provided evidence for a temporal gene expression cascade subdividing the CIV genes into three temporal classes: immediate-early (IE or α), delayed-early (DE, β), and late (L, γ) (Barray & Devauchelle, 1987, D'Costa *et al.*, 2001). The temporal expression of three classes of CIV genes during the course of infection suggests that both *cis*-acting DNA sequences and *trans*-acting regulatory factors interact at specific time points post-infection to initiate transcription of the appropriate mRNAs.

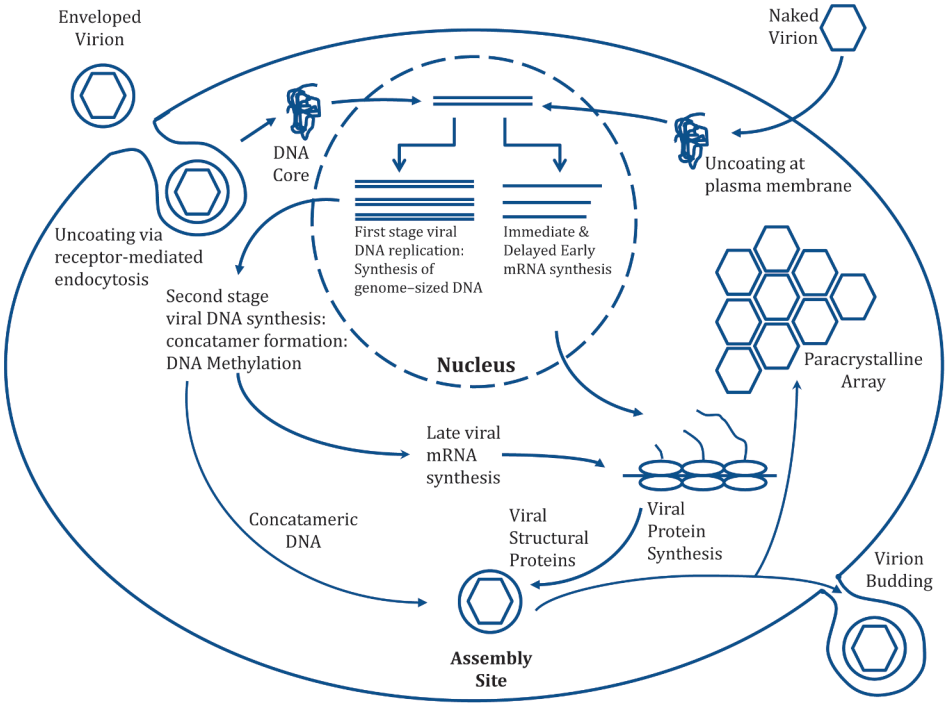

Figure 4. Schematic representation of iridoviral replication (This figure was obtained from the 9th report of ICTV with permission) (Chinchar *et al.*, 2009, King *et al.*, 2011).

The IE class was shown to give 38 transcripts, appearing around 0.5 h p.i. and being synthesized in the absence of *de novo* protein synthesis. The delayed-early class was transcribed in 34 mRNAs that appeared around 3 h p.i. and these transcripts were made in the presence of DNA synthesis inhibitors, but not in the presence of protein synthesis inhibitors, meaning these DE genes require at least one earlier gene product for their expression. The late class with 65 discovered transcripts that appeared around 6 h p.i. contained the genes transcribed only in the absence of both DNA and protein synthesis inhibitors (D'Costa *et al.*, 2001). The precise coding function of all these transcripts, however, became not clear from these studies and the total did not count up to the total number of non-overlapping genes.

Iridoviral naked DNA is not infectious. For example, purified FV3 DNA is not able to start infection unless complemented with UV-inactivated (not heat-) virus particles. Only then successful transcription of IE viral mRNA occurred and a productive virus infection followed, suggesting that initiation of viral transcription for instance requires one or more virion-associated protein (Willis *et al.*, 1990, Willis & Granoff, 1985). This phenomenon is not unique to FV3, since non-genetic reactivation has also been observed with CIV (Cerutti *et al.*, 1989).

Promoter elements and transcription initiation sites

Very little is known about the promoter elements of iridovirus genes (Eaton *et al.*, 2007). Promoter studies have been performed with *Frog virus 3* (FV3) and *Bohle iridovirus* (BIV) in genus *Ranavirus* for two IE genes of FV3 (ICR-169 and ICR-489) (Beckman *et al.*, 1988, Willis, 1987), and two early genes ICP-18 and ICP-46 putative promoter regions and the *mcp* gene of BIV (Pallister *et al.*, 2005). The transcription profile and promoter structures of the DNA polymerase (*DNApol*; 037L) and major capsid protein genes (*mcp*; 274L) of CIV have been analyzed in more detail (Nalcacioglu *et al.*, 2003). Infection of *Bombyx mori* SPC-BM-36 cells in the presence of inhibitors of DNA or protein synthesis showed that *DNApol* is an immediate-early gene and confirmed that *mcp* is a late gene.

Transcription of *DNApol* initiated 35 nt upstream of the translational start site and that of *mcp* 14 nt. The helicase gene was also shown to be expressed as an IE gene (Nalcacioglu *et al.*, 2003). CIV *DNApol*, *helicase*, and *mcp* transcripts most likely do not have poly A tails, because they could not be amplified when an oligo-dT primer was used in the reverse transcriptase reaction (Nalcacioglu *et al.*, 2003) and a clear polyadenylation signal downstream of CIV ORFs is not found. This is also the case for FV3 transcripts (Willis *et al.*, 1984a, Willis & Granoff, 1976).

The CIV promoters tested so far need viral proteins to be active. Reporter assays showed that without virion proteins the promoters are inactive upon transfection of reporter plasmid constructs carrying these promoters, indicating that at least one viral protein is needed to activate viral transcription (Nalcacioglu *et al.*, 2007). Two important questions remain to be answered: Which protein(s) from the virion are responsible for the promoter activities, and how do CIV gene transcripts obtain their 3' ends without using polyadenylation signals?

Inhibition of host macromolecular synthesis and induction of apoptosis

Studies on interactions between CIV and cells have shown that inoculation of vertebrate or invertebrate cells with CIV rapidly leads to massive formation of syncytia (Cerutti & Devauchelle, 1979). This suggests that fusogenic proteins are expressed very early after infection. In addition, CIV rapidly inhibits cellular RNA, DNA and protein synthesis in permissive and non-permissive vertebrate and invertebrate cell lines (Cerutti & Devauchelle, 1980). The integrity of the viral genome is not required for inhibiting host gene expression and host DNA replication, since viral proteins solubilized from CIV by freezing and treatment with EDTA exhibited inhibitory properties similar to those of intact virions (Cerutti & Devauchelle, 1980). This inhibition of host genes and cellular DNA replication is similar for FV3, since this virus also depresses cellular RNA and protein synthesis, and as a consequence DNA replication under conditions non-permissive for virus replication (Braunwald *et al.*, 1972, Guir *et al.*, 1971, Williams, 1995). In another study, the CIV internal membrane proteins were solubilized and then reconstituted, which led to the formation of vesicles behaving like viral particles with respect to cell fusion and these exhibited extensive inhibition of synthesis of macromolecules in host cells (Cerutti & Devauchelle, 1982). The mechanism of this fusogenic response is not known, but it is clearly differ from cell fusion that requires *de novo* synthesis of envelope fusion proteins (personal communication).

CIV virion protein extracts induced mortality in neonate boll weevil larvae (Bilimoria, 2001) and apoptosis in boll weevil and *C. fumiferana* cells (Bilimoria & Sohi, 1977, Paul *et al.*, 2007, Stiles *et al.*, 1992). It was also shown that apoptosis inhibition occurred under conditions permitting early viral gene expression (Chitnis *et al.*, 2008).

Based on homology searches the candidate anti-apoptotic genes in CIV are ORFs 157L, 193R, 332L, and 284R. ORF 193R is the most promising candidate as it contains both structural elements characteristic for inhibitor of apoptosis (*iap)* genes: the Baculovirus IAP Repeat (BIR) domain and a Really Interesting New Gene (RING) finger domain. These domains are present in all active IAP proteins (Clem, 2007), except for the African swine fever virus IAP, which contains a zinc finger instead of a RING finger (Nogal *et al.*, 2001).

Scope of the thesis

Iridoviruses cause major diseases in vertebrates and invertebrates, but little is known about their structure, replication and gene expression. Fundamental knowledge of iridoviruses is important in view of their possible use as biological control agents for pest insect, thereby replacing chemical insecticides. CIV is the prototype of the genus *Iridovirus* and the most promising candidate to control weevils, for instance in tea and hazelnut cultures in Turkey. In the case of vertebrate iridoviruses, fundamental information on the structure and replication of iridoviruses may lead to novel intervention strategies to mitigate or prevent the disease in fish aquaculture. Iridoviruses have not only been found in insect of agricultural importance, but also infect insects that transmit plant pathogens and/or parasites of medical importance, such as mosquitoes, whiteflies and grasshoppers (Fukuda, 1971, Hunter *et al.*, 2003, Hunter *et al.*, 2001, Kleespies *et al.*, 1999), but their potential as biocontrol agent has been underexplored.

Although data have been collected over the past years about the iridovirus infection cycle, many fundamental questions remain to be answered, for instance concerning the structure and scaffolding of the virus particles and the nature of virus-host interactions, including the initial steps in virus infection such as cell entry mechanisms and the onset of transcription of viral genes. Viral structural proteins are likely to play crucial roles in these processes and although the complete genome sequence of CIV is known for several years, it is not known, which of the encoded proteins form the virus particle. Virion proteins may not only be important to build the structure of the virions, but may also play crucial roles in the initial stages of infection. Such information is for instance important to determine which proteins are the crucial players in spreading the virus infection from cell-to-cell inside the insect body, how the virus particles interact with the cell and which structural components of the virions are needed to start the transcription cascade. The major aim of this thesis is to reveal the protein composition and structure of the CIV particles and to understand underlying mechanisms of the infection process. Therefore, I will first focus on the identification of the CIV virion components by using a proteomic approach (**Chapter 2**), in which a combination of protein separation using one dimensional SDS-polyacrylamide gel electrophoresis, liquid chromatography and tandem mass spectrometry (LC-MS/MS) is applied. The available CIV computational protein database will facilitate accurate protein predictions from the output of the protein analysis.This approach will provide a fast and highly sensitive method for the identification of proteins through the sequences of the corresponding genes and serve as a starting point to decipher the functions of these virion proteins. In **Chapter 3,** the focus is on the transcriptional analysis of the virion protein genes identified in Chapter 2. From this study it may be possible to derive information on their promoters and the coordination of virion assembly. In **Chapter 4,** the presence of these virion proteins in infected cells during the course of infection will be studied.

In **Chapter 5,** the promoter region of the CIV DNA polymerase gene is analyzed in detail. Previous studies had already classified *DNApol* as a delayed early gene. Here, a series of precise deletion mutants is used to determine the core promoter sequence. The importance of this sequence is further studied by introducing point mutations. It will also be investigated which viral proteins are interacting with this domain. There are no obvious conventional transcription polyadenylation signals in CIV messenger RNA such as AAUAAA or AUUAAA. Therefore, a new method is needed to analyze these messengers.

In many viral infections, the cell reacts by the onset of apoptosis in the early stages of infection in order to minimize viral replication and to prevent cell-to-cell transmission of progeny virus. This is a major defense mechanism in insects in response to viral infections. In **Chapter 6** the aim is to investigate whether ORF 193R, the most promising *iap* gene candidate, encodes a functional anti-apoptotic protein that prevents virus-induced apoptosis early in infection.

In **Chapter 7,** the general discussion, I will evaluate the importance of this work for understanding the structure and scaffolding of the CIV virus particles and the nature of virus-host interactions, and will describe perspectives for future research.

Chapter 2

Proteomic analysis of *Chilo iridescent virus*

Abstract

The genome of *Chilo iridescent virus* (CIV) contains 468 open reading frames (ORFs), half of which are non-overlapping with other ORFs in the genome. Which of the predicted genes encode proteins present in the virus particle is largely unknown. In this first proteomics study of an invertebrate iridovirus, 46 CIV-encoded proteins were identified in CIV virions based on the presence of 2 or more distinct peptides; an additional 8 proteins were found based on a single peptide. Thirty-six of the 54 identified proteins have homologs in another invertebrate and/or in one or more vertebrate iridoviruses. The genes for five of the identified proteins, 22L (putative helicase), 118L, 142R (putative RNaseIII), 274L (major capsid protein) and 295L, are shared by all iridoviruses for which the complete nucleotide sequence is known. These five genes may therefore be considered as iridovirus core genes. Three proteins have homologs only in ascoviruses. The remaining 15 proteins in the proteome are so far unique to CIV. In addition to broadening our insight in the structure and assembly of CIV virions, this knowledge is also pivotal to unravel the initial steps in the infection process and the role of virion proteins therein.[2]

[2] This chapter was published in Virology 405: 253-258 (2010).

Introduction

Chilo iridescent virus (CIV), also known as *Invertebrate iridescent virus 6*, belongs to the family *Iridoviridae* and is the type species of the genus *Iridovirus* (Chinchar *et al.*, 2005, Fauquet *et al.*, 2005, Williams, 1996, Willis, 1990). Iridoviruses are large, cytoplasmic, icosahedral viruses with a linear double-stranded DNA genome, which is both circularly permuted and terminally redundant (Darai *et al.*, 1983, Goorha, 1982, Goorha & Murti, 1982). The CIV virion consists of an unusual three layer structure containing an outer proteinaceous capsid, an intermediate lipid membrane, and a core DNA-protein complex containing the 212.482 bp genome (Chinchar *et al.*, 2005, Jakob *et al.*, 2001, Williams, 1996). Up to May 2011, fifteen complete sequences of iridovirus genomes have been published, including CIV (Eaton *et al.*, 2010). The availability of the CIV sequence facilitates the identification and functional analysis of the proteome of CIV virions. Replication of CIV occurs in the nucleus of infected cells and the assembly takes place in the cytoplasm (Goorha, 1982, Goorha & Murti, 1982).

Many questions remain to be answered concerning the structure and scaffolding of the virus particles, the nature of virus-host interactions and the initial steps in virus infection, including the mechanism behind the onset of transcription of CIV genes. Viral structural proteins are likely to play crucial roles in these processes. Initiation of viral transcription for instance requires one or more virion proteins, since CIV DNA alone is not infectious, similar to what has been shown for the vertebrate iridovirus *Frog virus 3* (Willis & Granoff, 1985). In previous studies, efforts have been made to characterize the polypeptides in CIV virions by one- or two-dimensional SDS-PAGE. The presence of 21-28 polypeptides was revealed by one-dimensional SDS-PAGE, while 35 polypeptides were observed in two-dimensional SDS-PAGE (Barray & Devauchelle, 1979, Barray & Devauchelle, 1985, Cerutti & Devauchelle, 1985, Kelly & Tinsley, 1972, Orange & Devauchelle, 1987). The size of these polypeptides ranged from 11 to 300 kDa. However, most of these proteins were not further characterized and it is unknown, except for the major capsid protein MCP, which CIV genes encode them.

In the current study, we identified the CIV virion proteins by a proteomic approach, based on a combination of one-dimensional SDS-PAGE and LC-MS/MS. The data obtained were analyzed by searches against a CIV ORF database. This provided a fast and highly sensitive method for the identification of genes through the sequences of the encoded proteins (Pandey & Mann, 2000).

Experimental procedures

Preparation of virus particles and gel electrophoresis

CIV was propagated in larvae of the wax moth, *Galleria mellonella*, isolated as described (Marina *et al.*, 1999) and further purified by 25-65 % sucrose density gradient centrifugation. The purified CIV particles were checked for quality by transmission electron microscopy and quantified by UV spectroscopy. The purified particles were denatured and the proteins were separated by 12% one-dimensional SDS-PAGE. The gel was stained with colloidal blue and the gel lane containing the virion proteins was cut into six segments based on a comparison with molecular markers.

Each gel piece was sliced and dehydrated with 100% acetonitrile (ACN). After vacuum drying, the gel segments were incubated in 10 mM dithiothreitol (DTT) in 50 mM ammonium bicarbonate (ABC buffer) at 57°C for 1 h and subsequently in 55 mM iodoacetamide (IAA) in ABC buffer at room temperature for 1 h. After a final wash step with ABC buffer the gel material was dried.

Trypsin digestion and LC-MS/MS

In-gel protein digestions were performed using sequencing grade modified porcine trypsin (Promega, Madison, WI) in ABC buffer at 37°C for 15 h, after which the digests were centrifuged at 6000 g. The supernatants were collected, and the remaining gel pieces were extracted with 5% triflouroacetic acid (TFA) and then with 15% ACN/1% TFA. The extracts were combined with the supernatants of the original digests, vacuum-dried, and the dried material was dissolved in 20 µl 0.1% formic acid in water. The peptides resulting from this digestion were analyzed by LC-MS/MS. To this aim, 18 µl of the samples were concentrated over a 0.10 * 32 mm Prontosil 300-5-C18H (Bischoff, Germany) pre-concentration column at a flow of 6 µl/min for 5 min. The peptides were eluted from the pre-concentration column and loaded onto a 0.10 * 200 mm Prontosil 300-3-C18H analytical column with a gradient of 10 to 35% ACN in 0.1% formic acid at a flow of 0.5 µl/min for 50 min. After that, the percentage of ACN was increased to 80% (with 0.1% formic acid) in 3 min as a column-cleaning step. Between the pre-concentration and analytical column, an electrospray potential of 3.5 kV was applied directly to the eluent via a solid 0.5 mm platina electrode fitted into a P875 Upchurch microT. Full scan positive mode Fourier transform mass spectra (FTMS) were measured between mass-to-charge ratios of 380 and 1400 with a LTQ-Orbitrap spectrometer (Thermo electron, San Jose, CA, USA).

MS/MS scans of the four most abundant doubly and triply charged peaks in the FTMS scan were recorded in a data dependent mode in the linear trap (MS/MS threshold = 10.000). All MS/MS spectra obtained with each run were analyzed with Bioworks 3.1.1 software (Thermo Fisher Scientific, Inc.). A maximum of single differential modification was allowed per peptide was set for oxidation of methionines and de-amidation of asparagine and glutamine residues. Carboxamidomethylation of cysteines was set as a fixed modification. Trypsin specificity was set to fully enzymatic and a maximum of three missed cleavages with monoisotopic precursor and fragment ions. The mass tolerance for peptide precursor ions was set to 10 parts per million (10 ppm = 0.01 Da at m/z 1000 Da) and for MS-MS fragment ions to 0.5 Da.

An Invertebrate iridescent virus 6 protein database was used for the analysis (AF303741; created July 31, 2001 and downloaded from www.ncbi.nlm.nih.gov) after adding a list of commonly observed contaminants like: BSA (P02769, bovine serum albumin precursor), trypsin (P00760, bovine), trypsin (P00761, porcin), keratin K22E (P35908, human), keratin K1C9 (P35527, human), keratin K2C1 (P04264, human) and keratin K1CI (P35527, human). A decoy database was created by adding the reversed sequences using the program Sequence Reverser from the MaxQuant package (Cox & Mann, 2008) resulting in a total of 1058 proteins in the database. To identify the proteins in the CIV virions, the MS-MS peptide spectra obtained from the LC-MS/MS were searched against the CIV ORF database using Bioworks 3.3.1 (Table 1).

The peptide identifications obtained were filtered in Bioworks with the following filter criteria: [3]ΔCn > 0.08, [4]Xcorr > 1.5 for charge state 2+, Xcorr > 3.3 for charge state 3+ and Xcorr > 3.5 for charge state 4+ (Peng *et al.*, 2003). Only those protein`s which have the Bioworks Score factor (Sf) higher than 0.6 were considered.

Results

To identify the virion proteins of CIV, the proteins of purified virion particles were separated by one-dimensional SDS-PAGE. Staining of the gel with colloidal blue revealed at least 21 proteins ranging from 10 to 250 kDa (Fig. 1) much in line to what has been found previously (Barray & Devauchelle, 1979, Barray & Devauchelle, 1985, Cerutti & Devauchelle, 1985, Kelly & Tinsley, 1972, Orange & Devauchelle, 1987). The gel lane was divided into 6 slices containing proteins with a molecular mass lower than 26 kDa, ranging from 26-34 kDa, 34-43 kDa, 43-55 kDa or 55-95 kDa and higher than 95 kDa, respectively. The proteins were digested with trypsin and analyzed by LC-MS/MS. A decoy database strategy (Elias & Gygi, 2007) was used which, after applying the appropriate filters, resulted in 89 protein hits: 54 CIV proteins, 34 contaminants and 1 decoy hit giving a false discovery rate of 1.1%. Out of the 54 CIV proteins, 46 of the more abundant proteins were identified with two or more peptides (Table 1), while relatively small proteins like ORFs 342R, 227L or 104L as well as some less abundant proteins could be identified with one peptide only (Table 2). The proteins, for which only one peptide was observed, were manually verified to correlate well to the theoretical b+y ion spectrum and to be unique for that particular protein.

In Figure 1, the proteins identified are indicated. 1. A genomic map of CIV ORFs that encode polypeptides represented in the proteome of CIV particles is shown in Fig. 2. For individual CIV virion proteins, 2.7 to 70% of the amino acid sequence was covered with peptides retrieved from the analysis. The major capsid protein (MCP) encoded by ORF 274L is one of the most abundant CIV proteins (Barray & Devauchelle, 1979, Barray & Devauchelle, 1985, Cerutti & Devauchelle, 1985, Kelly & Tinsley, 1972, Orange & Devauchelle, 1987) and this is clearly reflected by its relative abundance in the current analysis compared to all other CIV proteins (Table 1).

Functional domains alluding to possible functions were found in fifteen other identified virion proteins, including three putative serine/threonine kinases (ORFs 209R, 380R and 439R), one dual specificity phosphatase (123R), a protein with homology to the N- terminal domain of viral DNA polymerases (232R), carboxy-terminal domain (CTD) phosphatase (355R), nucleoside triphosphatase (NTP I) (22L), fasciclin (96L), ribonuclease III (142R), tyrosine protein kinase (179R), cathepsin (361L), DNA binding protein (401R), protein disulfide isomerase (453L), lysosome associate membrane glycoprotein (061R), and homolog of a ranavirus envelop protein (118L). For the 38 remaining proteins in the virion, we have no clear idea about their specific function at this moment (Table 1 and 2). Some of these show partial homology to viral proteins of poxvirus, coronavirus or baculovirus origin.

[3] ΔCn; SEQUEST uses the difference the first- and second-ranked sequences
[4] Xcorr; SEQUEST computes a cross correlation for evaluation of the quality of peptide spectra matches.

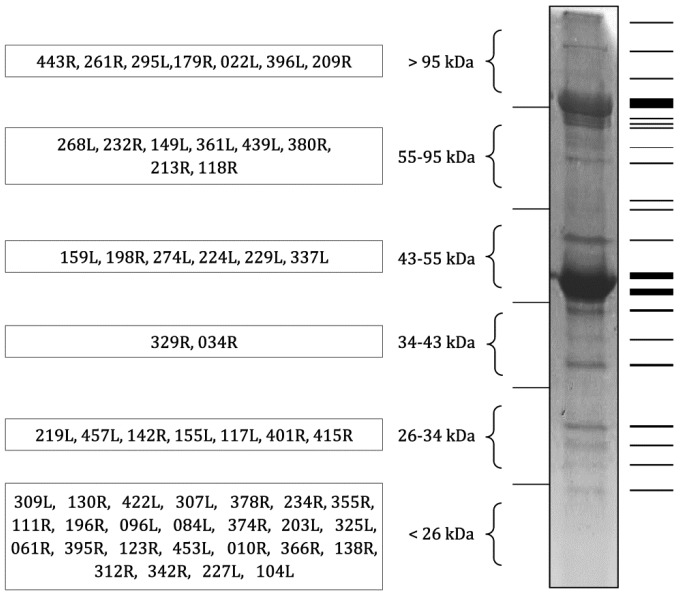

Figure 1. SDS-PAGE profile and LC-MS/MS identification results of purified CIV virion proteins. CIV proteins were separated by 12% one-dimensional SDS-PAGE and stained with colloidal blue. The SDS-PAGE gel was divided into 6 slices, which, based on comparison to a molecular marker, ranged from higher than 95 kDa, 55-95 kDa, 43-55 kDa, 34-43 kDa, 26-34 kDa to lower than 26 kDa. Proteins were in-gel-digested with trypsin, extracted and subjected to LC-MS/MS. The column on the right schematically indicates the relative abundance of the proteins as visualized by SDS-PAGE. The boxes on the left give the ORF numbers of the identified proteins in a particular gel slice in order of the predicted mass (see Table 1 and 2). Underlined numbers represent single peptide hits. Indications R and L point towards the direction of transcription from the CIV genome (see Fig. 2).

Recent cryoelectron microscopy studies on the capsid of CIV revealed, in addition to MCP, a group of relatively less abundant capsid proteins (Yan *et al.*, 2009). These proteins form a complex, which contains a 'finger' protein, a 'zip' protein, a pentameric complex and an anchor protein. The molecular mass estimations for the finger and zip proteins, the anchor protein and the monomer of the pentameric complex were estimated to be 19.7, 11.9, 32.4 and 39.3 kDa, respectively. For the finger protein, the standard deviation was 1.5 kDa, giving a size range of 18.2-21.2 kDa (Yan *et al.*, 2009). Based on this range, seven candidate genes for the finger protein were found in the CIV proteome; ORFs 234R, 111R, 096L, 374R, 325L, 203L, and 084L from large to small (Table 1). The zip protein with an expected size range of 10.5 to 13.3 kDa (1.4 kDa standard deviation) may correspond to three candidate ORFs represented in the proteome: 010R, 138R and 312R. The monomer of the pentameric complex estimated at 39.3 kDa corresponds most closely in size to ORFs 329R and 219L. Anchor protein candidate genes in the CIV proteome could be 457L or 142R, with sizes close to 32.4 kDa (Table 1).

Table 1. Structural proteins of CIV identified by LC-MS/MS with 2 or more distinct peptides.*

ORF	Accession No	Mol. Mass (kDa)	Protein coverage (% by amino acids)	Peptide hits on first rank	Relative abundance (% peak area)[5]	Predicted domains/function
443R	AAK82303	237.22	8.10	15	0.30	
295L	AAK82156	156.42	24.70	43	0.22	Bipartite nuclear localization signal
179R	AAB94478	137.93	14.70	24	0.06	Putative lipopolysaccharide-modifying enzyme, tyrosine protein kinase
022L	AAD48148	135.34	32.20	34	0.20	Putative nucleoside triphosphatase I, DEXDc; DEAD-like helicase superfamily
261R	AAK82122	129.06	2.70	30	2.20	Potential repetitive protein
209R	AAK82071	118.34	39.20	52	0.69	Serine/threonine protein kinase
396L	AAK82256	111.28	21.90	29	0.16	Potential repetitive protein
268L	AAK82129	83.22	46.10	74	2.06	
149L	AAB94464	76.41	36.60	72	0.91	
232R	AAK82093	75.56	49.00	75	1.63	DNA polymerase N-terminal domain
439L	AAK82299	63.45	12.10	8	0.02	Protein kinase domain
361L	AAK82221	60.58	50.70	55	1.06	Cathepsin B
380R	AAK82240	59.91	54.50	73	2.05	Serine or threonine-specific kinase
213R	AAK82075	58.42	29.70	22	0.16	Putative peptidoglycan bound protein
118L	AAB94444	55.29	55.10	65	1.77	Putative envelop protein
198R	AAK82060	52.15	42.60	26	0.32	
274L	AAK82135	51.29	63.20	157	17.97	Major structural protein
229L	AAK82090	50.64	22.30	15	0.25	
337L	AAK82199	46.13	27.20	25	0.21	Poxvirus protein of unknown function
159L	AAB94468	45.76	34.90	58	3.98	
329R	AAK82190	42.74	28.80	16	0.29	
219L	AAK82081	34.64	19.00	5	0.01	
142R	AAB94459	33.64	33.60	16	0.25	dsRNA-specific ribonuclease
457L	AAK82317	33.13	25.90	47	2.97	
155L	AAB94465	29.81	39.20	23	0.40	
401R	AAK82261	28.23	25.50	11	0.04	HMG-box superfamily of DNA-binding proteins
117L	AAB94443	27.45	29.70	43	0.93	
415R	AAK82275	26.66	63.20	70	1.34	
309L	AAK82170	24.83	70.00	12	0.10	
422L	AAK82282	22.73	49.50	19	0.20	*Cydia pomonella* granulovirus ORF34
378R	AAK82238	22.21	47.70	12	0.10	2-cysteine adaptor domain
355R	AAK82216	22.01	52.70	10	0.02	Catalytic domain of ctd-like phosphatases
234R	AAK82095	21.09	62.70	63	3.07	
111R	AAB94438	20.01	35.40	9	0.05	
096L	AAB94430	19.69	33.30	11	0.05	Fasciclin domain
374R	AAK82234	19.12	22.40	3	0.00	Bat coronavirus spike protein
325L	AAK82186	18.91	24.50	5	0.08	
203L	AAK82065	18.53	18.80	7	0.02	
084L	AAB94426	18.40	25.50	15	0.04	
061R	AAB94416	17.91	31.60	17	0.01	Lysosome associate membrane glycoproteins
123R	AAB94448	16.38	7.70	3	0.00	Dual specificity phosphatases
453L	AAK82313	15.91	26.10	12	0.05	Protein disulfide isomerase
034R[6]	AAK81969	14.63	16.40	5	0.03	
366R	AAK82226	13.66	17.50	2	0.01	
138R	AAB94455	13.03	16.70	7	0.02	
312R	AAK82173	10.60	20.70	3	0.01	

*The ORFs are ordered by the mass of the encoded proteins (column 3).

[5] The relative abundance was calculated by Bioworks as % peak area over all peaks (including contaminants observed) shown after applying the following filter settings: ΔCn > 0.08, Xcorr > 1.5 for charge state 2+, Xcorr > 3.3 for charge state 3+ and Xcorr > 3.5 for charge state 4+, Sf > 0.6

[6] This protein was identified in the 34-43 kDa gel piece.

Table 2. Structural proteins of CIV identified by LC-MS/MS with single peptide. The ORFs are ordered by the mass of the encoded proteins (column 3).

ORF	NCBI Accession No	Molecular mass (kDa)	Peptide sequence	Protein coverage (% by amino acids)	MH+	Δ m/z (ppm)	z	Xcorr
317L	AAK82178	43.95	IVNLIPQGQFQAK	3.11	1455.832	-0.30	2	1.77
130R	AAB94451	23.18	ICFSEQPLLDDFSNK	7.46	1812.847	1.04	2	2.86
307L	AAK82168	22.86	LKPLGYLNSLQ	5.58	1245.720	0.33	2	1.81
395R	AAK82255	17.28	YAINNENQYR	6.62	1284.597	-0.72	2	2.54
010R	AAK81948	12.84	TGSMVCSSTR	8.33	1085.471	3.19	2	2.34
342R	AAK82203	9.33	IQAQNYATMGIYNQGSQIR*	21.59	2156.055	2.74	2	3.73
227L	AAK82088	7.72	TFAYEVPIR*	14.30	1095.583	1.49	2	2.61
104L	AAB94434	7.05	RVACSPR*	12.30	845.441	2.01	2	2.78

*The same peptide was measured multiple times in different gel slices

Discussion

The CIV proteome revealed 54 proteins. The genes encoding these virion proteins are scattered over the genome (Fig. 2). It is not known which of the identified proteins are engaged in the scaffolding and assembly of CIV virions, and which are not essential for building the virion structure, but may be important for other processes, such as the initial stages of the infection and the regulation of gene expression. It is possible that one of these additional proteins is involved in chaperoning the viral DNA into the nucleus to initiate DNA replication (Willis & Granoff, 1985). To get a better clue about their importance, the conservation of the CIV virion protein genes in the complete genomes of members of the family *Iridoviridae* as well as *Ascoviridae* was assessed. The latter family was included since a common ancestry between iridoviruses and ascoviruses has been inferred from phylogenetic analysis (Stasiak *et al.*, 2000).

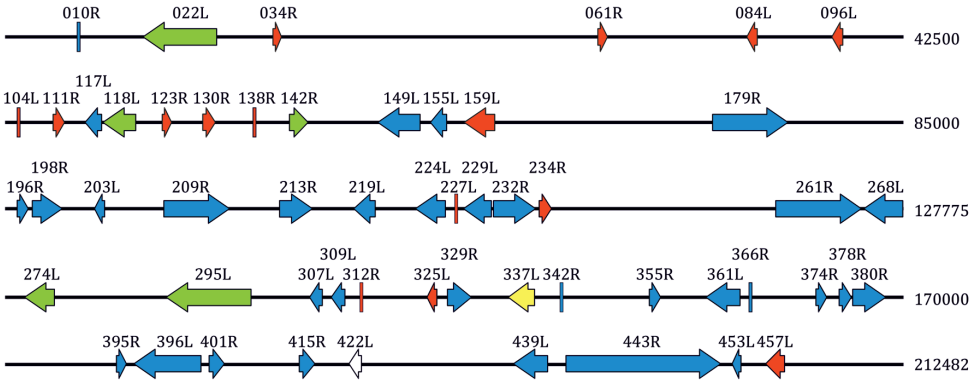

Figure 2. Linearized genomic representations of the 54 CIV structural protein ORFs determined by LC-MS/MS. Arrows indicate the positions and the direction of gene transcription (R or L). Red arrows are ORFs unique to CIV and green arrows represent ORFs present in all sequenced iridovirus genomes. The yellow and the white ORFs represent an entomopox and a baculovirus homolog's, respectively. The remaining ORFs are indicated in blue. Genomic positions are indicated on the right in base pair number.

Of the 54 ORFs encoding CIV virion proteins identified in the current study, thirty-four have homologs in *Invertebrate iridovirus 3* (IIV-3), which belongs to the genus *Chloriridovirus* (Table 3, column 2) (Chen *et al.*, 2008, Song *et al.*, 2004). Fifteen of the 34 ORFs with homologues in IIV-3 also have homologues in one or more vertebrate iridoviruses.The CIV proteome shares five ORFs with all iridoviruses: 022L, 118L, 142L, 274L (MCP) and 295L, and these may be considered to belong to the iridovirus core genes. The *Rana grylio virus* (RGV) ORF 53R, which is a homolog of the putative core gene 118L, has been shown to encode an iridovirus envelop protein (Zhao *et al.*, 2008). The CIV proteome shares 13 viral protein homologs with *Singapore grouper iridovirus* (SGIV) virion proteins identified by two independent mass spectrometric approaches (Chen *et al.*, 2008, Song *et al.*, 2004).

Previous phylogenetic studies on ascoviruses were based on comparative analyses of the capsid protein, DNA polymerase, thymidine kinase, and ATPase III led to the hypothesis that ascoviruses may have evolved from invertebrate iridoviruses (Stasiak *et al.*, 2003). The proteomic analysis of CIV performed here showed that 16 ORFs encoding CIV virion proteins have homologs in one or more ascoviruses (Asgari *et al.*, 2007, Bideshi *et al.*, 2006, Bigot *et al.*, 2008, Stasiak *et al.*, 2000, Wang *et al.*, 2006). Three of the identified CIV ORFs only have ascovirus homologs. Nine CIV structural proteins have homologs in *Heliothis virescens* ascovirus 3e (HvAV3e), thirteen have homologs in *Trichoplusia ni* ascovirus 2c (TnAV2c), eleven in *Spodoptera frugiperda* ascovirus 1a (SfAV1a) and six in *Diadromus pulchellus* ascovirus 4a (DpAV4a). The gene products of six of the eleven SfAV1a homologs were also found in the proteome of SfAV1a virions (Tan *et al.*, 2009). Only one of the determined CIV ORFs (422L) has a homolog in baculoviruses (*Cydia pomonella* granulovirus ORF34; genus *Betabaculovirus*), and one ORF (337L) has homology to an entomopoxvirus gene (Table 1, Fig. 2). These results underscore the evolutionary distance of iridoviruses from, both baculoviruses and entomopoxviruses and the closely related ascoviruses.

Although the morphology of the virions of members of the family *Ascoviridae* differs considerably from that of viruses of the family *Iridoviridae*, evidence is mounting that the ascoviruses and iridoviruses shared a common ancestor. Phylogenetic analyses based on proteins found in most enveloped dsDNA viruses provide strong evidence that ascoviruses evolved from iridoviruses, despite the marked differences in the characteristics of the virions belonging to these two families and differences in their cytopathology (Bigot *et al.*, 2008). The conservation of structural proteins between CIV and ascoviruses further supports the hypothesis of common ancestry.

In conclusion, this is the first detailed study towards the determination of the virion proteins of an invertebrate iridovirus. This study will contribute to a better understanding of the molecular mechanisms underlying CIV virion assembly, CIV entry into cells, the initial steps of early iridovirus gene expression and the cell to cell movement of this virus.

Table 3. CIV virion proteins identified by MS with homologs in other iridoviruses and/or ascoviruses*

Invertebrate		Vertebrate											
Irido -virus	Chloriridо -virus	Ranavirus						Lymphocystivirus		Megalocytivirus			Ascoviridae‡
CIV	IIV-3	ATV	TFV	FV3	SGIV	GIV	STIV	LCDV-C	LCDV-1	ISKNV	RBIV	OSGIV	
443R	91L												
295L	**16R**	**72R**	**45R**	**41R**	**57L**	**29L**	**45R**	**234R**	**92R**	**76L**	**72L**	**75L**	**[a]144R, [b]43R, [c]84L**
179R	35R	60R	29R	27R	78L	44R	31R	172R	110R				[a]129L, [b]58R, [c]90R
022L	**87L**	**7L**	**9L**	**9L**	**60R**	**30L**	**11L**	**75L**	**70L**	**63L**	**59L**	**63L**	**[a]15R, [b]161R, [c]9R**
261R	91L												
209R													[a]76R, [b]115L, [c]64R
396L	91L, 8L												
268L	74L												
149L	113L												
232R	84L	84L					21R						[b]141R
439L	35R								110R	114L		111L	[c]90R
361L	24R							223L	23R				[a]101L, [b]102R, [c]114R
380R	10L, 11L	84L	19R	19R	39L	17L	21R	13L	5L				
213R	51L												
118L	**6R**	**53L**	**55R**	**53R**	**88L**	**49L**	**55R**	**157R**	**35L**	**7L**	**8L**	**8L**	**[b]157L, [d]5L**
198R	69L												
274L	**14L**	**14L**	**96R**	**90R**	**72R**	**39R**	**96R**	**43L**	**80L**	**6L**	**7L**	**7L**	**[a]55R, [b]153R,[c]41R,[d]19R**
229L	46R	3R	4R	229L	16L	2L	5R						
337L	47R	1L	2L	2L	19R	4R	2L	38R	89L		85L		[b]129L, [c]54R
329R	99R												
219L	36R, 91L												
142R	**101R**	**25R**	**85L**	**80L**	**84L**	**46L**	**87L**	**186R**	**74R**	**87R**	**83R**	**85R**	**[a]26R, [b]8R, [c]22R, [d]18L**
155L	113L												
401R	68R												
117L	107R	83L	20R	20R	038L	16L	23R	73R	109R				
415R	18L												
309L	63R												
422L													[d]8R, [d]9R, [d]14L
307L	33L	11R	100L	94L	98R	56R		152L	9R	86R			[a]142R, [c]86L, [d]4R
378R	100L	84L	19R	19R	39L	17L	21R	13L	50R				[b]141R
355R	104L	67R	40R	37R	61R	31L	41R	147L	43L	5L	6L		[a]108R, [b]93L, [c]109L
374R													[b]1R
203L	85L												
395R	1R												
453L	41R												
366R		63R	33R	32R			35R						
010R	43R												
342R	115R												

*ORFs in bold are conserved in all analyzed iridio- and ascovirus genomes. ‡ The a-d indices for the ascovirus ORFs refer to the following species: [a]HvAV3e, *Heliothis virescens* ascovirus 3e (Asgari *et al.*, 2007), [b]TnAV2c, *Trichoplusia ni* ascovirus 2c (Wang *et al.*, 2006), [c]SfAV1a, *Spodoptera frugiperda* ascovirus 1a (Bideshi *et al.*, 2006), [d]DpAV4a, *Diadromus pulchellus* ascovirus 4a (Bigot *et al.*, 2008, Stasiak *et al.*, 2000). The names of the other viruses are abbreviated as follows; IIV-3, *Aedes taeniorhynchus iridescent virus* (Delhon *et al.*, 2006); ATV, *Ambystoma tigrinum stebbensi virus* (Jancovich *et al.*, 2003); TFV, *Tiger frog virus* (He *et al.*, 2002); FV3, *Frog virus 3* (Tan *et al.*, 2004); SGIV, *Singapore grouper iridovirus* (Song *et al.*, 2004); GIV, *Grouper iridovirus* (Tsai *et al.*, 2005); STIV, *Soft-shelled turtle iridovirus* (Huang *et al.*, 2009); LCDV-C, *Lymphocystis disease virus* - isolate China (Zhang et al., 2004); LCDV-1, *Lymphocystis disease virus* - 1 (Tidona & Darai, 1997); ISKNV, *Infectious spleen and kidney necrosis virus* (He *et al.*, 2001); RBIV, *Rock bream iridovirus* (Do *et al.*, 2004); OSGIV, *Orange-spotted grouper iridovirus* (Lü *et al.*, 2005).

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Chapter 3

Transcriptomic analysis of *Chilo iridescent virus* virion proteins using a novel RT-PCR strategy

Abstract

The sequence of *Chilo iridescent virus* (CIV) genome is known for some time, however knowledge on the transcriptional regulation of viral gene expression is limited. Fifty-four CIV virion proteins have recently been identified (İnce *et al.*, 2010). In the current study, the temporal regulation of the corresponding genes was unraveled by combining drug treatments that inhibit protein or DNA synthesis with a novel RT-PCR strategy, especially designed for non-polyadenylated mRNAs. This method is based on the ligation of an oligonucleotide to the 3' end of a 5' capped mRNA to generate a uniform 3' terminus, which can be recognized by a complementary oligonucleotide primer for PCR amplification. By combining this second primer with gene specific forward primers cDNAs for individual genes can be obtained. With this method named Ligation-based Amplification of cDNA Ends (LACE), all CIV virion protein mRNAs were investigated. Contrary to the general assumption that virion proteins would belong predominantly to the late gene class, i.e. genes requiring DNA replication for their expression, CIV virion protein genes fall into all three predetermined temporal classes (i.e. immediate-early, delayed-early and late). The fact that many virion protein genes are of the early gene class, supports the idea that CIV virion proteins may not only have crucial functions in virion formation and structure, but also may play crucial roles in the initial stages of infection. As such, a certain number of these proteins may be required upon entry to initiate infection. Alternatively, some of these proteins may be a reflection of the intracellular path that the virus followed during infection.[7]

[7] This chapter is in preparation for submission.

Introduction

The species *Chilo iridescent virus* (CIV), officially assigned as *insect iridovirus 6* (IIV6), is the type member of the genus *Iridovirus* within the family *Iridoviridae* (Fauquet *et al.*, 2005). The family contains both vertebrate and invertebrate-infecting viruses. The genus *Iridovirus,* accommodating the invertebrate-infecting iridoviruses, is divided into two groups based on cladistic analyses. CIV belongs to Group I (Wang, *et al.*, 2003). Up to now, the genomes of 15 members of the family *Iridoviridae* have completely been sequenced (Eaton *et al.*, 2010) including the most recently sequenced, *Wiseana iridescent virus* (WIV) or *insect iridovirus 9* (IIV-9), which belongs to group II within the genus *Iridovirus* (Wang *et al.*, 2003). The CIV genome has a size of 212,482 base pairs of linear, double-stranded DNA (Jakob & Darai, 2002, Jakob *et al.*, 2001), consisting of 211 non-overlapping and putative protein-encoding open reading frames (ORFs) instead of the previously computationally predicted 468 (Eaton *et al.*, 2007, Tsai *et al.*, 2007).

CIV infection occurs in a coordinated, cascaded fashion (D`Costa *et al.*, 2001, D`Costa *et al.*, 2004), but the control mechanisms are still largely elusive. The information that we have about the replication mechanism of iridoviruses mainly comes from studies on *Frog virus 3* (FV3), the prototype of the genus *Ranavirus* (Granoff, 1984). Replication starts in the nucleus, while assembly and maturation of virions occurs in the cytoplasm (Goorha, 1982, Goorha & Murti, 1982), hence the term nucleocytoplasmic DNA viruses. The transcriptional regulation of gene expression of vertebrate iridoviruses, both *in vivo* and *in vitro,* has received increasing attention over the past years and has been studied in detail for FV3 (Majji *et al.*, 2009), *Singapore grouper iridovirus* (SGIV), also a member of the genus *Ranavirus* (Chen *et al.*, 2006, Teng *et al.*, 2008), and for Red Sea bream iridovirus, the latter within the genus *Megalocytivirus* (Lua *et al.*, 2005, Lua *et al.*, 2007). So far the only extensive transcriptional study for an invertebrate iridovirus was conducted for IIV-9 (McMillan & Kalmakoff, 1994). This study focused on identifying transcriptionally active regions of the genome, but did not classify individual transcripts in temporal classes or assign these to ORFs.

The occurrence of CIV polypeptides during the course of infection (Barray & Devauchelle, 1987, Kelly & Tinsley, 1972) has previously been studied by different research groups and provided evidence for three temporal classes of gene expression: immediate-early (IE or α; 0-2 h post infection, p.i.), delayed-early (DE, β; 2-4 h p.i.) and late (L, γ; from 6 h p.i.). However, at the time, the CIV genome sequence was not known, and most of the detected polypeptides could not be functionally assigned. By definition, IE gene expression does not require *de novo* protein synthesis, in contrast to DE gene expression, and occurs in the presence of inhibitors of protein synthesis, like cycloheximide (CHX). Late gene expression occurs after the onset of DNA replication and can be blocked by DNA synthesis inhibitors, such as cytosine-1-β-D-arabinofuranoside (Ara-C). FV3 IE genes are transcribed by host RNA polymerase II (Goorha, 1981) and it is likely that this is also the case for invertebrate iridoviruses. In addition, transcription of IE genes requires the presence of a virion-associated protein, since purified DNA is not infectious by itself, as was shown for both CIV and FV3 (Nalcacioglu *et al.*, 2003, Willis & Granoff, 1985). Infectivity can be restored by adding virion proteins in the form of UV-inactivated virus particles (Cerutti *et al.*, 1989).

An initial study of CIV transcripts in *Choristoneura fumiferana* (IPRI-CF-124T) cells identified 137 transcripts dispersed over the CIV genome encompassing thirty-eight IE, thirty-four DE and sixty-five L transcripts (D`Costa *et al.*, 2001), but again it was not clear from which genes these transcripts originated. For CIV the transcription profile and promoter structure has been determined for the DE gene DNA polymerase (*DNApol*; 037L) and for the late *mcp* gene (274L) (Nalcacioglu *et al.*, 2007, Nalcacioglu *et al.*, 2003). CIV transcripts most likely do not have polyA tails, because cDNA copies could not be made, when a conventional oligo-dT primer is used in the reverse transcription reaction (Nalcacioglu *et al.*, 2003). In accordance with this, canonical polyadenylation signals (AATAAA) are not found downstream of most CIV ORFs. The absence of polyA tails has also been reported for FV3 transcripts (Willis *et al.*, 1984a, Willis & Granoff, 1976). Therefore, a novel strategy is required to amplify these transcripts in an RNA-specific manner.

Recently, we have annotated 54 CIV genes encoding virion proteins by using a proteomic approach (LC-MS/MS) (İnce *et al.*, 2010). In the present study, we aimed to delineate the temporal classes of these CIV virion protein genes. For this purpose, a novel reverse transcriptase-PCR technique was developed, which we named Ligation-based Amplification of cDNA Ends (LACE) and allows amplification of non-polyadenylated transcripts.

Material and Methods

Viral infections

Chilo iridescent virus was kindly supplied by Dr. C. Joel Funk (USDA-ARS Western Cotton Research Laboratory, USA) and originally came from Dr. James Kalmakoff (University of Otago, Dunedin, New Zealand). The virus was propagated in larvae of the wax moth *Galleria mellonella* and quantified after purification using UV spectroscopy as described (Marina *et al.*, 1999). *Drosophila* Schneider 2 (S2) cells (Invitrogen) were grown to 80–90% confluency in 'Express Five' serum free medium (Invitrogen) at 27°C in T25 flasks and infected with 5 µg CIV per ml culture or mock-infected. For the determination of the temporal transcript classes, infections were conducted in the presence or absence of compounds inhibiting either *de novo* polypeptide synthesis (Cycloheximide, CHX (Sigma), 200 mg/ml), or viral DNA replication (cytosine-1-β-D-arabinofuranoside, Ara-C (Sigma), 100 µg/ml). The cultures were pretreated with the inhibitors for 1 h prior to infection. Cultures were maintained with or without the metabolic inhibitors until three pre-defined time points (1, 3, 6 h p.i.) and used for total RNA isolation. RNA samples of non-drug treated samples were harvested 0, 12, 24, 48 h and 96 h p.i. The cells were centrifuged at 1500 rpm for 2 min, and washed twice with 0.1 M Tris/HCl pH 7.6 containing 0.1 M DTT, and RNA samples were prepared using Trizol reagent (Invitrogen) according to the manufacturer's instructions.

Ligation-based amplification of cDNA ends

To detect the transcripts of individual virion protein genes a novel technology was used (Fig. 1), for which we propose the name Ligation-based Amplification of cDNA Ends (LACE). This method was especially developed to allow analysis of non-polyadenylated mRNAs without the risk of amplifying traces of viral DNA.

In the first step, the 5' phosphorylated oligonucleotide P1 (5'-PO4-GACCACGCGTATCGA TGTCGAC$(T)_{15}$VN-3') was ligated to the 3'end of the transcripts. To this aim, 10 µg samples of total RNA were incubated with 2 µl of 100 mM primer in the presence of 5 µl 0.1 mg/ml BSA, 5 µl ligase buffer (50 mM Tris-HCl pH7.5 at 25°C, 10 mM MgCl2, 10 mM DTT), 5 µl of 1 mM ATP, and 6 µl of 10U/µl T4 RNA ligase (Fermentas) in a total volume of 40 µl for 16 h at 4-8°C. After primer ligation, first-strand cDNA synthesis was conducted. To this aim 40 µl of the oligonucleotide-ligated RNA, 10 µl primer P2 (from 5 µM stock), which the sequence 5'-ATCG ATACGCGTGGTC-3' and 20 µl of 2 mM dNTPs were first incubated at 65°C for 5 min and chilled on ice. The mixture was completed with 20 µl first-strand buffer (Invitrogen), 5 µl of 5 mM DTT (Invitrogen), 1 µl of 2 U/µl RNasin (Promega) and 4 µl of 10 U/µl Superscript III reverse transcriptase (Invitrogen) in a 100 µl total reaction volume and incubated for 60 min at 55°C. The reaction was stopped by heating for 15 min at 72°C. Aliquots of first strand cDNA were used for PCR amplification with gene-specific forward primers (Table 3) and the reverse primer P2 described above. After 2 min at 95°C, 30 cycles of amplification were performed (95°C for 20 s, 55°C for 20 s, 72°C for 30 s), followed by a final elongation step at 72°C for 10 min.

For every virion gene, the primers were chosen in such a way that the expected amplicons would be approximately 500 bp. For smaller sized genes, the complete coding sequence was included. PCR products were analyzed in 1.5% agarose stained with ethidium bromide. Control reactions, in which the RT step was omitted, were also performed. The nature of the amplified fragment was confirmed by automated sequencing (Macrogen Inc.) and compared to the CIV ORF database.

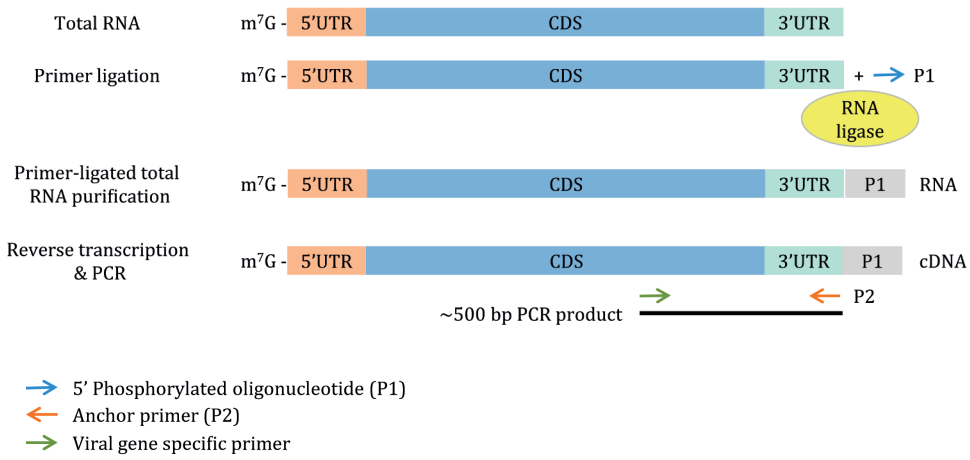

Figure 1. Schematic representation of Ligation-based Amplification of cDNA Ends (LACE), a method for rapid amplification of non-polyadenylated RNA.

Results and discussion

Choice of cell line

CIV replicates *in vitro* in a large number of cell lines derived from insects mainly belonging to the orders Lepidoptera, Coleoptera and Hymenoptera (Constantino *et al.*, 2001, D'Souza *et al.*, 1997, Funk *et al.*, 2001, Hunter & Lapointe, 2003, Monnier & Devauchelle, 1976, Monnier & Devauchelle, 1980, Ohba, 1975). The *in vitro* replication of CIV in a wide range of insect cell lines directed us to choose a cell line of an organism, which is genomically well characterized. Replication of CIV in *Drosophila melanogaster* cells has been reported before (Constantino *et al.*, 2001) and the genome has been sequenced. In the future, this will allow us to determine the infected-cell protein profile, thereby discriminating between viral and host proteins, and to study the host response to CIV infection in more detail. Therefore, we selected *D. melanogaster* cells for our studies.

Detection of non-polyadenylated transcript

Iridoviruses contain a large DNA genome and show a complex replication strategy involving both the nucleus and the cytoplasm of infected cells (nucleocytoplasmic viruses). In the current study, a transcriptomic analysis was carried out to delineate the temporal classes of the 54 virion protein genes that were identified previously (İnce *et al.*, 2010). In order to reach this aim, S2 cells were infected in the presence or absence of protein and DNA synthesis inhibitors, and transcripts were analyzed by a modified RT-PCR approach. Since the 3' end of CIV transcripts does not contain a polyA tail, conventional 3' Rapid Amplification of cDNA Ends (RACE) methods are not suitable for PCR-based transcript detection (Frohman *et al.*, 1988). In order to amplify the CIV transcripts also in a gene-specific manner, thereby avoiding amplification of viral DNA segments, we developed a new strategy, which we called LACE (see Material and Methods; Fig. 1).

The assay condition was verified for known immediate early (inhibitor of apoptosis; *iap*, 193R) (İnce *et al.*, 2008), delayed early (*DNApol*; 037L), and late (*mcp*; 274L) (Fig. 2) gene transcripts (Nalcacioglu *et al.*, 2003). From this, we concluded that LACE serves as a robust, time and cost effective technique for transcriptomic analysis of non-polyadenylated transcripts.

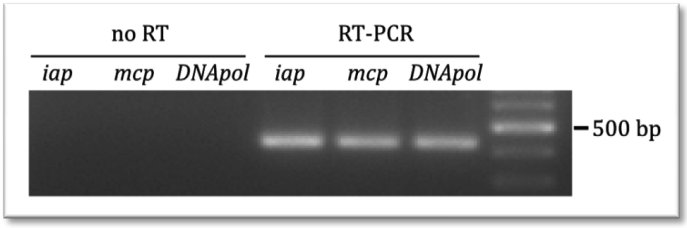

Figure 2. Amplification of non-polyadenylated viral transcripts. *iap*; inhibitor of apoptosis 193R; IE gene, *mcp*; Major capsid protein, 274R; L gene, *DNApol*; DNA polymerase, 037L; DE gene.

Temporal classes of CIV virion protein genes

With the LACE method, we analyzed whether we could detect the individual transcripts in total RNA samples isolated from cells treated with or without protein and DNA synthesis inhibitors. The transcripts for the *iap* (193R), *DNApol* (037L) and *mcp* (274L) genes served as internal controls for this analysis. This analysis revealed that CIV virion protein genes were divided over the three predetermined temporal classes i.e. IE, DE and L (Table 1). In total, 25 immediate-early, 11 delayed-early and 6 late virion transcripts were classified. For two ORFs, 219L, a putative component of a pentameric complex (Yan *et al.*, 2009) and 295L, a core CIV gene with a bipartite nuclear localization signal (Gene ontology; integral to membrane), we found expression in the presence of the protein synthesis inhibitor, but not in the presence of the DNA synthesis inhibitor. This is difficult to explain, but may be these genes use proteins remaining from the parental virion for their early expression. Twelve of the 54 virion protein gene transcripts could not be detected using LACE irrespective whether metabolic inhibitors were present or not. These transcripts could also not be detected in non-drug treated cells at any time point between 0 to 96 h post infection (Table 1). Twelve ORF transcript signals were not detected. Among these 010R, 061R, 084L, 096L (Fasciclin), 203L, 307L (UvR helicase), 325L and 355R (ctd-like phosphatase) have functional domains indicative of integration into viral membrane structures. Four of these non-detected transcripts encode proteins without any predicted domains (111R, 130R, 366R and 395R).

In general, it is assumed that virion protein genes dominantly would belong to the late temporal class, as they need to be incorporated in a coordinated fashion in the virion particle. In contrast, our data show that CIV virion genes are found in all three temporal classes and that the majority is expressed as immediate-early genes, meaning that they do not require protein or DNA synthesis in order to be expressed. The presence of proteins encoded by IE genes in the virion may indicate that they have a role in the very early stages of infection, for instance to act as initiator of viral gene expression as of viral DNA is not infectious by itself (Willis & Granoff, 1995; Nalcacioglu *et al.*, 2003). It is conceivable that upon entry the infection is boosted as more of these IE proteins are being made.

Since many CIV virion proteins have conserved domains characteristic for proteins with well-established roles in DNA replication and transcription (022L, IE; 142R, IE; 232R, IE; 307L, ND; 401R, L shown in Table 1), the virion proteins encoded by IE genes may be involved in regulation of *late* events in CIV infection directly or through modulation of viral DNA replication. Alternatively, their presence in the virion may be a footprint of the path that the virion followed during synthesis and maturation. Although many virion protein transcripts were assigned to the *early* class using metabolic inhibitors, this does not imply that the corresponding genes may not be transcribed at later time points of infection as well, as is for instance the case for the *Autographa californica* nucleopolyhedrovirus *ie-1* gene (Chisholm & Henner, 1988). In order for the encoded proteins to be incorporated in the CIV virions, this may be a possible scenario explaining the presence of IE proteins in the CIV virion. A similar phenomenon was observed for *early* transcripts in FV3 infections (Chinchar & Yu, 1992). Complete disappearance of *early* transcripts is probably also not a prerequisite for *late* CIV transcription, in contrast to the situation in for instance poxvirus infections (Cooper & Moss, 1979) where no early genes are expressed late.

Table 1. Temporal classification of CIV virion protein gene transcripts based on inhibitor studies.

Immediate Early				Delayed Early				Late				Not detected (ND)*		
ORF	**CHX**	**Ara-C**	**Predicted function**	**ORF**	**CHX**	**Ara-C**	**Predicted function**	**ORF**	**CHX**	**Ara-C**	**Predicted function**	**ORF**	**CHX**	**Ara-C**
022L	+	+	Nucleoside triphosphatase I	**117L**	-	+	**?**	**159L**	-	-	?	**010R**	ND	ND
034R	+	+	**?**	**149L**	-	+	**?**	**234R**	-	-	Integral to membrane	**061R**	ND	ND
104L	+	+	Integral to membrane	**198R**				**274L**	-	-	Major structural protein	**084L**	ND	ND
118L	+	+	Envelop protein	**229L**	-	+	**?**	**317L**	-	-	**?**	**096L**	ND	ND
123R	+	+	Dual specificity phosphatases	**329R**	-	+	**?**	**342R**	-	-	**?**	**111R**	ND	ND
138R	+	+	Integral to membrane	**337L**	-	+	Myristylated membrane protein	**401R**	-	-	HMG-box DNA-binding protein	**130R**	ND	ND
142R	+	+	dsRNA-specific ribonuclease	**374R**	-	+	Bat coronavirus spike protein					**203L**	ND	ND
155L	+	+	**?**	**378R**	-	+	2-cysteine adaptor domain					**307L**	ND	ND
179R			Tyrosine protein kinase	**396L**	-	+	Repetitive protein					**325L**	ND	ND
209R	+	+	Serine/threonine protein kinase	**422L**	-	+	*Cydia pomonella* granulovirus ORF34					**355R**	ND	ND
213R	+	+	Peptidoglycan bound protein	**457L**			**?**					**366R**	ND	ND
219L			**?**									**395R**	ND	ND
227L	+	+	Integral to membrane											
232R	+	+	Transcription termination											
261R	+	+	Repetitive protein											
268L	+	+	?											
295R			Bipartite nuclear localization signal											
309L	+	+	?											
312R	+	+	?											
361L	+	+	Papain cysteine protease											
380R	+	+	Serine/ threonine-specific kinase											
415R	+	+	?											
439L	+	+	Protein kinase											
443R	+	+	Electron transport											
453L			Protein disulfide isomerase											

*ND means that these gene transcripts could not be detected using LACE at none of the time points sampled between 1 and 96 h post infection.

Comparison to transcriptome data of other iridoviruses

The temporal classification of CIV virion protein genes was compared to the available transcriptome data from other iridoviruses (Table 2). This analysis showed that although the ORFs may be conserved, the temporal class assignment varies, e.g. for 022L (putative helicase) and 142R (RNaseIII), 380R and 439L (viral protein kinases), and 118L (putative envelop protein) and 274L (major capsid protein). In this context, we can speculate that although the ORFs are conserved the promoters may be different. In addition, it is likely that depending on the test system the findings may differ.

Table 2. Comparison of the temporal class of conserved virion protein between CIV and other iridoviruses. ND; not determined, E-S; immediate early stably expressed gene, E; not distinguished whether immediate or delayed-early. 639R and 641L are overlapping genes.

CIV	Temporal Class	FV3	Temporal Class	SGIV	Temporal Class[1/2]	RSIV	Temporal Class
022L	IE	9L	ND	60R	E/E	407R&413R	E & E
117L	DE	20R	L	038L	L/L		
118L	IE	53R	DE	88L	L/E	374R	L
142R	IE	80L	DE	84L	L/IE	639R-641L	E
179R	DE	27R	L	78L	E/L		
229L	DE	3R	L	16L	E/E		
274L	L	90R	L	72R	L/L	380R	L
295L	E	41R	L	57L	L/E	639R-641L	E
307L	ND	94L	IE-S	98R	IE-L/E	600L	E
337L	DE	2L	L	19R	IE-L/E	575R	L
355R	ND	37R	L	61R	L/L	385R	E
366R	ND	32R	IE-S				
378R	DE	19R	ND	39L	L/L		
380R	IE	19R	IE-S	39L&150L	L/L & E/E		
439L	IE					463R	L

Frog virus 3, (Genus *Ranavirus*) (Tan *et al.*, 2004); SGIV, two isolates of *Singapore grouper iridovirus* (*Ranavirus*) ([1]Chen *et al.*, 2006; [2]Teng *et al.*, 2008; RSIV, *Red Sea bream iridovirus* (Genus *Megalocytivirus*) (Lua *et al.*, 2005; Thi *et al.*, 2007).

Conclusion and outlook

This study shows that the CIV virion gene expression pattern more variable than previously thought which suggest a complex virion assembly process. The challenge will be to delineate specific functions to each of these virion proteins. The information obtained on the temporal classes of individual genes will provide the basis for determining for instance whether members of the same temporal class contain common upstream regulatory motifs and may assist us to identify virion-associated proteins and virus-induced proteins that control viral gene expression. The LACE technology developed for CIV transcripts may have a much wider application in the study of non-polyadenylated transcripts.

Table 3. Primer list for transcriptome of virion protein genes

ORF	Primer sequence	T_M (°C)	ORF		Primer position		Primer length	Amplicon (bp)
			Start	stop	Start	End		
010R	ATGAATAATTTTAATTACTTTAATG	56	2498	2860	2498	2522	24	362
022L	CCTCTGTGGAATGAATGG	54	9277	5762	6242	6259	17	480
034R	ATGAAACAGAATTTATTAATCC	54	11997	12401	11997	12019	22	404
061R	ATGAACGATAGAGGTATAATTAT	58	27538	28005	27538	27563	25	467
084L	ATGGCAAAAGTCATTAAA	46	35091	35074	34594	35091	17	480
096L	GATGGTTTATATAGATTTTC	50	39395	39349	38871	39368	19	478
104L	ATGCCACATTACGTTGTTG	54	43008	42835	42990	43008	18	155
111R	ATGATTTTGGTTTTATTACTTAT	50	43911	44438	43911	43934	23	527
117L	GCTATGGCATTCAACCCT	54	46517	45768	46149	46166	17	381
118L	TGGGTAAATTCCGACCCA	52	48177	46630	47113	47130	17	483
123R	ATGAACCTACGAAAATAGTAG	56	49533	49961	49536	49554	18	425
130R	CTATTCAGGAAATACTACATTG	58	51751	52356	51855	51876	21	501
138R	ATGAATAATAATCAACAAATACTT	54	54168	54512	54168	54192	24	344
142R	TTTATTACAGCTACTTATGAT	52	55500	56378	55875	55895	20	503
149L	GTGATTTCTATTTTACACATG	54	61960	59960	60443	60463	20	483
155L	TACCTTGGATACGGAAATG	54	62770	62003	62356	62374	18	353
159L	ACAAGTAAAGTAGGTTCTATT	54	64967	63540	64029	64049	20	489
179R	TTGAATTATATATGGTTGCAC	54	75567	79127	78625	78645	20	502
198R	TTGCAGGTCAAACGTTAGA	50	85637	87055	86553	86571	18	502
203L	ATGTCCATTCAAACATTAATAA	54	89334	88852	89313	89334	21	461
209R	TGGGCGTTTTGTACTGGT	54	92277	95417	94917	94934	17	500
213R	GATAGTGGATATCATACGTT	54	98000	99568	99068	99087	19	500
219L	GCAGGATGGACACTATCT	54	102489	101458	101950	101967	17	492
227L	ATGAATCAGCTTAATCAATT	50	105597	105406	105578	105597	19	172
229L	GGAGCTTTTATATCTTGGCC	58	107921	106590	107071	107090	19	481
232R	TCACTACATTGCTTTAGAAC	54	108021	110036	109559	109578	19	477
234R	AGATGTAAAAGTAAGTGCTG	54	110163	110744	110163	110187	24	581
261R	TAATCCCAACACAACCGC	54	121519	125604	125106	125123	17	498
268L	GTCGTGATGACAAAATAATT	52	127775	125643	126135	126154	19	492
274L	CTATTAAAGCACTTTTCTTT	50	130158	128755	129248	129229	19	493
295L	TGTTTATGGTAGACAGTAAAA	54	139425	135394	135894	135914	20	500
307L	AGGTATAAAGGGATCTTGG	54	142846	142253	142754	142736	18	501
309L	TCGGATGTTGATAAAGGAC	54	143800	143147	143536	143554	18	389
312R	ATGAACCCCGCAATTGT	50	143926	144204	143926	143943	17	278
317L	CTTATGTCTGGAACGGT	50	147527	146271	146782	146766	16	511
325L	ATGTTTCTTTTGAGAATTTTC	50	148350	147871	148004	147984	20	133
329R	TGTTATTGATGGTGTTACTC	54	148473	149609	149087	149106	19	522
337L	GAATACTGTCTTCATAATCC	54	152755	151517	151971	151990	19	454
342R	ATGGATAAACCTCGCGAAC	52	153531	153797	153531	153549	18	266
355R	ATTTGAAGATATAACTGACT	50	158160	158708	158210	158229	19	498
361L	TCTCAAGTTCAAATACAGAC	54	162659	161031	161538	161557	19	507
366R	ATGCCATTATTAAGAAAACGAT	54	162769	163113	162769	162790	21	344
374R	ATGGATATAGAATTTGGAAAT	52	165568	166065	165568	165588	20	497
378R	TCATCTCCTAGAAGGTC	50	167305	167886	167473	167489	16	413
380R	GTGTCAGAAATGCAAAGGT	54	167936	169522	169014	169032	18	508
395R	ATGTCAGGATCTGGATATG	54	175856	176311	175856	175874	18	455

Acknowledgments

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Chapter 4

Proteomic analysis and label-free quantitation of *Chilo iridescent virus* virion proteins in infected cells

Abstract

The sequence of the *Chilo iridescent virus* (CIV) genome is known for ten years. Fifty-four virion proteins have recently been identified and assigned to the corresponding open reading frames (ORFs) (İnce *et al.*, 2010). However, the regulation of virion protein expression and virion assembly is not known. A proteomic - based approach (LC-MS/MS) was adopted to follow virion and other infected cell protein levels during the course of infection of *Drosophila* Schneider 2 (S2) cells. Proteomic data were processed with the MaxQuant algorithm using label-free quantitation approach, enabling us to quantify protein levels. In total, 95 viral proteins were identified, 37 of which were virion proteins. Comparative virion protein expression profiles over time fall into two main cluster; genes that are expressed early and reach high levels and those that are expressed late and reach moderate levels in the course of infection. Several hierarchical clusters were identified for the remaining virus-specific proteins. The latter allowed the assignment of 58 novel CIV genes. The fact that many virion protein genes belong to the early temporal class supports the idea that virion proteins have not only crucial roles in virion structure formation, but also at earlier stages of infection.[8]

[8] This chapter is in preparation for submission.

Introduction

Chilo iridescent virus is the type member of the genus *Iridovirus* within the family *Iridoviridae* (Fauquet *et al.*, 2005). The family *Iridoviridae* consists of nucleocytoplasmic DNA viruses. Replication starts in the nucleus with the formation of DNA concatemers, while assembly and maturation of virions occur in the cytoplasm (Goorha, 1982, Goorha & Murti, 1982). The CIV genome has a size of 212,482 base pairs of linear, double-stranded DNA (Jakob & Darai, 2002, Jakob *et al.*, 2001) and originally consisted of 468 protein encoding ORFs. Recently, the CIV genome sequence has been re-annotated and 211 putative protein encoding ORFs instead of the previously predicted 468 were assigned (Eaton *et al.*, 2007, Tsai *et al.*, 2007).

The temporal regulation of gene expression as evidenced from the synthesis of infected cell polypeptides and appearance of CIV virion particles (Barray & Devauchelle, 1979; 1987, Day & Mercer, 1964, Kelly & Tinsley, 1972, Reyes *et al.*, 2004) have previously been studied by different research groups. Studies on CIV infected cell-specific polypeptides provided evidence for three temporal classes of gene expression: immediate-early (IE or α; 0-2 h p.i), delayed-early (DE, β; 2-4 h p.i.) and late (L, γ; from 6 h p.i.) (Barray & Devauchelle, 1987) expression, and suggested a regulation cascade for the expression of these genes with both positive and negative control mechanisms (D`Costa *et al.*, 2001, D`Costa *et al.*, 2004). However, at the time, none of the detected polypeptides was annotated. The purified CIV DNA is not infectious by itself, but infectivity can be restored by adding virion proteins (Cerutti *et al.*, 1989) and transcription of CIV IE genes uses a virion-associated protein as an activator for early transcription. A time-dependent expression pattern of CIV proteins and assignment to ORFs would therefore, provide novel and detailed information on the temporal regulation of both virion and infected cell specific proteins encoded by CIV.

Recently, we have annotated 54 CIV genes encoding virion proteins by using a proteomic approach (LC-MS/MS) on in-gel digested of virion proteins (İnce *et al.*, 2010). In a parallel transcriptional study, we have determined to which temporal class the individual genes encoding these virion proteins belong. This was achieved by determining the ability to transcribe these genes in the presence or absence of inhibitors of protein and DNA synthesis (This thesis, Chapter 3). In the current study, we aimed at identifying and quantifying all 54 CIV virion proteins as well as other virus-specific proteins during the course of infection.

Material and Methods

Viral infections

Chilo iridescent virus was a gift from Dr. C. Joel Funk (USDA-ARS Western Cotton Research Laboratory, USA) and was originally obtained from Dr. James Kalmakoff (University of Otago, Dunedin, New Zealand). The virus was propagated in *Galleria mellonella* larvae and quantified as previously described (Marina *et al.*, 1999). *Drosophila* Schneider 2 (S2) cells (Invitrogen) were grown in Express Five -Serum Free Medium (EF-SFM, Invitrogen) at 27°C in T25 flasks and infected at a confluency of 80–90 % with 5 µg CIV per ml culture.

Western blot analysis

The protein factions were run on 12% SDS-PAGE gels and then transferred for 1 h to Immobilon-P at 0.8 mA per cm^3 (0.04 A/gel). Blots were blocked overnight in 2% skimmed milk/PBS-T20, washed three times for 10 minutes with 0.2 % skimmed milk/PBS-T20. Rabbit anti-CIV antibodies were used as the primary antibodies for Western blot analyses. Into the solution, the primary antibodies were added to final dilutions of 1:2000, and then incubated for 60 minutes at 25˚C and washed three times for 10 minutes with 0.2 % skimmed milk/PBS-T20. The blots incubated for a further 60 minutes with the secondary antibody, goat anti-mouse alkaline phosphatase (dil. 1:3000), followed by 2 times washing for 10 minutes with alkaline phosphatase buffer (AP buffer; 0.1 M Tris-HCl, 5mm $MgCl_2$, pH 9.5). The blot developed with 1% of Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (NBT/BCIP) in AP buffer.

Immunofluorescence microscopy

Glass slides coated with 0.05% w/v poly-L-lysine (smeared and let dried out). Infected cell suspension prepared by pelleting cells 10 min 2000 rpm and pellet resuspended the in 100 µl PBS. A drop of resuspended cells placed on the coated slide and incubated about 30 sec to maximum 1 min. The covered cells are fixed in 96% ethanol for 20 min and washed three times in PBS for 5 min. Glass slide surface blocked with 1% w/v BSA in Phosphate Buffered Saline pH 7.2 (PBS) in a wet box, 30 min at 37°C. Excess BSA removed with the help of a filter paper. Fixed cells coved with 100 µl primary antibody diluted in 1% BSA / PBS) and incubated 1 h at 37°C in a humid box. Glass slide gently washed with PBS in a beaker. Area of fixed cells covered approximately 100 µl secondary antibody GAR alexa-488 diluted in 1% BSA / PBS (1:400) and incubated 1 h at 37°C in a humid box. The coverslip washed gently with PBS and dried using filter paper so that the run-off excess PBS. The coverslips were mounted with Citifluor AF-1. The specimens stored at 4°C until examined. The confocal microscopic examinations were performed with LSM510 confocal laser-scanning microscope (Zeiss, Heidelberg-Germany)

Mass spectrometry

The Filter Aided Sample Preparation (FASP) method (Manza *et al.*, 2005, Wisniewski *et al.*, 2009) was used to prepare samples for identification of tryptic peptides by LC-MS/MS from the crude cell pellets of the infected-cell time course. To accomplish this, CIV-infected cells were harvested at 0, 1, 12, 24, 48, 72, 96 h post infection (p.i.), centrifuged at 1500 rpm for 2 min, and washed twice with 0.1 M Tris/HCl pH 7.6 containing 0.1 M DTT. The crude cell pellet was kept at -20°C. Mock-infected cells were used as control. The cells were suspended in 200 µl lysis buffer (0.1 M Tris/HCl pH 8.0, 0.1 M DTT, 8 M urea). Cells were disrupted and the DNA was sheared by sonication on ice with a 7-10 amplitude micron power three times for 15 seconds with 15 second intervals. Before tryptic digest of the infected cell protein samples, the resultant lysates were separated into soluble and insoluble fractions by centrifuged at 14000 rpm for 10 min and 100 µl from the soluble and 10 µl from the insoluble fractions were applied to Nanosep 3K Omega centrifuge filters (Pall Corporation, Ann Arbor, MI).

The filter units were centrifuged at 14000 rpm for 30 min at room temperature (20°C) and washed with 100 µl of 8 M urea in 0.1 M Tris/HCl pH 8.0 (UT), after which 100 µl of 0.05 M iodoacetamide in UT was added to the filter units, incubated for 1 h and centrifuged as before. Filter units were washed three times with 110 µl UT with increase in volume by 10 µl in each subsequent washing. After washing, 140 µl 0.05 M NH_4HCO_3 was added to the filter units, transferred to new Eppendorf tubes, centrifuged at 14000 rpm for 30 min, and the filtrate removed. For protein digestion, 100 µl 0.1 µg/µl sequencing grade modified porcine trypsin (Promega, Madison, WI) in 0.05 M NH_4HCO_3 was added to the filters followed by overnight incubation at room temperature with gentle shaking. Digested samples were centrifuged to collect the tryptic peptides. The peptide solutions were acidified by adding 3.5 µl 10 % trifluoroacetic acid and analyzed by LC-MS/MS as described before by İnce *et al.* (2010).

Identification and quantitation of the CIV virion proteins

The LTQ-Orbitrap raw data files were analyzed with MaxQuant software version 1.1.1.36 (Cox & Mann, 2008, Cox *et al.*, 2011). MS/MS spectra were searched against target-decoy databases. The databases used were downloaded from the Uniprot database (http://www.uniprot.org/; *Insect iridovirus 6* (Magrane & UniProt Consortium, 2011) and from Flybase (http://flybase.org/: *Drosophila melanogaster*; version FB2011-06, Indiana University) (Tweedie *et al.*, 2009). In addition, a contaminant database with commonly observed contaminants was included in the search.

Default MaxQuant 1.1.1.36 settings were used except that asparagine and glutamine de-amidation were added as variable modifications and the label-free quantitation as well as the "match between runs" options were enabled (with a default time window of 2 min). Proteins were considered identified when at least two peptides were observed of which at least one was uniquely assignable to the respective sequence. Bioinformatics analysis of the MaxQuant/Andromeda workflow output and the analysis of the abundances of the identified proteins were performed with the Perseus module (available at www.MaxQuant.org). Perseus is designed to perform all downstream bioinformatics and statistics on the MaxQuant output tables. It has increasingly been feasible to understand and interpret the results of quantitative proteomics experiments *in silico*. The publicly available Perseus program developed by Max Planck Institute of Biochemistry (Martinsried, Germany) combines generic quantitative bioinformatics methods with proteomics-specific algorithms in a single environment (http://www.perseus-framework.org).

The virion protein expression profile obtained using the label-free quantitation (LFQ) approach from MaxQuant (Perseus) was used to create a protein profile heat map of expression levels. This was done by first adding up the intensity data from MaxQuant for the soluble and insoluble protein fractions (not the LFQ intensity data). The Perseus analysis software was then used to show independent clustering of protein expression profiles without grouping the time points entered.

Results and discussion

The *in vitro* replication of CIV in a wide selection of insect cell lines (Constantino *et al.*, 2001, D'Souza *et al.*, 1997, Funk *et al.*, 2001, Hunter & Lapointe, 2003, Monnier & Devauchelle, 1976, Monnier & Devauchelle, 1980, Ohba, 1975) allowed us to choose a cell line from an organism with a well-characterized genome. Such a cell line allows the discrimination between viral and host proteins. In the current experiments the S2 cell line was chosen as a model system to study the expression profile of CIV encoded proteins during the course of infection.

CIV infected S2 cell culture samples were harvested from 0 to 96 h p.i., viewed in the light and confocal microscope and analyzed by SDS-PAGE. Infected cells became rounded and more granulated upon infection (Fig. 1, LM, control-1 panel). By confocal microscopy using an antibody against the major virion protein MCP of CIV, fluorescence cells were clearly observed in the cytoplasm of infected cells (Fig. 1, CF) at various times after infection. Sometimes the fluorescence was punctate.

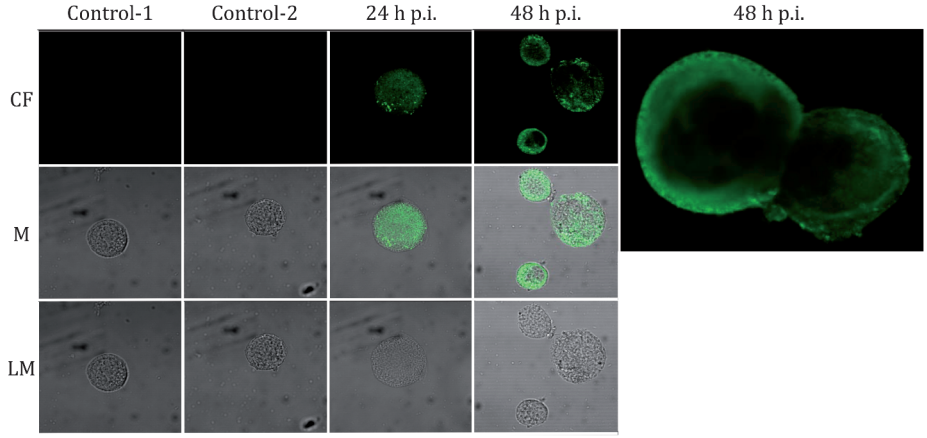

Figure 1. Immunoflourescent labelling of CIV virion particles. Control-1; non infected cell, control-2; 24 h p.i. testing pre-immun serum, CF; confocal microscopy, M; merged light and confocal microcopy images, LM; light microscopy.

To find out which viral proteins were produced, the samples taken at different time's p.i. were subjected to SDS-PAGE electrophoresis (Fig. 2). Coomassie brilliant blue staining did not reveal any dramatic changes in protein expression levels until 72 h p.i., from which point onwards., some of the proteins show differential expression as reflected by protein bands intensities in Fig. 2A. Most likely, these are virus-induced proteins. Overall, the changes were not as dramatic as could be seen in baculovirus-infected insect cells (Du & Thiem, 1997). To show that indeed virus-induced proteins were made, the SDS-PAGE gel was subjected to Western analysis using polyclonal antibodies against whole CIV virions (Fig. 2B). It can be seen that the antiserum strongly reacted with the major capsid protein MCP from 24 h p.i. onwards. The virions that are produced by the S2 cells were infectious to S2 cells. These experiments suggest that the infection of S2 cells with CIV is fully permissive and that viral proteins can be further investigated using proteomic techniques.

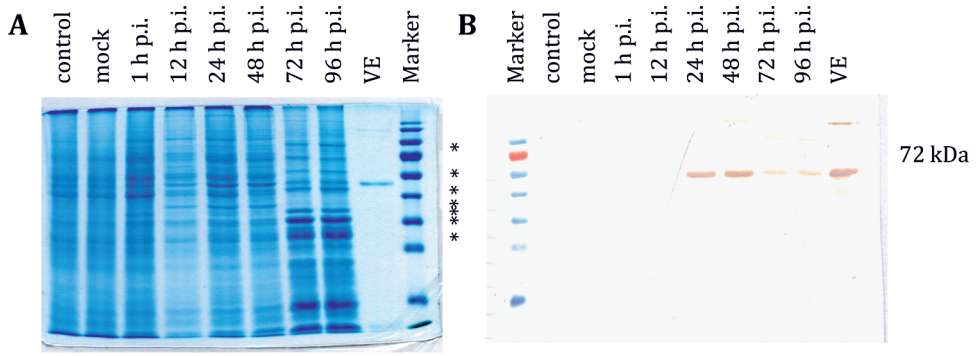

Figure 2. A; Protein profile of CIV infected *S2* cells, B; Western blot analysis with α-CIV antiserum showing successful CIV infection in S2 cell line. VE: virion extract, Marker: pre-stained Protein marker. The asterisks indicate proteins that showed differential expression levels at 72 h p.i.

For each time point, the soluble and insoluble protein fractions were applied to FASP and the extracted peptides were subjected to LC-MS/MS. Despite the big quantitative differences between host cells versus viral proteins, 68.5% (37/54) of the known CIV virion proteins (İnce *et al.*, 2010) were identified and quantified, among which was the major structural protein MCP (Table 1). The genes encoding for these proteins are depicted on the linear map of CIV DNA in Fig. 3 ('green' with black frame). However, 17 of previously identified virion proteins (İnce *et al.*, 2010) were undetectable in this study (Table 2 and Fig. 3) ('green' with light frame), a factor that can be attributed to their very low abundances in infected cells for LC-MS/MS detection. Interestingly, CIV genome contains two fully overlapping ORFs 255L within 254L and 375R within 374R. ORFs, 255L and 375R are short and share the same transcriptional direction with their overlapping genes. In addition to that, no transcripts and protein signals detected for these small overlapping ORFs, most probably they are not truly expressed ORFs. ORF 254L has been detected in infected cell proteome but not a part of CIV virion. ORF 374R detected in CIV virion with low abundance (İnce *et al.*, 2010) as a one of the candidate of the finger protein of the viral capsid proteins (Yan *et al.*, 2009), however not detected in infected cell proteome study. The heterologous protein of ORF 374R found in IIV-9 (141R) similarly in infected cell proteomic study (Wong *et al.*, 2011) and not in IIV-9 virion structure. In addition to these finding 374R transcript has been detected as a delayed early gene with a delayed early promoter motif (Chapter 3).

Recent advances in proteomics allow us to measure expression levels for thousands of genes simultaneously, across different conditions and over time. Analysis of the label free quantitation of the infected cell proteomic data unravels two main important phenomena. First of all, an opportunity to perform comparative global measurement of the relative protein expression levels in a related sample series (e.g. time course) offering a universal method so called expression proteomics. Secondly, this approach is technically similar with a microarray study in which transcriptome measurement is taken as a basis. However, expression proteomics obviously give a better insight as it looks at the end product of a gene expression cascade. This serves better than messenger RNA levels to unravel the biological functions of the protein(s) of interest.

Combining these two important outcomes of the approach provides us a powerful tool to identify and quantify the different proteome states with details on the primary structure of proteins, protein-protein interactions and provides further insight in cellular or organismal level proteomes.

As a consequence of a progressive infection over time, differences in viral protein expression profiles over the course of viral infection (0-96 h p.i) are to be expected. When looking at the presence and abundance of individual CIV virion proteins over time using label-free quantitation, clear expression pattern clusters were observed for the 37 virion proteins detected in infected cells. This allowed the construction of a so-called 'heat map' showing the relative abundance of the proteins over time (Fig. 4 & Fig. 6). It has to be noted that not all 37 virion proteins were detected at all times after infection (Tables 1 and 2). These expression patterns could be organized in hierarchical clusters, harboring proteins with similar expression patterns over time. The clustering may give us insight into the regulatory mechanisms of CIV gene expression.

Using hierarchical clustering techniques, three classes of virion proteins could be discerned (Fig. 4). Cluster I proteins are expressed at equal levels throughout the infection over time. Cluster II consists of proteins that gradually peaked at 48 h p.i., whereas cluster III represent proteins that were increasingly expressed over time.

When the 'heat map' was correlated to the information on transcripts of these 37 genes (Fig. 4, Table 1) no clear correlation could be found. All transcription classes (immediate-early, delayed early and late) were more or less randomly distributed over the various clusters of the 'heat map' Fig. 4 & Fig. 6). It may be that the infection became more asynchronous over time and cannot be easily compared to the transcription analysis using protein and DNA replication inhibitors (Chapter 3). It is possible that better correlations are found when transcription analysis was done on the same time course samples for which the proteomic analysis was done.

In the infected cell proteome study, we have identified 95 viral proteins in total (Table 3 and 4), 37 of which are virion proteins (İnce *et al.*, 2010). Fifty-eight new CIV-encoded proteins have thus been identified in the current study. When the 54 virion proteins are added, the total number of truly expressed viral ORFs is now 112. Assuming a total of 211 ORFs for CIV (Eaton *et al.*, 2007), this means that at least 53% of the putative ORFs are expressed into proteins and therefore functional. The detailed mass spectrometry analysis presented on Table 4.

When we view the temporal presence of all identified CIV proteins, CIV infection starts as early as 1 h p.i. and continues until 48 h p.i. before all 98 viral proteins are expressed. Using the comparative label-free quantitation approach, it is possible to group these proteins according to their relative abundance over time (1 h p.i. versus 48 h p.i.) (Fig. 5). In the scatter plot, it is clear that one group of proteins are abundantly expressed over this period (blue circles in the graph) and a second group has moderate expression levels without a significant change in this period (green circles). Only one gene, 439L has a decreased expression level. Both groups contain virion proteins as well as infected cell specific proteins. In this analysis, the major virion protein MCP (274L) is the most abundantly expressed protein and this is in line with the abundance of this protein in CIV virions (Ince *et al.*, 2010). It is interesting to note that other highly abundant virion proteins (Pink dots, Fig. 5) are either of the immediate early (380R and 415R) or late (401L) class based on the transcriptome studies and that they have relatively high values of sequence coverage (Table 1).

Table 1. Virion protein presence in time compared to transcript class*

ORF	Virion protein presence (h p.i.)	Coverage [%]	RNA class	Predicted function	Gene ontology
022L	0-1-12-24-48-72-96	27.9	IE	NTPase I; DEAD-like helicase	DNA replication
061R	0-12-24-48-72-96	58.4	ND	Lysosome associate membrane glycoproteins	Integral membrane protein
084L	0-1-12-24-48-72-96	36.6	ND		Integral membrane protein
104L	0-1-12-24-48-72-96	25	IE		
111R	0-12-24-48-72-96	30.5	ND		
117L	0-1-12-24-48-72-96	27.8	DE		
118L	0-1-12-24-48-72-96	35.2	IE	Myristylated membrane protein	Integral membrane protein
123R	0-1-12-24-48-72-96	18.4	IE	Dual specificity phosphatases	MAP kinase activity
130R	1-12-24-48-72-96	31	ND		
149L	0-1-12-24-48-72-96	43.6	DE		
155L	1-12-24-48-72-96	33.5	IE		
179R	0-1-12-24-48-72-96	6.8	IE	Tyrosine protein kinase	Protein phosphorylation
198R	0-1-12-24-48-72-96	25.1	DE		
203L	0-1-12-24-48-72-96	20.8	ND		Integral membrane protein
209R	0-1-12-24-48-72-96	23.3	IE	Serine/threonine protein kinase	Protein phosphorylation
229L	24-48-72-96	20.8	DE		
232R	0-1-12-24-48-72-96	51.6	IE	N- terminal domain of viral DNA polymerases	Transcription termination
234R	0-1-12-24-48-72-96	53.1	L		Integral membrane protein
261R	0-1-12-24-48-72-96	2.7	IE	Potential repetitive protein	
268L	1-12-24-48-72-96	14	IE		
274L	0-1-12-24-48-72-96	61.6	L	Major structural protein	Major viral capsid protein
295L	0-1-12-24-48-72-96	23.6	IE	Bipartite nuclear localization signal	Integral membrane protein
312R	0-24-48-72-96	15.4	IE		
317L	0-1-12-24-48-72-96	13.2	L		
325L	0-12-24-48-72-96	23.4	ND		Integral membrane protein
342R	12-24-48-72-96	52.9	L		
361L	0-1-12-24-48-72-96	20	IE	Cathepsin B	Cysteine-type endopeptidase activity
378R	0-12-24-48-72-96	34.9	DE	2-cysteine adaptor domain	
380R	0-1-12-24-48-72-96	50.9	IE	Serine/threonine kinase	Protein phosphorylation
395R	0-1-12-24-48-72-96	26.7	ND		
396L	0-1-12-24-48-72-96	15.8	DE	Potential repetitive protein	
401R	0-1-12-24-48-72-96	51.8	L	HMG-box superfamily	DNA metabolism
415R	0-1-12-24-48-72-96	49.8	IE		
439L	0-1-24-48-72-96	8.4	IE	Protein kinase domain	protein phosphorylation
443R	48-72-96	4	ND		Cell redox homeostasis
453L	12-24-48-72-96	26.3	IE	Protein disulfide isomerase	
457L	0-1-12-24-48-72-96	36.2	DE		

Table 2. Undetectable virion protein in infected cell proteome time course compared to transcript class*

ORF	Virion protein presence (h p.i.)	RNA class	Predicted function	Gene ontology
010R	ND	ND	zip protein	Integral membrane protein
034R	ND	IE		Integral membrane protein
096L	ND	ND	Fasciclin domain	
138R	ND	IE		Integral membrane protein
142R	ND	IE	dsRNA-specific ribonuclease	dsRNA processing
159L	ND	L		
213R	ND	IE	Putative peptidoglycan bound protein	Integral membrane protein
219L	ND	IE		
227L	ND	IE		Integral membrane protein
307L	ND	ND	Uvr/REP helicase	Integral membrane protein
309L	ND	IE		
329R	ND	DE		
337L	ND	DE	Myristylated membrane protein	Integral membrane protein
355R	ND	ND	Catalytic domain of ctd-like phosphatases	
366R	ND	ND		
374R	ND	DE	Bat coronavirus spike protein	
422L	ND	DE	*Cydia pomonella* granulovirus ORF34	

*IE, Immediate early; DE, delayed early; L, late; ND, not detected.

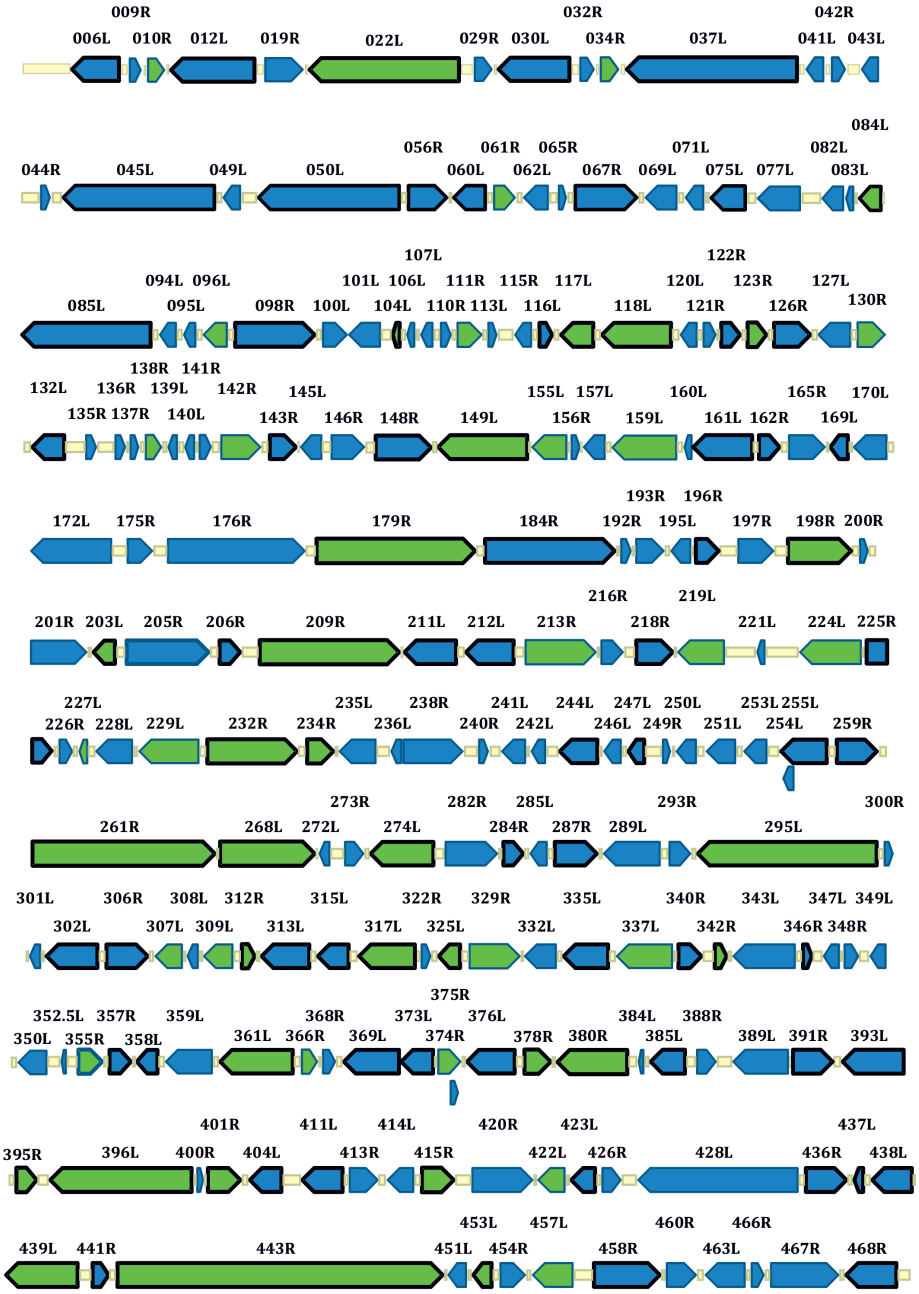

Figure 3. CIV linear genomic map presenting virion and infected cell viral proteins detected in mass spectrometry (Genome size 212.482 bp). Arrows representing the transcriptional direction of the ORFs. Green arrow represent virion proteins and black frame around the arrows represent detection of the corresponding protein of the ORF in infected cell proteomic analysis.

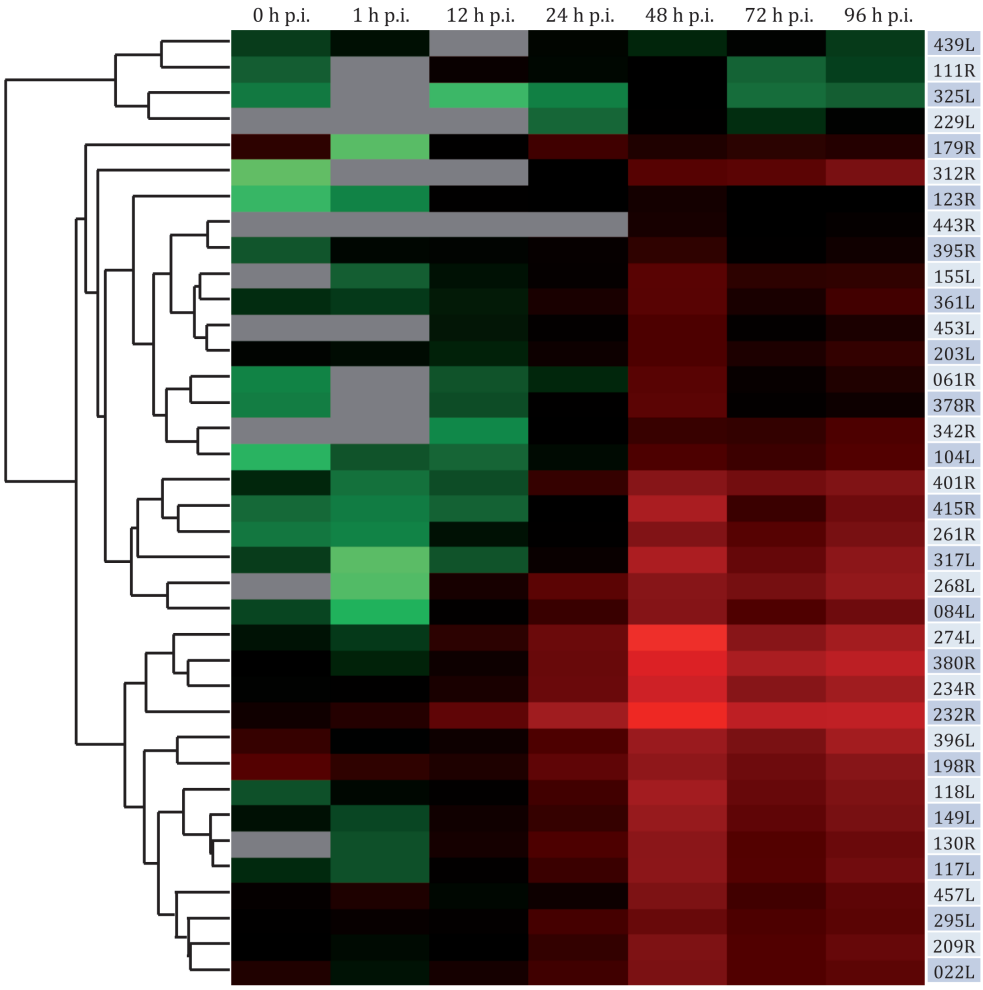

Figure 4. Virion protein heat map; The virion protein expression profile obtained using the label-free quantitation (LFQ) approach from MaxQuant (Perseus) showing protein profile comparative expression levels from 0 to 96 h post infection*

A global overview of the infected cell expression proteome shows the presence of various virus encoded kinases. They are involved in phosphorylation reactions, which are among the most abundant and important posttranslational modifications of viral (and host) proteins. Protein kinase activity as a post-translational modification regulates a broad range of cellular activities including the cell cycle, differentiation, metabolism, and communication. Virus-encoded serine/threonine protein kinases appear to be a feature that is unique to large DNA viruses. These kinases are thought to be related to virulence of viral infections (reviewed by Jacob *et al.*, 2011).

Table 4. The summary of mass spectrometry analysis

Time course	MS/MS Identified [%]		Identified Peptide Sequences		Average Absolute Mass Deviation		Mass Standard Deviation	
fractions	soluble	insoluble	soluble	insoluble	soluble	insoluble	soluble	insoluble
0 hpi	60.4	53.3	3971	2555	0.90	0.59	1.12	0.82
1 hpi	61.5	48.2	3859	1822	1.01	0.66	1.26	0.92
12 hpi	50.0	58.1	3357	2541	1.59	0.63	1.87	0.86
24 hpi	63.4	53.9	3977	2228	1.40	0.72	1.59	0.94
48 hpi	60.7	51.4	3909	2252	1.04	0.68	1.34	0.92
72 hpi	63.9	52.1	4004	2272	1.05	0.60	1.28	0.79
96 hpi	64	49.6	4065	1853	1.17	0.62	1.39	0.82
Average	**60.5**	**52.4**	**3877**	**2217**	**1.17**	**0.64**	**1.41**	**0.87**

We previously identified four protein kinases in the virion proteome including three putative serine/threonine kinases (ORFs 209R, 380R and 439R) and one tyrosine protein kinase (179R). One putative serine/threonine kinase (380R) is even present in high abundance in CIV virions (Fig. 5) In the CIV genome, nine ORFs encode putative kinases. However, in addition to the four known virion kinases from the virion, we have identified only one additional protein kinase in CIV infected cells (098R).

It is difficult to assign a function of the CIV kinases without expressing them individually in cell culture, unravel their subcellular localization and identify the proteins that are specifically phosphorylated. A recent study on the serine-threonine protein kinase 389L (so called iridoptin) suggested that this kinase is associated with the CIV virion structure and the purified form of the protein induced apoptosis in insect cells (Chitnis *et al.*, 2011).

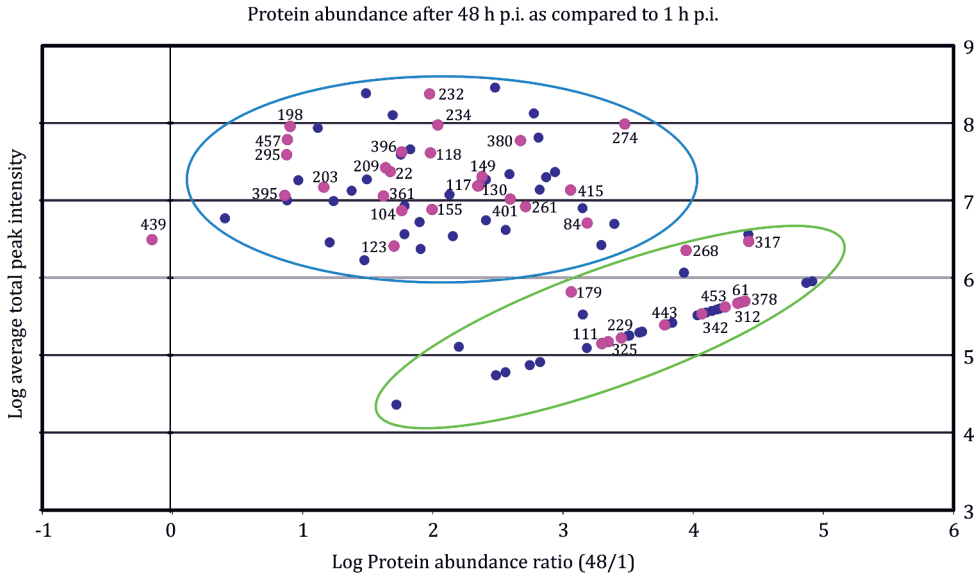

Figure 5. CIV protein abundances in infected cell system (1 h p.i. versus 48 h p.i.)

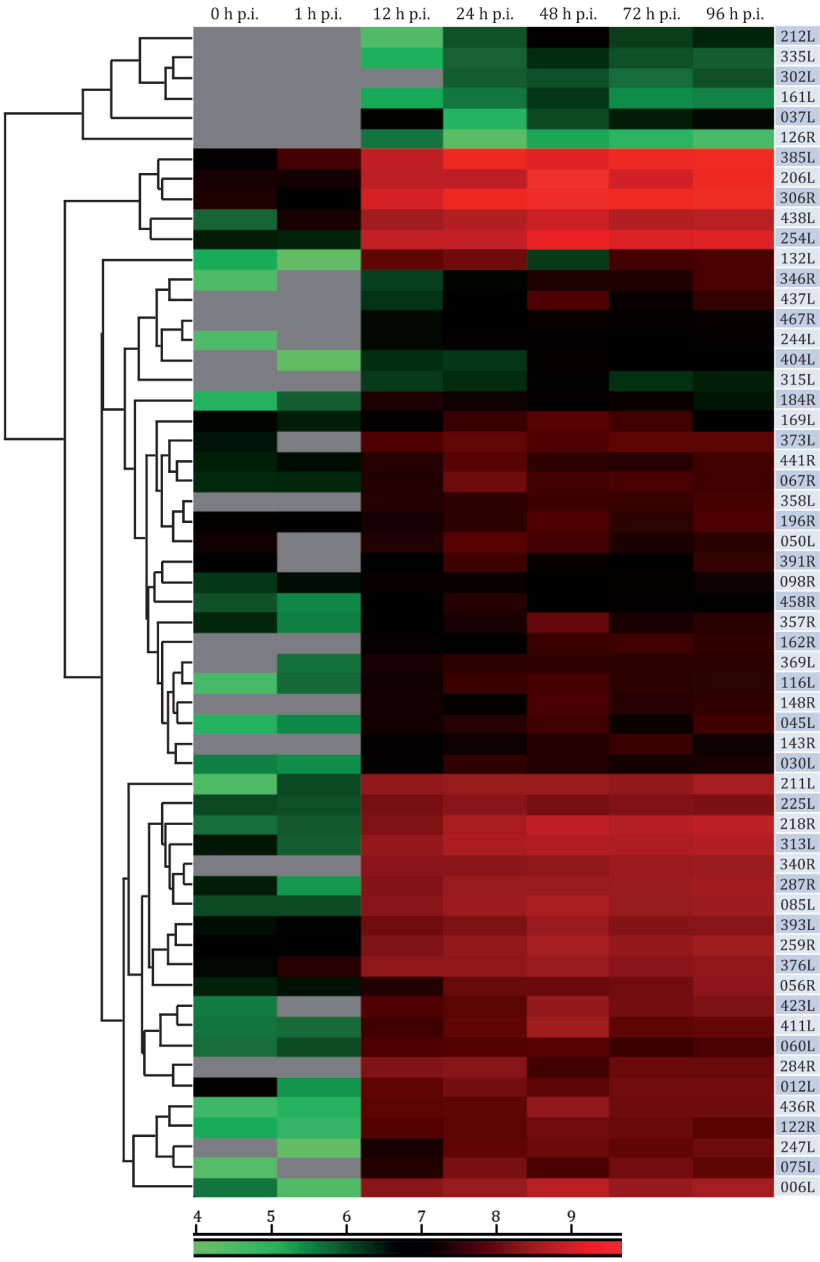

Figure 6. Infected cell viral proteins heat map showing expression levels from 0 to 96 h post infection. The colours represent comparative intensities of protein levels on a scale from 4-10, ranging from green to red, respectively, as indicated in the ruler. Grey bars indicate non-detected proteins. *The colours represent comparative intensities of protein levels on a scale from 4-10, ranging from green to red, respectively, as indicated in the ruler. Grey bars indicate non-detected proteins.

Table 3. Viral protein presence in time compared to transcript class.

ORF	Virion protein presence (h p.i.)	Coverage [%]	Predicted function	Gene ontology
006L	0-1-12-24-48-72-96	33.6	KilA N domain containing protein	Transcriptional regulation
012L	0-1-12-24-48-72-96	19.7	exoribonuclease 2	Intracellular protein involved in nucleic acid metabolism and morphogenesis
030L	0-1-12-24-48-72-96	12.5	DNA repair and recombination	Nucleoside-triphosphatase activity
037L	12-24-48-72-96	5.3	DNA polymerase	DNA-directed DNA polymerase activity
045L	0-1-12-24-48-72-96	11.7	DNA topoisomerase 2	DNA topoisomerase activity
050L	0-12-24-48-72-96	8.7	purine NTPase	dsDNA repair with exonuclease activity
056R	0-1-12-24-48-72-96	16		integral membrane protein
060L	0-1-12-24-48-72-96	16.2		integral membrane protein
067R	0-1-12-24-48-72-96	24.4		integral membrane protein
075L	0-12-24-48-72-96	21	ATPase	Small GTPase mediated signal transduction
085L	0-1-12-24-48-72-96	31.3	Ribonucleotide-diphosphate reductase alpha subunit	Involved in intein-mediated protein splicing pathway
098R	0-1-12-24-48-72-96	10.5	Serine threonine protein kinase	Protein kinase activity
116L	0-1-12-24-48-72-96	47.9		
122R	0-1-12-24-48-72-96	33.6		
126R	12-24-48-72-96	8.1		
132L	0-1-12-24-48-72-96	18.9	Putative zinc finger protein	Nucleic acid binding
143R	12-24-48-72-96	18	Thymidine kinase	Nucleic acid metabolism
148R	12-24-48-72-96	12.3	MSV199 domain	intracellular protein involved in DNA repair
161L	12-24-48-72-96	6.1	Helicase-like protein	DNA replication
162R	12-24-48-72-96	19.9		Unknown metabolic process; flags “precursor”
169L	0-1-12-24-48-72-96	48.9		integral membrane protein
184R	0-1-12-24-48-72-96	3.6	d5 family NTPase; helicase	DNA primase activity
196R	0-1-12-24-48-72-96	27.2	Thioredoxin	Redox homeostasis
206L	0-1-12-24-48-72-96	93.4		
211L	0-1-12-24-48-72-96	31.4	MSV199 domain	Transcriptional regulation
212L	12-24-48-72-96	4.2	MSV199 domain	Transcriptional regulation
218R	0-1-12-24-48-72-96	19.8		
225L	0-1-12-24-48-72-96	29.9	Thymidylate synthase	DNA biosynthesis
244L	0-12-24-48-72-96	9.3	Phosphoesterase	DNA repair; exonuclease
247L	1-12-24-48-72-96	16.4		
254L	0-1-12-24-48-72-96	55.5		
259R	0-1-12-24-48-72-96	44.3		integral membrane protein
284R	12-24-48-72-96	17.7		
287R	0-1-12-24-48-72-96	53.8		
302L	24-48-72-96	5.6	Zinc finger protein	intracellular protein
306R	0-1-12-24-48-72-96	78.1	SWIB domain containing protein	Protein binding
313L	0-1-12-24-48-72-96	38.7	KilA N domain containing protein	Transcriptional regulation
315L	12-24-48-72-96	10	KilA N domain containing protein	Transcriptional regulation
340R	12-24-48-72-96	55.8	Ribonuclease III	dsRNA processing
346R	0-12-24-48-72-96	60.3		
357R	0-1-12-24-48-72-96	25.8		
358L	12-24-48-72-96	23.8		
369L	1-12-24-48-72-96	18.6	Endonuclease	Host cell nucleus localized DNA repair protein
373L	0-12-24-48-72-96	22.7		
376L	0-1-12-24-48-72-96	46.8	Ribonucleoside-diphosphate α subunit	Deoxyribonucleoside diphosphate biosynthesis
385L	0-1-12-24-48-72-96	76.7		
391R	0-1-12-24-48-72-96	9.2		
393L	0-12-24-48-72-96	46.4	Immediate early protein	RNA ligase
404L	1-12-24-48-72-96	23.2		
411L	0-1-12-24-48-72-96	51.2		
423L	0-1-12-24-48-72-96	47.8		
436R	0-1-12-24-48-72-96	32.2		
437L	12-24-48-72-96	18.8		
438L	0-1-12-24-48-72-96	53.6	Deoxyuridine 5’ triphosphate nucleotidohydrolase	dUTP diphosphatase activity
441R	0-1-12-24-48-72-96	38.3		
458R	0-1-12-24-48-72-96	20.9	Myristoylated membrane protein	Integral membrane protein
467R	12-24-48-72-96	8		

However, in our studies on the virion proteome and the infected cell proteome we have not observed a signal for this protein (İnce *et al.*, 2010). It could be that this kinase is minor in the virion proteome or that is has been lost during the virion purification protocol. It remains to be elusive whether 389L is a virion-associated protein or not. Protein kinase activity as a post-translational modification regulates a broad range of cellular activities including the cell cycle, differentiation, metabolism, and communication (Jacob *et al.*, 2011). It is difficult to assign a function of the CIV kinases without expressing them individually in cell culture, unravel the subcellular localization and identify the proteins that are specifically phosphorylated. Basically, infected cell system proposed in this chapter is the most promising toll for unraveling the scientific question related to such a complex virus-host interaction events utilizing large-scale analysis of a phosphoproteome.

As aforementioned, these kinases may play crucial roles to guarantee successful viral infection by modulating pathways. For instance, apoptosis and inhibition of host gene expression (host-cell shutoff) may result from regulation of transcription factors, or the intracellular localization of the gene products, thus determining the fate of the host cell. The detailed roles of the kinases in virus-host interaction in general remain to be elucidated. These kinases are potential antiviral drug targets.

Previously we described virion-associated proteins, which are related to DNA replication, transcriptional regulation and RNA metabolism (Table1-3; Column 4-5). The virion also contains proteins, which may deal with viral DNA replication or mRNA synthesis, including a protein with homology to the N- terminal domain of viral DNA polymerases (232R), nucleoside triphosphatase (NTP I; 22L), and a putative DNA binding protein (401R). DNA binding proteins may be building blocks of the virion structure, but may also be trans-activator proteins that induce transcription of (a set of) genes. IIV genomes also contain other genes associated with RNA metabolism (e.g. Ribonuclease III; 142R), which was identified in the CIV virion structure and recently also in that of IIV-9 (Wong *et al.*, 2011). Ribonuclease III, one of the iridovirus core genes, is correlated to the microRNA pathway (Wong *et al.*, 2011) and may be one of the main players in virus-host interaction. The microRNA mechanism is relatively conserved among different organisms (Cullen & Umbach, 2009) and needs to be investigated in a variety of virus-host systems to obtain fundamental insights in viral pathogenesis in a background of host defence/immune responses.

In the current analysis, it alternative viral proteins with relatively similar functions such as being involved in DNA replication were also found in infected cells. This observation calls for further research as to why CIV would encode proteins with similar functions from different genes, proteins that end up in different appearances.

From our proteomic study we see that the protein levels for virion proteins determined to be expressed from early genes in the previous study (Table 1) remained substantial (e.g. 022L, 123R) over time even after the appearance of late proteins such as MCP (274L). Whether this reflects continuing transcription of early genes, translation of remaining early transcripts or persistence of earlier translation products cannot be concluded from the current experiments. From infections with the vertebrate iridovirus *Frog virus 3* we know that *early* transcripts can be present during *late* transcription (Chinchar & Yu, 1992), and this may also by the case for CIV. Pulse-chase experiments on specific viral proteins would answer this question.

The present study is the first to apply a label-free approach for virion protein detection and quantitation of individual protein levels in an iridovirus-infected cell model. For the virion proteins, it was not possible to cluster them in the same way as the transcripts in three classes using inhibitors (Chapters 3), probably due to the nature of the viral infection, which involves and requires protein input from the virus particle. CIV-infected cells treated with cycloheximide, which only allows expression of immediate early genes, showed for instance abundant late proteins such as MCP (274L). This may be residual protein from incoming virus. So the temporal class of the CIV virion protein genes could only be delineated by using transcriptome data (Table 1). However, the proteomic data may lead to a new way of CIV protein classification based on the protein presence profile over the course of infection and proteins can be divided different temporal expression subsets in protein expression level. Especially interesting protein cluster does the one comprise proteins that are present in the beginning and at later time points, but absent in the middle. These proteins may be required to fulfill crucial roles in the immediate early phase of infection.

Five proteins previously detected in the virion proteome (İnce *et al.*, 2010) were detected neither by the transcriptomic nor the proteomic approaches applied in this study. These are 010R (zip protein), 096L (fasciclin domain protein), 307L (Uvr/Rep helicase), 355R (ctd-like phosphatase) and 366R with unknown function. The possible explanation would be the low copy number of transcripts as well as very low concentration of the corresponding proteins, which are under the detection threshold of experimental applications.

This study shows that many CIV virion proteins are present early in infection, either as a result from early transcription or by introduction via the parental virus particle. The information obtained previously on the temporal classes of individual genes (see Chapter 3) and the present profile of the corresponding proteins may assist us to identify virion-associated proteins and virus-induced proteins that control viral gene expression.

Acknowledgments

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Chapter 5

The *Chilo iridescent virus* DNA polymerase promoter contains an essential AAAAT motif

Abstract

The delayed-early DNA polymerase promoter of *Chilo iridescent virus* (CIV), officially known as *Invertebrate iridescent virus 6,* was fine mapped by constructing a series of increasing deletions and by introducing point mutations. The effects of these mutations were examined in a luciferase reporter gene system using *Bombyx mori* cells transfected with promoter constructs and infected with CIV. When the size of the upstream element was reduced from position -19 to -15 relative to the transcriptional start site, the luciferase activity was reduced to almost zero. Point mutations showed that each of the five nucleotides (AAAAT) located between –19 and –15 were equally essential for promoter activity. Mutations at individual bases around the transcription initiation site showed that the promoter extended until position -2 upstream of the transcription start site. Southwestern analysis showed that a protein of approx. 100 kDa interacted with the -19 nt promoter fragment in CIV infected cells, this binding did not occur with a point mutant that lacked promoter activity. The AAAAT motif was also found in DNA polymerase promoter regions of other iridoviruses and in other putative CIV delayed early genes.[9]

[9] This chapter published in Journal of General Virology 88: 2488-2494, 2007.

Introduction

Chilo iridescent virus is the type member of the genus *Iridovirus*, family *Iridoviridae* (Fauquet *et al.*, 2005). Viruses within this family characteristically have linear double stranded DNA that is both circularly permuted and terminally redundant (Darai *et al.*, 1983, Delius *et al.*, 1984, Goorha & Murti, 1982). Up to now, 15 complete sequences of iridovirus genomes have been determined (Eaton *et al.*, 2010). One of these is the CIV genome, which has a size of 212,482 base pairs (Jakob & Darai, 2002, Jakob *et al.*, 2001). The *Iridoviridae* along with *Poxviridae* and *Asfarviridae* (Fauquet *et al.*, 2005) display complex replication and gene regulation strategies. For *Frog virus 3* (FV3), a vertebrate iridovirus belonging to the genus *Ranavirus,* it was shown that the initial round of DNA synthesis takes place in the host cell nucleus (Goorha *et al.*, 1978), while later in infection DNA concatemer formation and assembly into mature virions occur in the cytoplasm (Goorha, 1982, Goorha & Murti, 1982). Studies on infected cell-specific polypeptides provided evidence for a temporally regulated pattern of gene expression dividing the CIV genes into three classes: immediate-early (IE or α), delayed-early (DE, β) and late (L, γ) (Barray & Devauchelle, 1987) genes and demonstrated a regulation cascade with both positive and negative control mechanisms (D'Costa *et al.*, 2001, D'Costa *et al.*, 2004). The transcription cascade was analyzed in detail for Red Sea bream iridovirus (RSIV) belonging to the genus *Megalocytivirus* (Lua *et al.*, 2005). By definition, IE gene expression does not require *de novo* protein synthesis. DE and L genes on the other hand do require some IE or DE gene products, respectively, to be transcribed. Transcription of immediate early genes occurs also in the presence of inhibitors of protein synthesis, like cycloheximide, indicating that these genes are transcribed by a host DNA-dependent RNA polymerase. Host RNA polymerase II is required for the synthesis of ranavirus IE RNAs (Goorha, 1981) and it is likely that this is also the case for CIV IE genes. Transcription of CIV IE genes also requires a virion-associated protein, since purified CIV DNA is not infectious by itself, as is also the case for ranavirus DNA (Willis & Granoff, 1985), but can be reactivated by adding viral protein in the form of UV-inactivated virions (Cerutti *et al.*, 1989).

Promoter studies have been performed for two IE genes of FV3 (ICR-169 and ICR-489) (Beckman *et al.*, 1988, Willis, 1987), and for two early (ICP-18 and ICP-46) and one late (major capsid protein) gene of Bohle iridovirus (BIV) (Pallister *et al.*, 2005), which also belongs to the genus *Ranavirus.* Potential promoter regions have been determined for two CIV genes encoding the DNA polymerase (*DNApol*; ORF 037L) and the major capsid protein (*mcp*; ORF 274L), respectively (Nalcacioglu *et al.*, 2003). Transcription of the early DNApol gene initiated 35 nt upstream of the translational start site and the late MCP transcripts initiated 14 nt upstream of the AUG codon. Using a luciferase reporter gene assay (Nalcacioglu *et al.*, 2003) showed for *DNApol*, that sequences between position -27 and -6 relative to the transcriptional start site harbored promoter activity. For *mcp* this activity is located between position -53 and -29. Both the *DNApol* and the *mcp* promoters were not active in the absence of virus infection, suggesting that their activity was dependent on a protein expressed earlier in the cascade, as expected for delayed-early or late genes, or a protein associated with the virus particle.

In the present study, the promoter region of the CIV *DNApol* gene was analyzed in detail by generating a further series of deletion mutants and introducing point mutations in the core promoter region. Transcriptional analysis classified *DNApol* as a delayed-early gene and mutagenesis indicated that nucleotides from position -19 to -15 (AAAAT) are equally important for transcriptional activity. The interaction of viral proteins with the -19 promoter element was studied.

Materials and methods

Cells and virus

Bombyx mori SPC-BM-36 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and grown in Grace's insect medium supplemented with 10% fetal bovine serum (Invitrogen) at 27 C. *Chilo iridescent virus* (CIV or *Invertebrate iridescent virus 6*) was kindly supplied by C. Joel Funk (USDA-ARS Western Cotton Research Laboratory). The virus was propagated in larvae of the wax moth, *Galleria mellonella*, purified and quantified using UV spectroscopy (Marina *et al.*, 1999).

Transcript analysis in the presence of inhibitors

SPC-Bm-36 cells were infected with 5 g/ml of CIV particles (D'Costa *et al.*, 2001). Appropriate cultures were pretreated 1 h before infection with cycloheximide (CHX) or Ara-C at final concentrations of 200 and 100 g/ml, respectively to inhibit either protein or DNA synthesis. Total RNA was isolated from cells at 0 h p.i. or at 24 h p.i using Trizol (Invitrogen) according to the manufacturer's instructions. For RT-PCR analysis, 2 g of total RNA from CIV infected cells was reverse transcribed using 10 units of Superscript II reverse transcriptase (Invitrogen), 10 units of RNasin (Promega) and a specific reverse primer for *DNApol* (DNApol R) in a total reaction volume of 20 l. The cDNA's obtained were amplified by PCR using specific forward and reverse primers (DNApol F and DNApol R, see Table 1). PCR products were analyzed in a 1.5% agarose gel stained with ethidium bromide. Control reactions in which the RT step was omitted were also performed. To verify the activity of cycloheximide we also performed an RT-PCR for the late MCP transcripts (not shown).

Promoter constructs with deletions and point mutations

Upstream sequences for *DNApol* starting at positions -19 and -15 were amplified by PCR from the promoter construct, DNApol PC-247 (Nalcacioglu *et al.*, 2003), a derivative of pSPLuc+ (Promega), which contains a DNApol upstream element of 247 bp, its 5' untranslated region and the first nine nucleotides (nt) of the *DNApol* ORF fused to a luciferase reporter gene. The forward PCR primers (DNApol F Primers) introduced *Bgl*II restriction sites (see Table 1). At the 3' end a primer (Luc R *Nar*I) was used that annealed downstream of the *Nar*I site in the luciferase open reading frame (ORF) of the pSPLuc+ vector (Fig. 4A).

In this way, the size of the PCR products was increased to facilitate their cloning. The resulting DNA fragments were cloned between the *Bgl*II and *Nar*I sites of pSPLuc+, thereby generating constructs PC-19 and PC-15 (see Fig. 4A). The deletion constructs named as PC-86 and PC–27, which starting at -86 and -27 upstream of the transcription initiation site, have been constructed in a similar way (Nalcacioglu *et al.*, 2003). Point mutations in the upstream region of *DNApol* gene were introduced with using two-step PCR amplification. In the first step, the forward primer DNApol F-247 and a DNApol R Mut primer (see Table 1) were used to amplify mutant *DNApol* upstream regions from the original promoter construct, PC 247. The resulting DNA products were column purified from gel (Roche) and used as 5′ mutagenic primers in the second PCR together with a 3′-reverse primer annealing to the luciferase ORF (primer Luc R *Nar*I). The products of the second PCR were digested with *Bgl*II and *Nar*I, cloned in pSP-Luc+ and verified by automated sequencing (Baseclear). In this way, a series of plasmid constructs were obtained as outlined in Table 1 and Fig. 1B. Construct PC-19 was used as template for the mutant -19 (PC mut-19), in which the A at -18 was converted into a C. For this amplification the forward primer DNApol F mut-19 and the reverse Luc R *Nar*I primer were used (Table 1, Fig. 1B).

Transfections and luciferase assays

SPC-BM-36 cells were seeded at a density of 1.5 x 10^6 cells/35 mm tissue culture dish in Grace's supplemented medium without serum (Invitrogen). The cells were transfected using Cellfectin (Invitrogen) with 2 g of the DNApol plasmid constructs and 2 g of a control plasmid to normalize for variations in the efficiency of transfection. This control plasmid, pIC-IE-1 (Nalcacioglu *et al.*, 2003) contains the *Autographa californica* nucleopolyhedrovirus immediate early 1 (IE1) promoter (Jarvis *et al.*, 1996) upstream of the *Renilla* luciferase reporter gene in the pRL-null vector (Promega). Cells were infected with CIV at a concentration of 5 g/ml 18 h post transfection and further incubated at 27 C. Cells were harvested 6 h post infection with CIV. Transfections were carried out in triplicate, and firefly and *Renilla* luciferase activities were measured in cell extracts using the Dual luciferase reporter assay system (Promega) following the manufacturer's instructions.

Preparation of nuclear extracts

Nuclear extracts were prepared based on an existing protocol (Blissard *et al.*, 1992). A shaker culture of 250 ml containing 3x10^6 SPC-BM-36 cells per ml Grace's insect medium were either mock-infected or infected with CIV at a concentration of 5 g/ml culture. At 8 h post infection cells were pelleted at 2000 rpm and resuspended in five packed cell pellet volumes of buffer A (10 mM Tris-HCl pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM Pefabloc C) and incubated for 10 min on ice. The cells were collected as previously by centrifugation, then suspended in two packed cell pellet volumes of buffer A and lysed by 10 slow strokes in a Dounce homogenizer. At this step, a sample was checked microscopically for cell lysis using trypan blue. The homogenate was centrifuged for 10 min at 2500 rpm to pellet the nuclei. The pellet was resuspended in an equal volume of buffer C (20 mM Tris-HCl pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.2 mM Pefabloc C) by repetitive pipetting (10X) and homogenized with 15 slow strokes in a Dounce homogenizer. The resulting

homogenate was stirred slowly for 30 min at 4°C. Nuclear membranes were removed from the extract by centrifugation at 14.000 rpm for 30 min at 4°C. The supernatant was dialyzed against 70 volumes of buffer D (20 mM Tris-HCl pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20 % glycerol, 0.5 mM DTT, 10 mM 2-mercaptoethanol) for approximately 3 h at 4°C. After dialysis, the solution was centrifuged for 15 min at 14.000 rpm and aliquots of 50 l were frozen in liquid nitrogen and transferred to -80°C for storage. The protein concentration was determined by the method of Bradford (1976).

Table 1. Oligonucleotides used for the preparation of the various promoter constructs[a]

Primer Name	Sequences (5'-3')	Position	Construct names
DNApol F-247	GG**AGATCT**CGTGAAGGCAAATGATGA	-247/-230	PC-247
DNApol F-19	GG**AGATCT**AAAATTGATTATTTGTTTTC	-19/+1	PC-19
DNApol mut-19	GG**AGATCT**ACAATTGATTATTTGTTTTC	-19/+1	PC mut-19; ($18_{A\rightarrow C}$)
DNApol F-15	GG**AGATCT**TTGATTATTTGTTTTCG	-15/+2	PC-15
DNApol F-6	GG**AGATCT**GTTTTCGAAGAGATTTAAAAAA	-6/+17	PC-6
DNApol R Mut KKK	CTTCGAAAACAAATAATCAAKKKTTTATGGC TGTATTTTAAAACCAC	+5/-42	PC-18 $_{A\rightarrow C}$ PC-17 $_{A\rightarrow C}$ PC-16 $_{A\rightarrow C}$
DNApol R Mut MRR	CTCTTCGAAAACAAATAATMRRTTTTTTATG GCTGTATTTTAAAACCAC	+7/-42	PC-$15_{A\rightarrow C}$
DNApol R Mut KMR	GTACATTTAATTTTTTAAATCTCTKMRAAA ACAAATAATCAATTTTTTATGGCTG	+27/-28	PC+1,2,$3_{CGA\rightarrow TTC}$ PC+1,$2_{CG\rightarrow TT}$ PC+1 $_{C\rightarrow T}$
DNApol R Mut KMK	GTACATTTAATTTTTTAAATCKMKTCGAAA ACAAATAATCAATTTTTTATGGCTG	+27/-28	PC+ 5,6 $_{GA\rightarrow TC}$ PC+5 $_{G\rightarrow T}$
DNApol R Mut TTRR	CATTTAATTTTTTAAATCTCTTCGAARRCAA ATAATCAATTTTTTATG	+24/-24	PC-2 $_{T\rightarrow C}$ PC-1 $_{T\rightarrow C}$
DNApol R Mut RRTT	CATTTAATTTTTTAAATCTCTTCGRRAACAA ATAATCAATTTTTTATG	+24/-24	PC-4 $_{T\rightarrow C}$ PC-3 $_{T\rightarrow C}$
DNApol R Mut-19	CGAAAACAAATAATCAATTTGTTATGGCTGT ATTTTAAAACC	+2/-40	PC-19 $_{T\rightarrow C}$
DNApol R Mut TA	GTACATTTAATTTTTTAATACTCTTCGAAAA CAAATAATCAATTTTTTATGGCTG	+27/-28	PC+ 8,9 $_{AT\rightarrow TA}$
Luc R *Nar*I	GGAATGGCGCCGGGCCTTTCTTTATG	85/62 (Luc)	-
DNApol F	CAAGGAACAAGAACATATAAG		-
DNApol R	CACCTCTTTGTCCCATTTTAGG		-

[a]Restriction sites were added to the gene specific DNApol primers to assist cloning into the reporter vector pSP-Luc$^+$; *Bgl*II, printed in bold. The Luc R *Nar*I primer annealed to the pSP-Luc$^+$ vector downstream of the *Nar*I site. DNApol F and R were used for RT-PCR. Positions of primers are given relative to the transcript start site.

Southwestern analysis

Nuclear extracts (50 g) prepared as described above were subjected to electrophoresis in a 0.1 % SDS-12% polyacrylamide gel. Separated proteins were transferred to PVDF membrane. Blotted proteins were completely denatured in 6 M guanidinium hydrochloride solution prepared in PBS and then stepwise renatured by serially diluting the guanidinium chloride solution. Membranes were further incubated overnight in PBS solution containing 5% fat free milk and then washed in PBS solution containing 0.5% fat free milk. Protein-DNA binding reactions were performed in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 with 1% blocking powder containing DIG-labeled –19 and mut-19 probes overnight at 4°C.

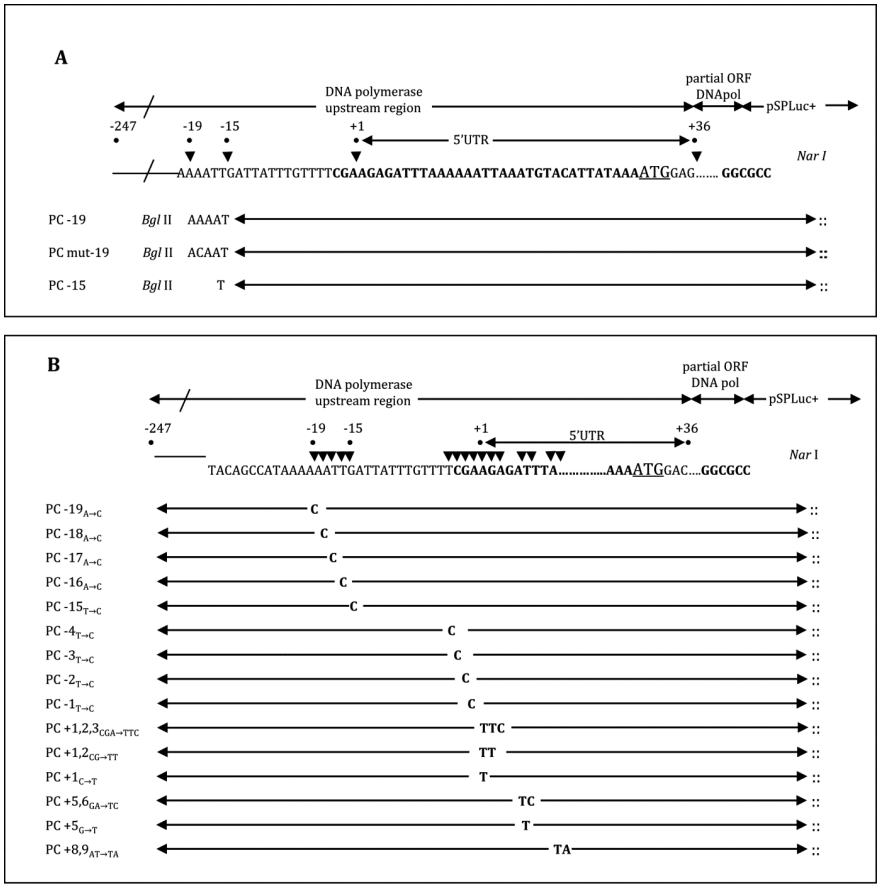

Figure 1. Representation of *DNApol* promoter deletion and point mutation constructs. Upstream DNA fragments decreasing in length (A) and with mutations at different points (B) were fused to a luciferase reporter gene. The sequences fused to luciferase included the original CIV translational start site and nine additional nucleotides derived from the *DNApol* ORF. The positions of the point mutations are given by the letters corresponding to the bases that were changed; the size of the DNA fragment included in the constructs is illustrated by the arrowed lines.

These probes were prepared by digesting the plasmids DNApol PC-19 and PC mut-19 with *Bgl*II and *Nar*I, followed by purification of the resulting in 113 bp fragments from agarose gel. Terminal transferase was used to label the DNA fragments (100 ng) at the 3'ends with DIG-11-ddUTP according to the DNA Gel Shift Kit protocol (Roche). For competition assays, the membranes were incubated with labeled -19 probes in the absence or presence of 1 or 10 fold excess of unlabeled -19 or mut-19 fragments. After the incubation with the probes, the membranes were washed at room temperature for 30 min with PBS containing 0.5% milk and 0.3% Tween-20. Membranes were further incubated for 1 h at room temperature with anti-DIG (1:20000) diluted in 0.5% milk in PBS and then washed 3 times 10 min with PBS-Tween-20, equilibrated with detection buffer. Chemiluminescent detection was performed with the CSPD® substrate (Roche Diagnostics).

Results

Classification of the DNA polymerase promoter

Previous studies suggested that the *DNApol* gene was controlled by an IE promoter (Nalcacioglu *et al.*, 2003). If this would be true, the CIV *DNApol* gene would behave different from its homologues in other large DNA viruses including vaccinia and baculoviruses (Blissard *et al.*, 1992). To elucidate this point, the transcriptional analysis based on RT-PCR was repeated using fresh batches of protein and DNA synthesis inhibitors and fresh RNA samples. Controls without a reverse transcriptions step were negative for all RNA samples (not shown). In the previous paper, this latter control was not performed and the PCR product observed in the presence of cycloheximide may have arisen from amplification of residual CIV DNA in the RNA sample. To verify that cycloheximide functioned properly we also checked that the late *mcp* transcript was not formed in the presence of this inhibitor (not shown). The experiments performed showed that *DNApol* was easily detected when no inhibitors were present or in the presence of Ara-C, an inhibitor of DNA synthesis. *DNApol* transcription was however inhibited by cycloheximide (Fig. 2). This new study classifies the *DNApol* gene as a delayed-early (DE) gene, since protein expression is needed for its expression. This brings the regulation of the CIV *DNApol* in line with the expression pattern of RSIV DNA polymerase (Lua *et al.*, 2005) as well as that of other large DNA viruses.

Mutational analysis of the *DNApol* promoter

Previous results revealed that sequences between -27 and -6 relative to the transcription initiation site were essential for promoter activity of the CIV *DNApol* gene by linking the *DNApol* promoter region to a luciferase gene copy (Nalcacioglu *et al.*, 2003). For further mapping the promoter region of this gene, sequences starting at -19 or -15 were fused to a firefly luciferase reporter gene and tested in a transient expression assay. These constructs included the 5' untranslated sequences as well as the first nine nucleotides of the open reading frame of *DNApol* to prevent loss of promoter activity in case the promoter region would extend over the ATG (see Fig. 1A). The luciferase reporter was expressed efficiently when it was linked to the promoter fragment starting at -19 (Fig. 3A; PC-19) and the level of expression was comparable to that observed for the constructs starting at -27 (PC-27). This level was about 25% lower than in the -247 and -86 constructs (PC-247 and PC-86), in agreement with previous results (Nalcacioglu *et*

al., 2003). Expression was drastically reduced (over 80%) when the length of the upstream region was reduced from -19 to -15 nt (PC-15; Fig. 3A). This result showed that the core promoter starts before position -15. To confirm the importance of the three adenine residues located between -19 and -15 for promoter activity (see Fig. 1A), these adenines were modified by changing the A's one by one into C's in a 247 nt long promoter construct (PC-247). In that way, the plasmids PC-$18_{A\rightarrow C}$, PC-$17_{A\rightarrow C}$ and PC-$16_{A\rightarrow C}$ were constructed (Fig. 1B).

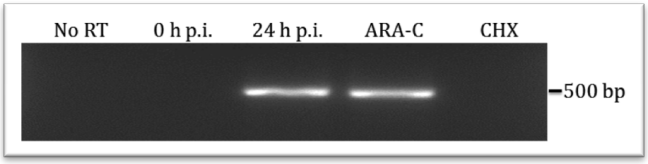

Figure 2. Determination of the temporal class of DNA polymerase transcripts. Total RNA was obtained from CIV-infected cells and amplified by PCR (lane 1) or by RT-PCR at 0 h p.i. (lane 2), 24 h p.i. (lane 3) and after 24 h in the presence of Ara-C (lane 4) or cycloheximide (lane 5) using primers specific for *DNApol* transcripts.

These mutations reduced the amount of luciferase synthesized by approximately 80-90% compared to the level found with the wild type 247 nt construct (Fig. 3B). This result confirmed the deletion studies (Figs. 1A, 3A) and showed that each of these three A's is required for promoter activity. In order to determine whether the core promoter starts at the A at -19 and includes the T at -15, these nucleotides were individually changed into a cytosine residue in the 247 nt promoter construct. Transfection results of the PC-19 $_{A\rightarrow C}$ and PC-15 $_{T\rightarrow C}$ constructs showed that the A at position -19 is the starting base essential for promoter activity and that the T at -15 is also crucial (Fig. 1B, 3B). The 3'end of the promoter unraveled by individual or in small groups mutation(s) at four bases upstream and six bases downstream of the transcriptional start site (position +1) resulting in 10 new mutant constructs (PC-$4_{T\rightarrow C}$, PC-$3_{T\rightarrow C}$, PC-$2_{T\rightarrow C}$, PC-$1_{T\rightarrow C}$, PC+1,2,$3_{CGA\rightarrow TTC}$, PC+1,$2_{CG\rightarrow TT}$, PC+$1_{C\rightarrow T}$, PC+5,$6_{GA\rightarrow TC}$, PC+$5_{G\rightarrow T}$, and PC+8,$9_{AT\rightarrow TA}$; see Fig. 1B). Mutations at each of the three bases from -4 to -2 relative to the transcription initiation site reduced promoter activity with 25-40% (Fig. 3B). Mutation on the base just before the transcription initiation site (PC-$1_{T\rightarrow C}$) did not affect promoter activity. Changing single bases downstream of the transcription initiation site did not reduce promoter activity either (Fig. 3B). These results showed that the *DNApol* promoter did not extend beyond the T at -2 position. The bases -4 till -2 played a less prominent role in promoter activity than the AAAAT motif located from -19 until -15. The role of the individual nucleotides between positions -14 and -5 has not been analyzed further in this study.

Southwestern Blot Analysis

To study whether viral proteins bind to the -19 promoter fragment Southwestern analysis was performed. Therefore, nuclear extracts were prepared of either mock or CIV-infected *Bombyx mori* cells. The proteins in these extracts were separated by SDS-PAGE and electroblotted to PVDF membranes. When the membranes were incubated with a -19 promoter fragment end-labeled with DIG-11-ddUTP, a protein was detected with an estimated size of 100 kDa (Fig. 4A). This protein-DNA interaction was not observed with nuclear extracts of mock-infected cells (Fig. 4A), indicating that a viral protein or a virus-induced protein interacted with

the *DNApol* promoter, in line with the finding that DNApol is a DE gene. In order to show that this interaction was specifically directed against the CIV DNA polymerase promoter and not to any DNA fragment, a mutated -19 fragment was used as probe, in which the A at -18 was changed into a C. In this case, no protein interaction was observed (Fig. 4B). A similar result was obtained with the -15 promoter fragment as probe (not shown). The unlabeled -19 probe was able to compete with the labeled -19 probe for binding in a competition assay (Fig. 4C), while the mutated -19 probes did not compete with the -19 probe (Fig. 4D).

Discussion

We previously reported that the CIV *DNApol* gene has a 35 nt region as 5'-untranslated region (UTR) and that sequences within 27 nucleotides relative to the transcriptional start site (at +1), were important for promoter activity (Nalcacioglu *et al.*, 2003). The study presented here reveals that a small region of 19 bp (AAAATTGATTATTTGTTT), located between -19 and -2 relative to the mRNA start site, is responsible for the promoter activity of the *DNApol* gene. Mutations in the AAAAT motif in this region have a major effect on promoter activity showing that this motif is an essential part of the core promoter structure. Mutations at the downstream side have less effect (-4, -3, -2). The role of individual nucleotides positioned at -14 to -5 was not analyzed in this study. The *DNApol* promoter as a whole does not show a common structure with other CIV genes. In a similar way, the critical promoter sequence of the ICR169 IE promoter of FV3 (ATATCTCACAGGGGAATTGAAAC) is also not conserved in other FV3 genes (Willis, 1987).

On the other hand, the critical AAAAT motif was found by *in silico* analysis of the CIV genome sequence (Jakob *et al.*, 2001) in the 100 nt upstream region of the putative translational start codons of several other putative CIV DE genes including two exonucleases (012L and 244L), a topoisomerase II (045L), an endonuclease homolog (369L), a helicase (161L), a ligase (205R), the largest subunit (176R) and the five small subunits (107L, 343L, 349L, 428L and 454R) of DNA-dependent RNA polymerase, two subunits of ribonucleoside diphosphate reductase (085L and 376L), thymidylate synthase (225R), thymidylate kinase (251L), nucleoside triphosphatase (022L), thioredoxin (453L) and two possible apoptosis inhibitors (157L and 193R). Many homologues of the genes listed here also have an AAAAT motif in close proximity of their start codon in the other completely sequenced IIV-3 (Delhon *et al.*, 2006).

A striking resemblance was also found when the region upstream of the DNA polymerase ORF was compared to the corresponding region of 12 other completely sequenced iridovirus genomes. Eight of these viral genomes showed a similar AAAAT motif in the DNA polymerase upstream region including IIV-3. The three sequenced ranavirus genomes shared the related TAAAT motif in their DNA polymerase promoter regions. Whether this points towards a conserved regulation of delayed early promoter activity in iridoviruses needs to be established. The distance of these motifs to the start of the *DNApol* ORF varied between 44 and 105 nt. The *DNApol* gene of *Lymphocystis disease virus* 1 (LCDV-1) lacks such a motif within reasonable distance of the ORF.

The temporal expression of three classes of CIV mRNA molecules during the course of infection (D'Costa *et al.*, 2001) suggests that both cis-acting DNA sequences and trans-acting regulatory factors interact at specific times post infection to initiate transcription of the appropriate mRNAs. Immediate early (IE) genes do not require *de novo* protein synthesis for

their expression. It has previously been shown that purified CIV DNA is not able to start infection unless complemented with UV-irradiated virus particles (Cerutti *et al.*, 1989), suggesting that for IE gene expression in CIV a virion-associated protein is required. Delayed early (DE) transcripts require at least one earlier gene product for their expression. In this study, we found a protein of approximately 100 kDa interacting with the active site of the DNApol promoter and may represent a transactivator protein. This protein is considered virus-specific or virus-induced because in mock-infected control cells, no binding was observed. Nuclear extracts prepared at different time points post infection and in the presence or absence of protein synthesis inhibitors may help to determine the timing of expression of this 100 kDa protein in the expression process. Although a single DNA binding protein was observed for the delayed-early DNApol promoter, its expression may require more transactivating proteins. This could be the case if these proteins form a multimeric complex with non-identical subunits in which only the 100 kDa protein has affinity for DNA. This kind of indirect interaction of proteins with DNA has been observed for instance in phage T4 systems where two phage-specified proteins bind DNA only if the T4 DNA polymerase is also present (Huang & Buchanan, 1974).

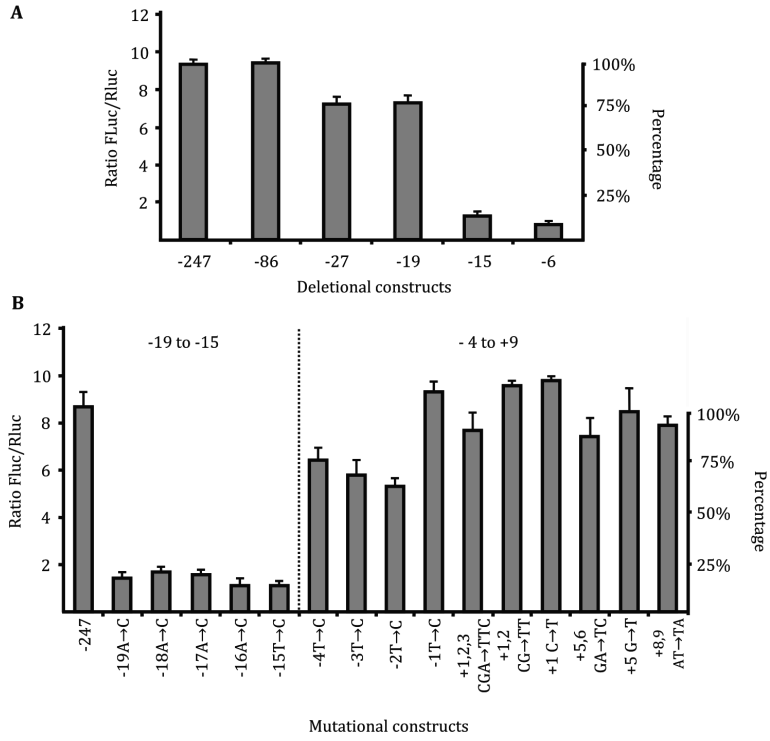

Figure 3. Promoter analysis of the CIV *DNApol* gene. SPC-Bm-36 cells were transfected with constructs containing deletion (A) or point mutations (B) of the DNApol promoter fused to a luciferase reporter gene. After transfection cells were infected with CIV. Firefly luciferase activities were normalized based on the activity of *Renilla* luciferase, which was expressed from a co-transfected control plasmid containing the baculovirus IE-1 promoter. The relative proportion gives the ratio firefly/*Renilla* luciferase versus the ratio obtained for the positive -247 control construct, which is set at 100%.

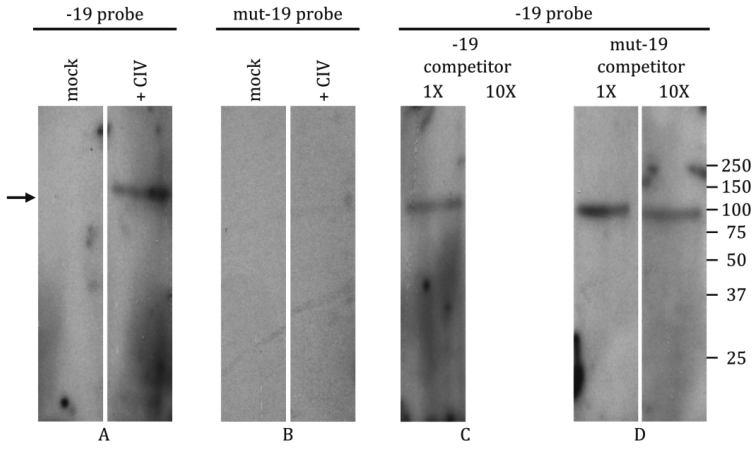

Figure 4. Interaction of the *DNApol* core promoter with nuclear proteins of mock– and CIV infected SPC-Bm-36 cells. Nuclear extracts were subjected to 12% SDS-PAGE and electroblotted on to PVDF membrane. The blots were probed with -19 (A) or mutant -19 (19*; B) promoter fragments, which were end-labeled with DIG-11-ddUTP. The arrow indicates an approximately 100 kDa protein which binds to the -19 promoter fragment. Competition assays were performed on blots probed with labeled -19 probes in the presence of 1 or 10 fold excess of unlabelled -19 (C) or mutant -19 (mut-19) fragments as competitor (D).

In addition, DNA binding proteins that require specific ions or cofactors for their binding to DNA or proteins that bind outside the region used for the probe may modulate the level of expression will be missed by the approach used. Such a protein is expected for the region between positions -86 and -47, removal of which has a negative effect on promoter activity (Nalcacioglu *et al.*, 2003) and may be identified in a similar way with nucleotide stretches of this region as probe.

Until now, there are only two reports about DNA binding proteins in the *Iridoviridae* family. Twelve virus-induced DNA binding proteins were found in FV3 infected cells by DNA affinity chromatography (Goorha, 1981). These proteins have molecular weights ranging from 14 to 119 kDa. The DNA binding protein interacting with the DNApol promoter in CIV-infected nuclear extracts has a molecular mass of approximately 100 kDa. In the CIV genome sequence a total of 468 ORFs (of which 211 non-overlapping) have been identified (Jakob *et al.*, 2001) and molecular masses around 100 kDa are predicted for 022L, 045L, 050L, 085L, 176R, 179R, 184R, 261R, 295R, 396L and 428L ORFs. When these gene candidates were examined for their possible roles as transactivator of the DNApol promoter, we noted that 045L (DNA topoisomerase II), 176R and 428L (the large and a small subunit of DNA-dependent RNA polymerase) have predicted functions associated with DNA. This hypothesis could be addressed by expressing the candidate proteins in a heterologous system and validating their binding specificity to the -19 mutation probe.

Acknowledgements

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Chapter 6

Open reading frame 193R of *Chilo iridescent virus* encodes a functional inhibitor of apoptosis (IAP)

Abstract

Programmed cell death or apoptosis is a major defense mechanism in insects in response to viral infections. The genome of *Chilo iridescent virus* (CIV) has three ORFs with homology to baculovirus *inhibitor of apoptosis (iap)* genes. The proteins encoded by the 157L, 193R, and 332L ORFs contain 152, 208 and 234 amino acids, respectively. While all three proteins contain C-terminal RING domains, only the protein encoded by ORF 193R contains a baculoviral IAP repeat (BIR) domain, indicative of a putative IAP protein. The 193R protein has 28 and 27 percent similarity in amino acid sequence to the *Orgyia pseudotsugata* MNPV and *Cydia pomonella* granulovirus IAP-3 proteins, respectively. ORF 193R from CIV is the only gene known to exist among members of the family *Iridoviridae* that encodes a BIR domain. 193R is transcribed early during CIV infection, and its transcription is not dependent on the synthesis of early viral proteins. When this putative CIV IAP was transiently expressed in SPC-BM-36 and Sf21 cells under the control of an immediate early baculovirus promoter it significantly reduced apoptosis induced by actinomycin-D. Silencing of the CIV *iap* gene (193R) in CIV-infected SPC-BM-36 cells with 193R-specific dsRNA resulted in apoptosis. Thus, CIV ORF 193R is the first *iap* gene identified in an iridovirus, which encodes a functional IAP protein.[10]

[10] This chapter published in Virology Virology 376: 124-131, 2008.

Introduction

Apoptosis, or programmed cell death, is a highly conserved and integral process necessary for tissue remodeling and normal organism development. It removes unwanted, damaged or mutated cells from the system. In addition it provides a cellular defense mechanism against oncogene expression and viral infection (Miller, 1997, Shi, 2002, Zimmermann *et al.*, 2001) and is therefore part of the innate immune system. Apoptosis is characterized at the cellular level by typical morphological features such as cell and nuclear shrinkage, cytoplasmic blebbing, and nuclear and cytoplasmic fragmentation, and in most cases genomic DNA is fragmented, as a result of apoptosis, producing a classical DNA ladder upon agarose gel electrophoresis (Clem & Miller, 1994, Deveraux & Reed, 1999).

In viral infection, apoptosis starts in the early stage of infection in order to minimize viral replication and to prevent cell to cell transmission of progeny virus. Many viruses, however, have evolved evasion mechanisms by producing anti-apoptotic proteins to secure the production of progeny virus and enhance the spread of viral infection to neighboring cells (Everett & McFadden, 2001, Razvi & Welsh, 1995).

The best studied viral anti-apoptotic genes to date are the baculovirus *p35* and inhibitor of apoptosis (*iap*) genes (Clem, 2007). P35 is found in the baculoviruses *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), *Bombyx mori* (Bm) NPV and in *Choristoneura occidentalis* granulovirus (ChocGV). The *p35* homologue *p49* is present in *Spodoptera litura* (Splt) MNPV and *Spodoptera littoralis* NPV (Pei *et al.*, 2002, Yu *et al.*, 2005). All baculoviruses appear to carry anti-apoptotic genes, but none of these individual genes is conserved throughout (van Oers & Vlak, 2007). Baculovirus *iap* genes are often found in multiple phylogenetically distant copies, but are also present in other viruses like entomopoxviruses (Li *et al.*, 2005a, Li *et al.*, 2005b) and nudiviruses (Wang *et al.*, 2007).

IAPs are characterized by the presence of one to three baculovirus inhibitor repeat (BIR) domains at the amino terminus and a C3HC4 RING finger domain at the carboxy terminus(Clem, 2007). All active *iap* genes determined until now, contain at least these two types of conserved domains, except the African swine fever virus IAP which contains a zinc instead of a RING finger (Nogal *et al.*, 2001).

CIV belongs to the family *Iridoviridae* and is the type species of the genus *Iridovirus* (Chinchar *et al.*, 2005, Williams, 1996, Williams *et al.*, 2005, Willis, 1990) (Williams *et al.*, 2005). The genome of CIV has been entirely sequenced (Jacob *et al.*, 2001). Iridoviruses are large, cytoplasmic, icosahedral viruses with a linear double-stranded DNA genome, that is both circularly permuted and terminally redundant (Darai *et al.*, 1983, Delius *et al.*, 1984, Goorha, 1982, Goorha & Murti, 1982). The CIV virion consists of an unusual three-layer structure containing an outer proteinaceous capsid, an intermediate lipid membrane, and a core DNA-protein complex containing the genome (Williams, 1996). CIV has a broad host spectrum and has, in general, a limited mortality effect on its hosts (Williams, 1996, Williams *et al.*, 2005, Williams *et al.*, 2005). Up to now, fifteen complete sequences of iridovirus genomes have been published (Eaton *et al.*, 2010). However, CIV is a unique member of the *Iridoviridae,* since it is the only member, containing putative *iap* genes. Three CIV ORFs have been identified (157L, 193R, and 332L) that show 17.5-19.5% identity and 22.9–40.6% similarity in predicted amino acid sequence to the functional IAP-3 protein of *Cydia pomonella* granulovirus (CpGV)

(Birnbaum, 1994, Jakob *et al.*, 2001). However, only 193R contains both a BIR domain and a RING finger domain, while 157L and 332L contain only a RING finger domain and may, therefore, not be functional as inhibitors of apoptosis. The aim of the current work is to investigate whether CIV 193R indeed encodes an anti-apoptotic protein that prevents virus-induced apoptosis early in infection.

Materials and methods

Cells and virus

Bombyx mori SPC-BM-36 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and grown in monolayer cultures at 28°C in supplemented Grace's insect medium (Invitrogen) containing 10% fetal bovine serum and 1% NaCl (TNM-FH medium). The *Spodoptera frugiperda* cell line IPLB-SF-21 (Sf21) (Vaughn *et al.*, 1977) was maintained at 28°C in Sf900 II SFM medium (Invitrogen) containing 10% fetal bovine serum (FBS), 5 U/ml penicillin G and 5 g/ml streptomycin. *Chilo iridescent virus* (CIV) (*invertebrate iridescent virus 6*) was a gift from C. Joel Funk (USDA-ARS Western Cotton Research Laboratory Phoenix, USA). The virus was propagated in larvae of the wax moth, *Galleria mellonella*, purified as described by (Marina *et al.*, 1999) and quantified by using UV spectroscopy (D'Costa *et al.*, 2001).

Virus infections

SPC-BM-36 cells were infected with a fresh preparation of 5 µg (low dose) or 50 µg (high dose) CIV particles/1×10^6cells as described (D'Costa *et al.*, 2001). Briefly, SPC-BM-36 cells were plated in 35 mm tissue culture dishes (10^6 cells) and incubated for 1 h at 28°C. The medium in each well was then removed and replaced with 500 µl of fresh medium without 10% FBS containing an appropriate amount of CIV particles. After 1 h gently rocking for at 28°C, 1 ml supplemented medium without FBS was added to each well. The cells were placed at 28°C for another 2 h, after which the inoculum was removed and replaced with 2 ml of fresh medium with FBS.

Computer-assisted analysis

Protein comparisons with entries in the updated GenBank and EMBL databases were performed with the FASTA and BLAST programs (Altschul *et al.*, 1997, Pearson, 1990). Sequence alignments were performed with the program ClustalW (EMBL European Bioinformatics Institute, http://www.ebi.ac.uk) and edited with Genedoc Software (Nicholas *et al.*, 2001).

RNA isolation and RT–PCR analysis of CIV *iap*

One million SPC-BM-36 cells were infected with 5 µg as described above. One hour prior to infection, appropriate cultures were pretreated with 200 µg/ml cycloheximide (CHX) inhibit protein synthesis or 100 µg/ml Ara-C to inhibit DNA synthesis. These inhibitors were maintained at the above levels throughout the infection as described before (Nalcacioglu *et al.*, 2007, Nalcacioglu *et al.*, 2003). Total RNA was isolated from cells from 0 to 36 h p.i. using Trizol according to the manufacturer's instructions. For RT-PCR analysis, 2 µg of total RNA from CIV

infected SPC-BM-36 cells was reverse transcribed using 10 units of Superscript III reverse transcriptase; 10 units of RNAsin; and 250 nM of a CIV *iap* specific reverse primer 5'-GAAAACGATGGAGGAGATAA-3' in a total reaction volume of 20 µl. The cDNA's obtained were amplified by PCR using the same reverse primer in combination with a CIV *iap* specific forward primer 5'-TAAAAACACATTCATTCATAACACGA-3'. 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 $MgCl_2$, GoTaq flexi buffer and 0.5 units of GoTaq DNA polymerase. PCR products were analyzed in a 1% agarose gel stained with ethidium bromide. Two controls were performed, in which RNA was used for PCR directly while omitting the RT step or in which the cDNA was obtained with RNA isolated from uninfected cells.

Plasmid constructions

For the construction of plasmid pFB-GFP (Fig. 4A) the AcMNPV *ie-1* promoter fused with the hr5 enhancer region was cloned as an *Xma*I/*Bgl*II fragment from pIEHr3, kindly provided by Dr. Donald Jarvis, University of Wyoming, Laramie, USA (Jarvis *et al.*, 1996) into the *Xma*I/*Bam*HI sites of pFastBac Dual (Invitrogen), thereby deleting the p10 and polyhedrin promoters in the vector. In the opposite direction, a marker gene was cloned by inserting an *Xho*I fragment containing EGFP under the control of the OpMNPV *ie-2* promoter. The *egfp*-(*ie2*) construct in pFB-GFP was made by cloning e*gfp* from pEGFP (Clontech) as *Bam*HI/*Xba*I fragment into pIB/V5-His (Invitrogen), followed by PCR amplification with primers IE2-FW 5'-TTCTCGAGTCATGATGATAAACAATGTATGGTG-3' and GFP-RV 5'-TTTCTCGAGGTCGACCCGCTT TACTTGTAC AGC-3' (underlined sequences introduce an *Xho*I site). For this and all other PCR amplifications, the proofreading Phusion DNA polymerase was used.

The plasmid pFB-CIV*iap* (Fig. 4A) was constructed by cloning CIV *iap* as *Spe*I/*Pst*I fragment into the pFB-GFP plasmid. To this end the CIV *iap* gene was PCR amplified using primers CIViap-FWI 5'-TTACTAGTATGGATACATGTGGAATTTATA-3' and CIViap-RVI 5'-TTCTGC AGTTATATAAAAAGATTGTTAATTTTGAT-3' (underlined sequences introduce *Spe*I and *Pst*I restriction sites, respectively) and genomic CIV DNA as template.

The OpMNPV *iap3* gene was cloned as *Eco47*III/*Pst*I fragment from pHSOpiap (kindly provided by Dr. Rollie J. Clem, Kansas State University, USA (Clem & Miller, 1994) into the *Stu*I–*Pst*I sites of pFB-GFP to obtain pFB-Op*iap3* (Fig. 4A). The AcMNPV *p35* ORF was PCR amplified using primers 5'-TTAACTAGTATGTGTGTAATTTTTCCGGTA-3' and 5'-AATCTGCAGTTATTTAATT GTGTTTAATATTACATTTTTG-3' (underlined sequence introduce a *Spe*I and *Pst*I site, respectively) and genomic AcMNPV-E2 DNA as template. The PCR product was ligated first into pGEM-T easy (Promega) and cloned from there as *Not*I/*Pst*I fragment, of which the *Not*I site was blunt-ended, into the *Stu*I/*Pst*I sites of pFB-GFP to obtain pFB-Ac*p35* (Fig. 4A).

In order to produce dsRNA a vector with two bidirectional T7 promoters and terminators was constructed. To this aim, the multiple cloning site (MCS) behind the polyhedrin promoter in pFastBac-dual (Invitrogen) was amplified with 5'-CCCTCAAGACCCGTTTAGAGGCCC CAAGGGGTTATGCTAGTTATTGCTCAGCGGATCCCGGTCCGAAGCGCGCGGAATTC-3' and 5'-CCCTCA AGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATTGCTCAGCGGAAGCTTGTCGAGACTGCAGC TCTAG-3' (underlined sequences introduce part of the T7 terminators). The PCR product was further amplified with primer 5'- TT*CCTAGG***TTAATACGACTCACTATAGG**CAAAAAACCCCTCAAG ACCCGTTTAGAGGCCCCAAGG-3' (italic, bold and underlined sequences introduce an *Avr*II site,

T7 promoter and the additional part of the T7 promoter respectively). The PCR product was cloned into the *Avr*II site of the vector pFB-gfp(p10)Δpolh, which was made by deleting the polyhedrin promoter and adjacent MCS from pFastBac-dual as *Bst*1107I/*Hpa*I fragment and subsequently insertion of a red-shifted GFP (smRS-GFP) (Davis & Vierstra, 1998) into the *Xma*I site. The CIV *iap* PCR product described above was cloned into the MCS between the two bidirectional T7 promoters as a *Spe*I/*Pst*I fragment to obtain pFB-T7/CIViap (Fig. 4B).

Transient expression assay

CIV *iap* and *egfp* both are placed under an immediate early, constitutive promoter to allow transient expression in the insect cell lines SPC-BM-36 and Sf21 (see above). The marker gene (e*gfp*) is simultaneously expressed with CIV *iap* in these insect cell lines. The assay was completed by two positive controls, Ac-*p35* and Op-*iap3,* and a vector without an anti-apoptotic gene (only *egfp*) as a negative control.

SPC-BM-36 and Sf21 cells were seeded into 35 mm tissue culture dishes (1×10^6 cells) and incubated for 24 h at 28°C. Cells were transfected with 10 µg of plasmids pFB-GFP, pFB-CIV*iap*, pFB-*Opiap3* or pFB-*Acp35* (Fig. 4A) with Cellfectin (Invitrogen), following the manufacturer's instructions. At 48 h post transfection, the number of GFP-expressing cells was counted by using an inverted microscope (Olympus) subsequently apoptosis was induced by adding actinomycin D (Sigma) to the medium at a final concentration of 0.5 µg/ml. The number of GFP-expressing cells was counted again 8 h after Act D addition and presented as percentage viable cells compared to those before the induction of apoptosis. Each data point represents an average of three independent assays with two replications. For DNA fragmentation assay, the cells were harvested 12 h post induction of apoptosis.

DNA fragmentation assay

Cultured cells were collected by centrifugation at 3000 rpm for 10 min at 4°C. The cell pellet was washed twice with PBS and stored frozen until use. Thawed cells were suspended in 1 ml TENS solution (10 mM NaCl, 0.1 M Tris-HCl, 10 mM EDTA pH 8.0 and 10% sarcosyl) containing 0.2 mg proteinase K and incubated overnight at 50°C. DNA was extracted three times with an equal volume of phenol and once with phenol/chloroform/isoamyl alcohol (25:24:1). Extracted DNA was precipitated using isopropanol and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0) containing 0.4 mg/ml RNAse. DNA samples were analyzed in a 1.2 % agarose gel.

dsRNA-induced silencing

The T7 RiboMAX Express RNAi System (Promega) was used to produce dsRNA of CIV *iap* using pFB-T7/CIV*iap* as template according to the manufacturer's instructions. SPC-BM-36 cells were seeded into 35 mm tissue culture dishes (0.5×10^6 cells) and incubated for 24 h at 28 °C. Cells were transfected with 0, 10 or 50 µg dsRNA using Cellfectin (Invitrogen). Twenty-four hour post transfection cells were either infected with CIV or mock infected. Cells were examined 1 and 2 d p.i. for apoptotic affects. Afterwards cellular DNA was purified for DNA fragmentation assays as described above.

Results

Cytopathic effect in SPC-BM-36 cells upon CIV infection

CIV replicates in several different cell lines including those derived from *Bombyx mori* (Constantino *et al.*, 2001, Nalcacioglu *et al.*, 2003), such as SPC-BM-36 cells (Fig. 1A). When these cells are infected with a high dose of CIV, vesicles resembling apoptotic bodies are produced at 24 h p.i. (Fig. 1B). However, these bodies disappear at later time point's p.i. (Fig. 1C). At 3 days, the infected cells seem to expand and form intracellular vacuoles as compared to mock infected cells (Fig. 1C). At the end of infection, the cells necrotize. This is in contrast to cells treated with actinomycin D, where apoptotic bodies are present in abundance over a long period of time (Fig. 1D). As the formation of apoptotic bodies has also seen upon infection of fish cells by the vertebrate-infecting *Red sea bream iridovirus* (RSBIV) (Imajoh *et al.*, 2004), the DNA was extracted from infected SPC-BM-36 cells and subjected to DNA fragmentation analysis. Total cellular DNA of CIV infected cells was purified at different time point's p.i. and analyzed by agarose gel electrophoresis along with DNA of SPC-BM-36 cells in which apoptosis was induced with Act D (Fig. 1E). The DNA of cells treated with Act D showed the classical DNA ladder (Fig. 1E, lane 2), whereas CIV-infected SPC-BM-36 cells (lanes 3-5) did not.

Characteristics of a putative CIV *iap* gene

Computational analysis of the CIV genome indicated that ORF 193R, located at nucleotide position 82,521 to 83,144 in the genome (Jakob *et al.*, 2001), is a putative *iap* gene (CIV *iap*). The CIV *iap* ORF contains 624 bp and encodes a putative protein of 208 amino acids (aa) with a predicted molecular mass of 22.8 kDa. Typically, IAPs contain one or more so-called Cys/His BIR domains represented by a $GX_{9\text{-}11}CX_2CX_{8\text{–}10}E/DX_5HX_{3\text{–}6}C$ domain, and often a carboxy-terminal RING finger (C_3HC_4). However, a RING domain is not always necessary for IAP function (Clem & Griffin, 2005).

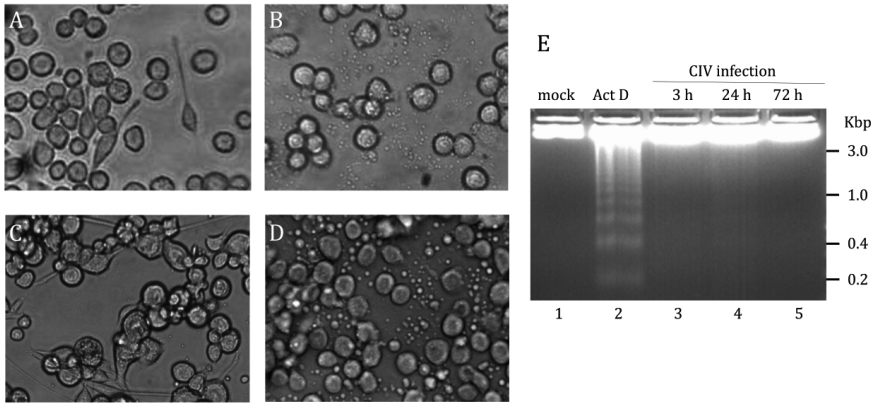

Figure 1. Cytopathic effect of *Chilo iridescent virus* on SPC-BM-36 cells. Cells were either mock infected (A), or infected with CIV (50 µg/ml) for 24 h (B) or 72 h (C). In control cells, apoptosis was induced by Act D treatment for 8 h (D). Cellular DNA was isolated from either mock infected (lane 1) or infected cells at different time points p.i. at 3 h (lane 3), 24 h (lane 4) and 72 h (lane 5) as well as from Act D treated cells (lane 2) and analyzed in a 1.2 % agarose gel for DNA fragmentation (E).

CIV ORF193R contains one BIR domain (aa positions of 62-105) at the N-terminal portion of the protein and a RING finger domain (aa positions of 163-196) at the C-terminus (Fig. 2). This ORF was designated as CIV *iap* because of the sequence homology with other *iap*s identified previously in CpGV (Crook, 1993), AcMNPV, *Orgyia pseudotsugata* (Op) MNPV (Birnbaum, 1994), *Epyphias postvittana* (Eppo) MNPV (Maguire *et al.*, 2000), *Hyphantria cunea* (Hycu) NPV (Ikeda *et al.*, 2004) and *Amsacta moorei* entomopoxvirus (AmEPV) (Li *et al.*, 2005b). The CIV IAP protein is most similar to baculovirus IAP-3 proteins (Fig. 2) and has 16 and 15 percent identity, and 27 and 28 similarity in its amino acid sequence to the OpMNPV and CpGV IAP-3 proteins, respectively. Most of the functional IAPs of baculoviruses belong to this IAP-3 family (Birnbaum, 1994, Carpes *et al.*, 2005, Crook, 1993, Ikeda *et al.*, 2004, Kim *et al.*, 2007). Based on these comparisons, we anticipate that CIV IAP is active and functions as an inhibitor of apoptosis in CIV infections.

Transcription of CIV *iap*

To investigate whether the putative CIV *iap* gene is transcribed, SPC-BM-36 cells were infected with CIV in the presence or absence of CHX, which inhibits *de novo* polypeptide synthesis, and Ara-C, an inhibitor of DNA replication. Total cellular RNA was extracted from cells at several time points p.i. and analyzed for the presence of CIV *iap* transcripts by RT–PCR. CIV *iap* transcripts were observed from 4 to 36 h p.i. (Fig. 3). CIV *iap* transcript levels (measured at 12 h p.i.) were not affected by the presence of Ara-C (Fig. 3, lane 13) or CHX (Fig. 3, lane 14). This indicates that CIV *iap* is transcribed before CIV DNA replication and does not require any *de novo* CIV protein expression. Therefore, the CIV *iap* should be classified as an immediate-early (*ie*) CIV gene.

Suppression of actinomycin-D induced apoptosis by CIV *iap*

In order to analyze the anti-apoptotic activity of the CIV *iap* gene, SPC-BM-36 and Sf21 cells were transfected with the dual plasmid pFB-CIV*iap*. This allowed transient expression of the CIV *iap* gene under the control of the AcMNPV *ie1* promoter and GFP under control of the AcMNPV *ie2* promoter (Fig. 4A). As a negative control, cells were transfected with a plasmid expressing GFP only (pFB-GFP). For positive controls, GFP together with OpMNPV IAP-3 (pFB-*Opiap3*) or AcMNPV P35 (pFB-*Acp35*) were used. At 24 h post transfection (p.t.) apoptosis was induced by actinomycin-D. GFP expressing cells were counted before and after induction of apoptosis to calculate the percentage of viable cells. The cell viability in the presence of CIV IAP was reduced to 69% and 46% in SPC-BM-36 and Sf21 cells, respectively (Fig. 5A), following Act D treatment. In the GFP-only control the amount of viable cells was reduced to 19% in SPC-BM-36 and 22% in Sf21 cells by Act D treatment. The anti-apoptotic effect observed in this assay was somewhat less with CIV IAP than with AcP35 and OpIAP-3. The anti-apoptotic effect was for all anti-apoptotic genes stronger in SPC-BM-36 cells than in Sf21 cells. DNA was purified from the cells transfected with the CIV*iap* construct or with pFB-GFP. DNA isolated from cells exposed to Act D in the absence of CIV *iap* was fragmented as shown by agarose gel electrophoresis, while DNA of cells expressing CIV *iap* was mostly intact (Fig. 5B, lanes 1-2). These results show that CIV IAP can inhibit, at least to a large extend, apoptosis induced by actinomycin-D.

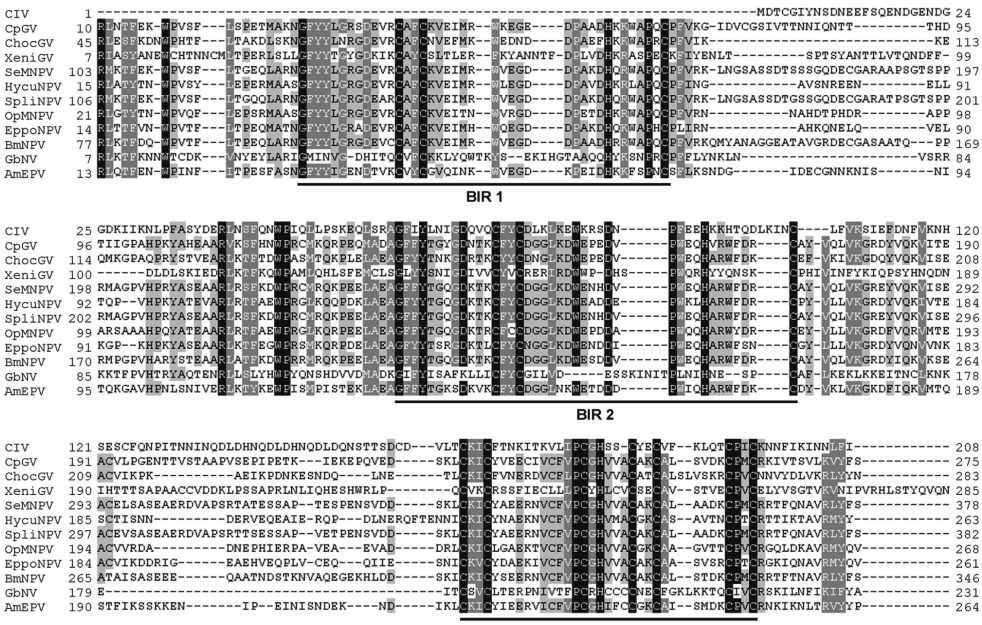

Figure 2. Multiple alignment of CIV IAP with partial C-terminal amino acid sequences of viral IAP-3 homologues. Black, dark grey and light grey columns represents amino acids with ≥ 95% identity, ≥ 80% similarity and ≥ 60% similarity respectively. The two BIR domains represented by the sequence $GX_{9-11}CX_2CX_{8-10}E/DX_5HX_{3-6}C$ and the RING finger domain characterized by the C_3HC_4 motif are underlined. CIV: *Chilo iridescent virus* [NP_149656], CpGV; *Cydia pomonella* GV [NP_148801], ChocGV; *Choristoneura occidentalis* GV [YP_654505], XecnGV; *Xestia c-nigrum* GV [NP_059285], SeMNPV; *Spodoptera exigua* MNPV [ABA62322], HycuNPV; *Hyphantria cunea* NPV [YP_473308], SpliNPV; *Spodoptera litura* NPV [CAM96614], OpMNPV; *Orgyia pseudotsugata* MNPV [NP_046191], EppoNPV; *Epiphyas postvittana* NPV [NP_203195], BmNPV; *Bombyx mori* NPV [NP_001037024], GbNV; *Gryllus bimaculatus* nudivirus [NP_001111365], AmEPV; *Amsacta moorei* entomopoxvirus [NP_0643802]. GenBank Accession numbers are shown between brackets.

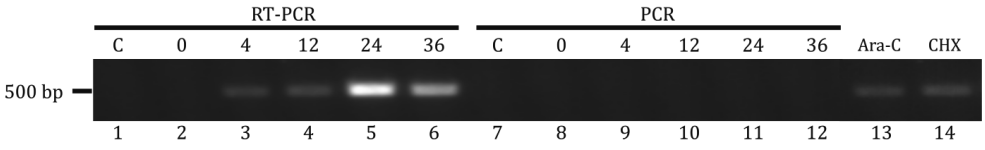

Figure 3. Transcriptional analysis of the CIV *iap* gene by RT-PCR. Total cellular RNA of mock infected (lane C) and CIV-infected SPC-BM-36 cells at 0, 4, 12, 24 and 36 h p.i. was purified and subjected to RT-PCR (lanes 1 and 7) or PCR only (lane 7-12). RT-PCR was also performed on total RNA of cells infected with CIV in the presence of the DNA synthesis inhibitor Ara-C (lane 13) or the protein synthesis inhibitor CHX (lane 14) and harvested 12 h p.i.

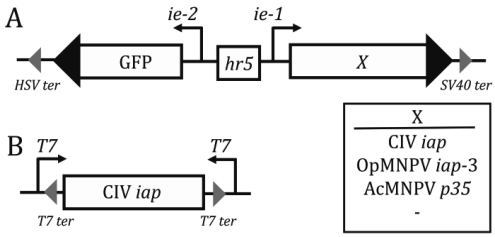

Figure 4. Schematic representation of pFB-GFP, pFB-CIV*iap*, pFB-*Opiap*, pFB-*Acp35* (A) and pFB-T7/CIV*iap* (B). The *gfp* gene is under transcriptional control of the OpMNPV *ie-2* promoter and *Herpes simplex virus* terminator (HSV ter), while the *inhibitor of apoptosis* genes and *p35* (X) are under the control of the OpMNPV *hr5* enhancer / *ie-1* promoter and Simian Virus 40 terminator (SV40 ter). pFB-T7/CIV*iap* is used for dsRNA transcription using the bidirectional T7 promoters (T7) and terminators (T7 ter).

CIV *iap* prevents apoptosis of CIV infected cells

To examine if the product of ORF 193R (CIV *iap*) prevents apoptosis in a CIV infection, the CIV *iap* gene was knocked down by RNA silencing (Fig. 6). To this aim SPC-BM-36 cells were transfected with different amounts (0, 10 and 50 µg) of *in vitro* produced CIV *iap* dsRNA (Fig. 4B). Twenty-four hour post transfection with dsRNA, the cells were infected with CIV (Fig. 6). This treatment resulted in the formation of apoptotic bodies, observed from 1 d p.i (Fig. 6E-F) onwards. The amount of apoptotic bodies increased at 2 d p.i. (Fig. 6K-L). Transfection with CIV *iap* dsRNA without a subsequent CIV infection did not result in an apoptotic response in SPC-BM-36 cells (Fig. 6B-C, H-I), neither did transfection with dsRNA of GFP (not shown). dsRNA against GFP had no apoptotic effect on SPC-BM-36 cells and did not affect CIV infection. These results indicate that apoptosis is not induced by dsRNA as such but is specifically observed when 193R is silenced during infection. The analysis of DNA by agarose gel electrophoresis (Fig. 6M) showed DNA fragmentation in cells transfected with CIV *iap* dsRNA followed by CIV infection (Fig. 6M, lane 4), while this phenomenon was not found in cells that were either uninfected, not transfected before CIV-infection, or not infected with CIV after dsRNA transfection (Fig. 6M, lanes 1-3, respectively). Thus, CIV IAP appears to be a functional inhibitor of apoptosis during CIV infection.

Discussion

CIV replicates in several insect cell lines and this assists in the study of CIV gene function and regulation (Constantino *et al.*, 2001, D'Costa *et al.*, 2001, Nalcacioglu *et al.*, 2003). CIV infection of SPC-BM-36 cells results in a specific cytopathology. A notable feature early after infection is the formation of vesicles resembling apoptotic bodies upon high dose (50 µg/ml) of CIV infection (Fig. 1B) suggesting the partial absence of an anti-apoptotic response. Also in *Choristoneura fumiferana* Cf124T cells, a similar high dose results in a massive apoptotic response (Chitnis *et al.*, 2008). Probably only a minority of cells indeed underwent apoptosis early in infection in the current study, which would explain the absence of apparent DNA laddering in Fig. 1E. These vesicles, even at a high dose infection, disappeared at later time points p.i. (Fig. 1C), when virus infection proceeded in the majority of cells, suggesting an anti-apoptotic response upon virus infection (Constantino *et al.*, 2001, D'Costa *et al.*, 2001,

Nalcacioglu *et al.*, 2003). The level of apoptosis observed appears, however, to be cell line and CIV dose dependent, as at an equal dose the apoptotic response in Cf124T cells seems to be a lot stronger than in SPC-BM-36 cells. The vesicles seen early after CIV infection are different from those observed for RSBIV, where apoptotic vesicles are formed late in infection as well, a process that may facilitate cell-to-cell dissemination of progeny virions within the host (Alnemri, 1992, Imajoh *et al.*, 2004, Teodoro, 1997). This is consistent with the absence of any putative anti-apoptotic genes in RSBIV. In baculovirus infections apoptosis can also be triggered by early as well as late events (LaCount, 1997).

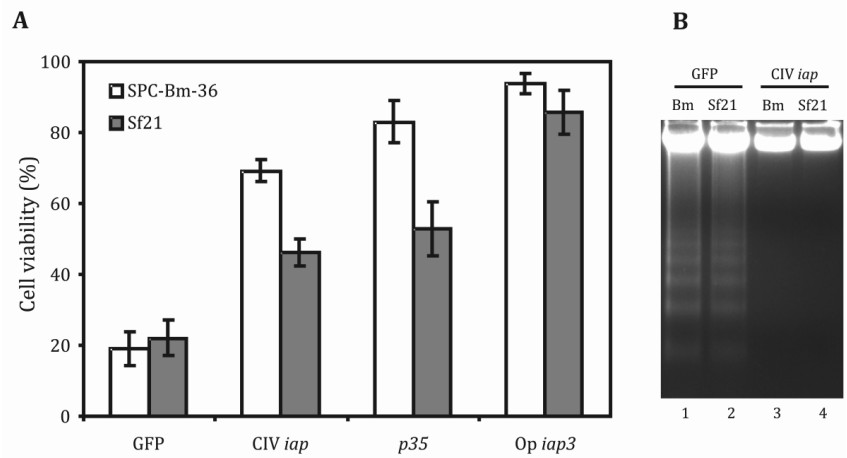

Figure 5. Inhibition of apoptosis by CIV IAP in Sf21 and SPC-BM-36 cells. Cells were transfected with plasmids expressing either GFP only or GFP together with CIV-IAP, AcP35 or OpIAP3. Apoptosis was measured as described in Material and Methods. Bars represent standard error of the mean (SE) (A). Total DNA was isolated from cells transfected with plasmids expressing GFP only or GFP together with CIV IAP 8 h after induction of apoptosis with actinomycin D. The DNA fragmentation was analyzed in a 1.2 % agarose gel.

In the current study, we focused on the question whether CIV has a functional anti–apoptosis system based on the expression of functional anti-apoptotic genes. IAPs are characterized by the presence of one to three baculovirus iap repeat (BIR) domains at the amino terminus and often a C3HC4 RING finger domain at the carboxy terminus (Clem, 2007). All active baculovirus *iap* genes determined until now contain at least these two conserved domains, but not all proteins that contain a BIR domain inhibit apoptosis. CIV open reading frame 193R contains a BIR domain and a RING finger domain, while 157L and 332L contain only a RING finger domain (Fig. 2). We have not tested the CIV 157L and 332L genes, but anticipate that they are not functional as IAPs since they lack BIR domains. CIV is the only iridovirus virus known containing putative *iap* genes in its genome and CIV ORF 193R is the only iridovirus gene so far with a BIR domain. Other iridoviruses may have other mechanisms to counteract apoptosis, such as the vertebrate grouper iridovirus (GIV), where a B-cell lymphoma *bcl-2* like gene prevents apoptosis (Lin *et al.*, 2008).

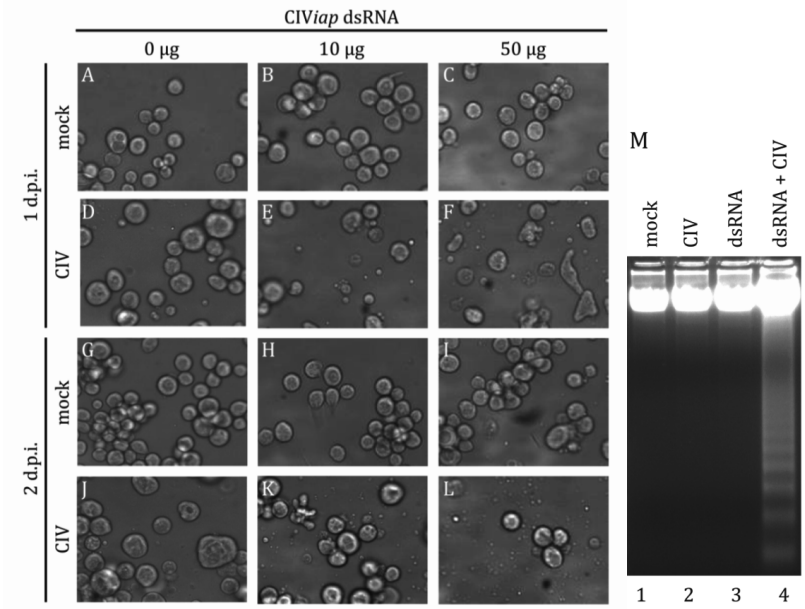

Figure 6. RNA silencing of CIV *iap* in CIV-infected SPC-BM-36 cells. Cells were transfected with 0 µg (A,D,G,J), 10 µg (B,E,H,K) or 50 µg (C,F,I,J) CIV-*iap* dsRNA. One day after the transfection cells were either mock infected (A-C,G-I) or infected with 5 µg of CIV (D-F, J-L) and observed 1 and 2 d p.i. for apoptotic effects. (M) Total DNA was purified from mock infected (G, lane 1), CIV infected (J, lane 2), CIV *iap* dsRNA transfected (I, lane 3) and CIV iap dsRNA transfected and CIV infected cells (L, lane 4) and DNA fragmentation was analyzed on a 1.2 % agarose gel (M).

The immediate early transcription of the CIV *iap* gene (Fig. 4) is in agreement with recent studies in Cf124T cells suggesting the presence of an early anti-apoptotic function upon CIV infection (Chitnis *et al.*, 2008). In this regard this CIV *iap* gene behaves in a similar fashion as *iap* gene family members in baculoviruses, such as AgMNPV *iap*-3 (Carpes *et al.*, 2005). The anti-apoptotic gene *bcl-2* that identified in GIV is also expressed as in the immediate early phase of infection (Lin *et al.*, 2008).

Transient expression assays performed in SPC-BM-36 and Sf21 cells showed that in both cases CIV IAP was able to block apoptosis induced by Act D (Fig. 5). The difference observed in CIV IAP activity in the two cell lines may be due to variations in expression level or in differences in affinity for IAP antagonists in these cell lines. The pivotal role of the CIV *iap* gene in the anti-apoptotic response upon CIV infection of SPC-BM-36 cells was further demonstrated by RNA interference experiments. Only when dsRNA specific for CIV 193R was used a strong apoptotic response was seen (Fig. 6). All the results obtained so far show that the putative CIV *iap* gene studied here (193R) encodes a functional anti-apoptotic protein. Genetic (knockout) mutants of CIV and other iridoviruses are difficult to generate since iridovirus DNA is not infectious by itself. The potential of the RNAi approach was shown before in another iridovirus study, where RNA interference effectively inhibited the expression of the major capsid protein (*mcp*) gene of tiger frog virus (Xie *et al.*, 2005). The RNAi approach also proved to be a good strategy to study CIV gene function.

Acknowledgments

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Chapter 7

General discussion

Background

The members of the family *Iridoviridae* are large, cytoplasmic DNA viruses with an icosahedral outer capsid, an internal lipid membrane, and an electron dense core containing a circularly permuted and terminally redundant double stranded DNA genome. Iridoviruses cause major diseases in vertebrates (fish, amphibians) and invertebrates, but little was known about their structure, replication and gene expression. Fundamental knowledge of iridoviruses is important in view of their potential use as biological control agents for pest insects as a means for replacing chemical insecticides. *Chilo iridescent virus* (CIV) or *insect iridovirus 6* (IIV-6) is the prototype of the genus *Iridovirus* within the family *Iridoviridae,* which also includes the invertebrate iridoviruses in the genus *Chloriridovirus.* Iridoviruses can infect weevils and caterpillars that cause major problems in agro-ecosystems. Specifically, CIV is a potential biocontrol agent of weevils in tea and hazelnut in the North-East of Turkey and in cotton in the South-Eastern and Mediterranean parts of Turkey. Iridoviruses have also been found to infect insects, such as mosquitoes, whiteflies and grasshoppers, that transmit plant pathogens and/or parasites of medical importance (Fukuda, 1971; Hunter *et al.* 2003; Hunter *et al.* 2001; Kleespies *et al.* 1999), but the potential of IIVs as biocontrol agents for these insects has not been fully explored. Fundamental information on the structure and replication of iridoviruses in general may also lead to novel intervention strategies to mitigate or prevent disease caused by vertebrate iridoviruses in fish and amphibians.

Although data had been collected about the iridovirus infection cycle, many fundamental questions remained to be answered for instance, concerning the structure and scaffolding of the virus particles, the nature of virus-host interactions including cell entry and the initial steps of viral infection, and the spread of the virus infection from cell-to-cell. Viral structural proteins are likely to play crucial roles in these processes. This is exemplified by the fact that the viral DNA by itself is not infectious (Cerutti *et al.* 1989), and that a component of the virus particle is required to initiate viral entry and/or early gene expression (Willis *et al.* 1990; Willis & Granoff, 1985). In the past, efforts have been undertaken to characterize the polypeptides present in CIV virions by one or two dimensional SDS-PAGE (Barray & Devauchelle, 1979; Day & Mercer, 1964; Reyes *et al.* 2004). The polypeptides were not linked to identification of the corresponding genes because of the limited information present at the time on IIV genomics. Only when the complete genome sequence of CIV and two other IIVs became available (Delhon *et al.* 2006; Jakob *et al.* 2001; Wong *et al.* 2011), and sensitive proteomic techniques (LC-MS/MS) were developed in combination with modern software that allowed the analysis of large mass-spectrometric data sets, precise delineation of the genes that encode proteins present in the virus particle and in infected cells became possible and this was a major subject of this thesis.

Virion proteins and putative functions

The first study described in this thesis was directed towards the identification of the CIV virion components by using a proteomic approach (**Chapter 2**). Here, protein separation by one dimensional SDS-polyacrylamide gel electrophoresis was combined with peptide analysis by liquid chromatography and tandem mass spectrometry (LC-MS/MS). The available CIV protein database was generated based on 211 predicted ORFs (Eaton *et al.* 2007) and facilitated accurate protein identification from the output of the peptide analysis. This approach provided a fast and highly sensitive method for the identification of proteins through the sequences of the corresponding genes and served as a starting point to decipher the functions of these virion proteins. The proteomic analysis of the CIV virions revealed 54 proteins and the genes encoding these virion proteins are scattered over the genome (Chapter 2; Figure 2).

Four CIV virion proteins were shown by cryoelectron microscopy to form a multimeric complex containing a 'finger' protein, a 'zip' protein, a pentameric complex and an anchor protein (Yan *et al.* 2009). When the molecular mass estimation from the capsid structure of CIV was connected to the virion proteome results from the current study, the putative candidate gene encoding these proteins could be ORFs 234R, 111R, 096L, 374L, 325L, 203L and 084L for the 'finger' protein; 010R, 138R and 312R for the 'zip' protein, ORFs 329R and 219L for the monomer of the pentameric complex, and 457L and 142R for the anchor protein (Chapter 2; Table 1). Domains that suggest possible functions were found in a number of virion proteins, and this led to the identification of three putative serine/threonine kinases (ORFs 209R, 380R, and 439R), one tyrosine protein kinase (179R), one dual specificity phosphatase (123R), and a carboxy-terminal domain (CTD) phosphatase (355R). These enzymes may play crucial roles to secure successful viral replication in the sense that they determine the fate of the host cell by affecting pathways that regulate for instance host cell shut off or apoptosis. These enzymes may act by modulating the intracellular localization and movement of viral or host gene products or by controlling host or viral gene expression by modulating transcription factors.

The virion also contains proteins, which may deal with viral DNA replication or mRNA synthesis, including a protein with homology to the N-terminal domain of viral DNA polymerases (232R), nucleoside triphosphatase (NTP I; 22L), and a putative DNA binding protein (401R). DNA binding proteins may be building blocks of the virion structure, but may also be trans-activator proteins that induce transcription of (a set of) genes. IIV genomes also contain other genes associated with RNA metabolism (e.g. Ribonuclease III; 142R), which was identified in the CIV virion structure and recently also in that of IIV-9 (Wong *et al.*, 2011). Ribonuclease III, one of the iridovirus core genes, is correlated to the microRNA pathway (Wong *et al.*, 2011) and may be one of the main players in virus-host interaction. The microRNA mechanism is relatively conserved among different organisms (Cullen & Umbach, 2009) and needs to be investigated in a variety of virus-host systems to obtain fundamental insights in viral pathogenesis in a background of host defence/immune responses. Other identified proteins with putative conserved domains include fasciclin (96L), cathepsin (361L), protein disulphide isomerase (453L), lysosome associated membrane glycoprotein (061R), and a homolog of the ranavirus envelop protein (118L), but their functions are less clear.

Protein disulphide isomerase may be an example of a protein that is not incorporated on purpose but a relic of the cellular history of the virion. The same may be true for the DNA polymerase mentioned above. Intriguingly, the identified CIV virion proteins include virion-associated kinases, surface fibres, and RNA processing enzymes, but should also include the not yet identified trans-activator protein(s) responsible for initiating viral gene expression.

CIV virion proteins and evolutionary relationships

Of the 54 ORFs encoding CIV virion proteins identified in this study, thirty-four have homologs in the IIV-3 genome. The species IIV-3 belongs to the genus *Chloriridovirus* and shares the highest degree of similarity with CIV in terms of gene content and homology. Recently, the virion protein set of *Wiseana iridescent virus* (IIV-9) has been identified showing great consistency with the CIV virion proteins (Wong *et al.*, 2011). IIV-9 also belongs to the genus *Iridovirus*. Infected cell proteome analysis of IIV-9 revealed 94 viral proteins, including 64 virion proteins, out of a total of 193 proteins predicted from the genome sequence (NCBI accession number; NC 015780). The 64 virion proteins have 39 homologs with the CIV virion proteins. The discrepancy in number of homologs between these two viruses is due to the fact that several CIV ORFs have more than one homolog in the IIV-9 genome (Wong *et al.*, 2011), which may be the consequence of gene duplications.

When compared with vertebrate iridoviruses, the CIV virion proteome shares 13 homologs with *Singapore grouper iridovirus* (SGIV) virion proteins, when compared to two independent mass spectrometric analyses (Chen *et al.*, 2008, Song *et al.*, 2004). The CIV proteome suggests five ORFs shared with all iridoviruses for which the genome has been sequenced: 022L, 118L, 142L, 274L, and 295L. These virions ORFs are currently considered to be iridovirus virion core genes, out of the 26 genomic core genes recognized within the family *Iridoviridae*. The functions of the encoded proteins are largely elusive except for 274L, which encodes the major capsid protein (MCP), and ORF118L, which is a homolog of the *Rana grylio virus* (RGV) (genus *Ranavirus*) ORF 53R. The latter has been shown to encode an envelope protein (Zhao *et al.*, 2008).

Recent genomic sequence information on IIV-9 (Wong *et al.*, 2011), which is the third completely sequenced member of the lesser known group of IIVs in the family *Iridoviridae,* has led to an updated phylogeny using the 26 known core IV genes sequences. IIV-9 demonstrated a closer phylogenetic relationship to IIV-3 than to CIV (Table 1). However, IIV-9 and CIV are currently classified in the same genus *Iridovirus* (Chinchar *et al.*, 2009, Wong *et al.*, 2011), while IIV-3 is classified in the genus *Chloriridovirus.* Even though, current phylogenetic studies suggest that CIV may form a clade distant from other IIVs, the comparison of virion proteomes showed that a high number of virion proteins are shared by CIV and IIV-9. Without further genome sequence, proteome and biological data for other IIVs, further conclusions on the classification on IIVs cannot be drawn. In conclusion, the current representation of only these three members of IIVs is not sufficient for understanding the full phylogenetic relationship between the members of the IIVs.

Table 1. CIV virion proteins identified by LC-MS/MS with homologues in IIV-9 and other iridoviruses*

Invertebrate			Vertebrate												
CIV	**IIV-9**	**IIV-3**	**ATV**	**TFV**	**FV3**	**SGIV**	**GIV**	**STIV**	**EHNV**	**LCDV-C**	**LCDV-1**	**ISKNV**	**RBIV**	**RSIV**	**OSGIV**
443R	**038L***	91L													
295L	**143L**	16R	72R	45R	41R	57L	29L	45R	77R	234R	92R	76L	72L	639R	75L
179R	**016R**	35R	60R	29R	27R	78L	44R	31R	62R	172R	110R				
022L	**055L**	87L	7L	9L	9L	60R	30L	11L	8L	75L	70L	63L	59L		63L
261R	**038L***	91L													
396L	**067L***	91L*													
268L	**069R**	74L													
149L	**150R**	113L													
232R	**085R***	84L	84L					21R	89L*						
439L		35R*									110R	114L		463R	111L
361L	**177R**	24R								223L	23R				
380R	**023L**	10L	84L	19R	19R	39L	17L	21R	89L	13L*	5L*				
213R	**115L**	51L													
118L	**005R**	6R	53L	55R	53R	88L	49L	55R	53L	157R	35L	7L	8L	374R	8L
198R	**019L**	69L													
274L	**010R**	14L	14L	96R	90R	72R	39R	96R	14L	43L	80L	6L	7L	380R	7L
229L	**084R**	46R	3R	4R	229L	16L	2L	5R	3R						
337L	**031R**	47R	1L	2L	2L	19R	4R	2L	1L	38R	89L		85L	575R	
329R	**086L**	99R													
219L	014L*	36R*													
142R	**034L**	101R	25R	85L	80L	84L	46L	87L	24R	186R	74R	87R	83R	639R	85R
155L	**150R**	113L													
401R	**169L**	68R													
117L	**183R**	107R	83L	20R	20R	038L	16L	23R		73R	109R				
415R	**159R**	18L													
309L	**091L**	63R													
307L	**104L**	33L	11R	100L	94L	98R	56R		11R	152L	9R	86R		600L	
378R	**085R**	100L	84L	19R	19R	39L	17L	21R	89L	13L	50R				
355R	**036L**[1]	104L	67R	40R	37R	61R	31L	41R	72R	147L	43L	5L	6L	385R	
374R	**141R**														
203L		85L													
395R	**153R**	1R													
453L	**062R**	41R													
366R			63R	33R	32R			35R	68R						
010R	066L	43R													
342R	**174L**	115R													
325L	**027L**														
159L	**038L***														
317L	**136L**														
234R	**140L**														
111R	**188R**														

*ORFs labeled with asterisk have more than one heterologous gene in corresponding species. IIV-9 proteins identified by proteomic study represented in bold and virion proteins were underlined (Wong *et. al*., 2011). CIV virion proteins detected in infected cell proteomic study represented in bold.

The conservation of CIV virion protein genes in the genomes of members of the family *Ascoviridae* was also assessed. This family was included in the analysis since a common ancestry between iridoviruses and ascoviruses had been inferred from phylogenetic analysis, based on comparative analyses of the capsid protein, DNA polymerase, thymidine kinase, and ATPase III. This has led to the hypothesis that ascoviruses may have evolved from invertebrate iridoviruses (Stasiak *et al.*, 2000, Stasiak *et al.*, 2003) despite disparity of virion structure and infection

characteristics (Bigot *et al.* 2008). The proteomic analysis showed that CIV virion protein genes have a considerable number of homologs in one or more ascovirus genomes (Asgari *et al.*, 2007, Bideshi *et al.*, 2006, Bigot *et al.*, 2008, Stasiak *et al.*, 2000, Tan *et al.*, 2009, Wang *et al.*, 2006). This conservation of virion proteins between CIV and ascoviruses further supports the hypothesis of a common ancestry. Very few CIV virion proteins show (only partial) homology to viral proteins of poxvirus, coronavirus or baculovirus origin, which underscores the evolutionary distance of iridoviruses from these other virus groups.

In summary, in Chapter 2, a first step was taken to understand the structure and composition of the virion particle of invertebrate iridoviruses. This information is crucial for deciphering the molecular mechanisms underlying the CIV replication cycle, including virion assembly, cell entry, egress, and the initial steps leading to early iridovirus gene expression, as well as providing clues for interactions between CIV and host proteins.

Transcriptomic analysis of virion protein genes via a novel amplification approach

The aim of **Chapter 3** was to determine the temporal class of the individual virion protein genes identified in Chapter 2. Barray & Devauchelle (1987) previously showed that iridovirus gene expression follows a temporal cascade in which immediate-early (IE, α), delayed-early (DE, β) and late (L, γ) genes are sequentially expressed. In previous related studies the detected CIV transcript signals could not be assigned to the corresponding ORFs (D`Costa *et al.*, 2001) since CIV genomic fragments that often spanned more than one ORF were used as probes to characterize the transcripts. So far, transcriptionally active regions of the genome have also been identified in IIV-9 (McMillan & Kalmakoff, 1994), but this study did not allow a precise distribution of the genes over the three temporal classes. Hence, there was no consistent correlation between IIV genes, transcripts and observed proteins.

In the current study a novel strategy was followed to detect individual transcripts**.** Since the 3' end of CIV transcripts does not contain a polyadenylation signal, conventional 3' RACE analysis was not suitable for PCR-based transcript detection. To be able to amplify the CIV transcripts in a specific manner, thereby avoiding amplification of viral DNA segments, the LACE (Ligation-based amplification of cDNA ends) technique was developed. The principle of this technique is the enzymatic addition of a small specific stretch of DNA to the 3' end of the transcript, followed by an RT-PCR based on this stretch and an internal primer in the ORF (Chapter 3, Figure 1). LACE showed to be a robust and effective technique for transcriptomic analysis of non-polyadenylated transcripts.

It is generally believed, that virion protein genes may predominantly belong to the late temporal class since they need to be present in the cell during virion particle assembly at the end of the infection. In contrast to this hypothesis, our data showed that CIV virion genes are distributed over all three temporal classes and that the majority are expressed as immediate-early genes, meaning that to make their mRNA, they do not require *de novo* protein or DNA synthesis. Since CIV DNA is not infectious by itself, one or more virion-associated, transcriptional transactivators in addition to host RNA Polymerase II conceivably manipulate the initial phases of viral infection after entry of the virus into the cell.

Early viral transcripts in general encode regulatory and catalytic proteins such as the viral DNA polymerase, the helicase or viral kinases. As both IE and DE transcripts were observed for CIV virion protein genes, the participation of the encoded proteins in virion formation is

likely. The fact that many transcripts were defined in an early temporal class does not imply that these mRNAs may not be present or even synthesized (and translated) at later time points during infection as well. In CIV, many early gene transcripts were indeed found at late time points in infection together with late transcripts, as was also the case for FV3 infection (Chinchar & Yu, 1992, İnce *et al.*, 2008). The long presence of early gene transcripts might be linked to the presence of the encoded proteins in the virion complex. The early gene products may be continuously involved in transcriptional regulation or viral replication during the course of infection as they were apparently not strongly down regulated when late transcription starts. Differences were observed between iridoviruses when comparing the temporal classes of homologous virion protein genes, indicating that iridoviruses implement diverse mechanisms to regulate the course of infection (Chapter 3; Table 4). The transcriptome data may be used to unravel virion protein gene promoters and their expression in cohorts may reveal information on subsequential steps in virus infection and virion assembly and maturation.

CIV Promoters

In **Chapter 5,** we have used a CIV delayed-early (DE) gene as a model to find out trans-activator protein(s) in viral infection. The promoter region of DNA*pol* gene (ORF 037L) was fine mapped to a small region of 19 bp (AAAATTGATTATTTGTTT) located between 19 and -2 relative to the mRNA start site. Changes in any of the nucleotides in the underlined AAAAT nucleotide motif in this region had a major effect on *DNApol* promoter activity showing that this motif is an essential part of the core promoter structure. The second important finding was that the critical AAAAT motif was found in the 100 nt upstream of the translational start codons of several other putative CIV DE (including 149L, 179R, 374R, 396L and 422L from virion protein genes) as derived from the CIV genome sequence (Jakob *et al.*, 2001). Many homologues of these putative DE genes also have an AAAAT motif in close proximity of their start codon in the other two completely sequenced IIVs, IIV-3 and IIV-9 (Delhon *et al.*, 2006, Wong *et al.*, 2011). Interestingly, the three sequenced *Ranavirus* genomes shared the related TAAAT motif in their DNA polymerase promoter regions. When this search is extended to the DE gene identified among CIV virion protein genes TAAAT motifs are also observed (incl. 117L, 179R, 329R, 337L, 374L, 378R and 457L) like AAAAT motif in some others mentioned above.

Whether this is pointing to a conserved regulation mechanism involved in delayed early gene expression in iridoviruses needs to be further established. As a start, the importance of these putative promoter motifs in other genes needs to be determined, as part of the homology may be the consequence of the AT-richness of the CIV genome.

Another hallmark finding of this study was the detection of a virus-specific or virus-induced protein of approximately 100 kDa, which interacted with the active site (AAAAT) of the DNA*pol* promoter and that may represent a trans-activator protein for DE genes. As *DNApol* is a DE gene, its promoter is most probably activated by a protein expressed earlier in infection, which is encoded by one of the predicted CIV ORFs that code for a protein with a molecular mass around 100 kDa (i.e. **022L**, 045L, 050L, 085L, 176R, **179R**, 184R, **261R**, **295R**, **396L** and 428L, in which the core genes are indicated in bold letters). Among these, ORFs 045L (DNA topoisomerase II), 176R and 428L (the large and a small subunit of DNA-dependent RNA polymerase) and 184R (primase-helicase domain) have predicted functions associated with

DNA. The transactivator may also consist of a multimeric protein complex, with a DNA-binding subunit of 100 kDa.

Because the expression of trans-activator genes can be controlled, transactivation can be used to turn groups of genes on and off. So far, we do not know which of these proteins is responsible for activating these genes and may be other DE CIV promoters. The interacting protein could be unraveled using an RNA interference approach to knock down the expression of candidate genes to show an eventual effect on DE (and probably also indirectly late) gene expression. This may be done with the same luciferase reporter plasmid used previously to delineate the promoter. For RNAi inhibition, a similar method may be followed as described by (Sample *et al.*, 2007), where an antisense morpholino oligonucleotide was used to down regulate the synthesis of the FV3 18-kDa IE protein. In that case, the authors showed that this protein did not act as a key viral regulator. An alternative approach would be an infected cell proteomic approach to map protein profiles, to know which proteins are present in the IE phase. Transiently expressing these candidate genes in a heterologous system followed by testing the ability of trans-activation of DE promoters is may be less attractive, as several viral proteins may be simultaneously required to allow DE gene expression, but such heterologous proteins may be analyzed in promoter binding assays.

Determining the infected cell proteome

Identification of proteins in complex biological samples by LC-MS/MS is a well-established strategy, which further exploits recently developed powerful data analysis packages such as MaxQuant (Cox & Mann, 2008). The mass spectrometry data should be analyzed with downstream bioinformatics tools using an extensive database including the proper genomic information of the input sample and globally accepted search settings. Database search algorithms can facilitate the conversion of peptide sequence information into protein identification. Success depends on a proper dataset containing all proteins potentially present in the sample, hence not using incomplete genomic data; otherwise the output search results may be misleading. The excellence of the genomic dataset is defined by the coverage achieved with the protein sample. Incorrect identification results can also be produced by the use of incorrect search engine parameters, or accepting too low score thresholds instead of global standards.

For the proteomic analysis of infected cells (**Chapter 4**) an organism was chosen for which public genome data were available and that would allow downstream bioinformatic analysis with globally accepted standards. This directed us to choose a cell line from an organism with a well-characterized genome and thus a viral infection model system was established using S2 cell line. This selection enabled the reliable delineation of viral and host proteins, and will in the future assist in understanding virus-host interactions. The same cell system was used for the transcriptome analysis (described in Chapter 3) in order to be able to compare proteome and transcriptome data.

The global profiling or differential protein expression of a target protein or protein groups in complex systems, involving for instance microbes and hosts, requires high throughput proteomic approaches and sophisticated bioinformation technologies. Developments are rapidly progressing to meet the increasing demand for rapid, highly reproducible and accurate quantitation strategies. The study presented in this thesis was the first to apply a label-free approach for virion protein detection and quantitation during the course of infection in an

iridovirus-infected cell model. The virion proteins could not be clustered using inhibitors in the same way as the transcripts, probably due, among others, to the nature of virus infection, which involves and requires protein input from the virus particle to get started. The input proteins would be measured and cannot be compared to newly synthesized proteins. Cycloheximide treated, CIV-infected samples showed, for instance, abundant late proteins such as the major capsid protein MCP (274L). As a consequence, the temporal class of the CIV virion proteins could so far only be delineated by using transcriptome data. Several labeling techniques of *de novo* synthesized proteins could be tested for this purpose. Nevertheless, the proteomic data did lead to a new way of grouping based on the presence of common protein presence profile (kinetics) over the course of infection. The virion proteins were subdivided into three subsets. Especially interesting are the group 3 proteins, present in the beginning and at the later time points, but absent in the middle of the infection process. These "starting set" of proteins may be present in the virus particle to fulfill crucial roles in the immediate early phase of infection. Unexpectedly, five known virion proteins were detected neither by the transcriptome nor by the proteomic approaches applied in these studies. These are 010R (zip protein), 096L (fasciclin domain protein), 307L (Uvr/Rep helicase), 355R (ctd-like phosphatase) and 366R with unknown function. It may be that the level of these proteins is too low to be detected by this LC-MS/MS and quantitation technique. The CIV virion gene expression is more variable than previously thought and many virion proteins are present early in infection, either as a consequence of early transcription or by introduction from the parental virus particle. The information obtained on the temporal classes of individual genes and the protein presence profile of the corresponding proteins during the course of infection will provide the basis for determining whether members of the same temporal class contain common upstream regulatory motifs and may assist to identify virion-associated proteins and other virus-encoded proteins, that control viral gene expression.

An intriguing example of virus host interaction: the 'struggle' between virus and host

In many viral infections, the cell reacts by the onset of apoptosis in the early stages of infection. This apoptosis induces the synthesis of caspases, which in turn degrade the interior of the cell including the chromosome. This response minimizes viral replication and prevents cell-to-cell transmission of progeny virus. This is a major defence mechanism in insects in response to viral infections. Iridovirus infection is often associated with induction and inhibition of apoptosis in consecutive phases (Chitnis *et al.*, 2008). Viral proteins inducing or enhancing apoptosis were postulated, but ORFs were not assigned. In **Chapter 6** the aim was to investigate whether or not ORF 193R, the most promising candidate gene, encodes a functional inhibitor of apoptosis (IAP) that can prevent virus-induced apoptosis during early infection.

Viruses in general deal with pro and anti-apoptotic regulation during their infection cycle to facilitate successful infection. Viruses encode anti-apoptotic genes (e.g. *iap*, *blc*-2, p*35*) that can bypass host defence aimed at limiting replication by killing infected cells (Clem, 2007, Hay & Kannourakis, 2002). The expression of these anti-apoptotic genes provides prolonged infected cell survival, which increases the production of progeny virus and/or may lead to virus persistence. Furthermore, viruses contain genes that may stimulate apoptosis (protein tyrosine phosphate or viral kinases), which may contribute to viral dissemination to neighboring cells. Fish iridoviruses, for instance, appear to exploit the host-defence mechanism apoptosis for their

own benefits. Late in infection with *Red sea bream iridovirus* (RSIV) vesicles are formed that resemble apoptotic bodies and that carry progeny virus particles. RSIV-infected cells also show the typical DNA fragmentation pattern, characteristic of apoptosis. The virion containing vesicles are then taken up by phagocytosis by neighboring cells (Imajoh *et al.*, 2004), which thereby get infected.

A CIV virion protein extract has been shown to induce apoptosis in *Choristoneura fumiferana* Cf124T cells (Paul *et al.*, 2007). Chitnis *et al.* (2008) showed that apoptosis requires entry and endocytosis of virions or virion proteins, and that inhibition of apoptosis under certain conditions permitted early gene expression. Which viral protein(s) is responsible for the induction of apoptosis is not known, however, from the current study CIV virion particles are now known to contain protein tyrosine phosphates (123R) and viral kinases ORFs 179R, 209R, 380R and 439R), each of which is a good candidate for pro-apoptotic activity. Very recently, Chitnis *et al.* (2011) published a paper showing that the product of CIV ORF 389L, so called iridoptin, encodes a 48 kDa protein which pure form exhibits serine/threonine kinase activity and ultimately induces apoptosis, and inhibition of host protein synthesis *in vitro* in budworm and boll weevil cell cultures (Chitnis *et al.*, 2011). The iridoptin protein may have potential for insect control. However, this protein was not among the 54 virion proteins identified in the CIV proteome (Chapter 2), although Chitnis *et al.* (2011) showed it was part of the virion. So, it remains to be elucidated, which protein in the virion is responsible for the apoptosis upon entry of the virion into cells.

On the other hand, three CIV ORFs show homology to *iap*-genes (ORF157, ORF193 and ORF332). The presence of *iap* genes in an insect iridovirus genome is of particular interest since it seems to conflict with the proposed model for cell to cell spread of vertebrate iridoviruses described above. CIV infection of SPC-BM-36 cells at a high dose (50 µg/ml) resulted in a specific cytopathology early after infection, characterized by the formation of vesicles resembling apoptotic bodies (Chapter 5; Figure 1). These vesicles disappeared at later time points post infection, when virus infection proceeded in the majority of cells, suggesting the induction of an anti-apoptotic response (Constantino *et al.*, 2001, D'Costa *et al.*, 2001, İnce *et al.*, 2008). Whether the vesicles observed early after CIV infection may also facilitate cell-to-cell dissemination of virions within the host is unknown, but the major difference with RSBIV is that the vesicles occur early instead of in the late phase (Alnemri, 1992, Imajoh *et al.*, 2004, Teodoro, 1997). Electron microscopy and/or immunolabeling will be required to show whether these CIV-induced vesicles are loaded with virus particles or not.

The question tackled in **Chapter 6** was whether CIV has a functional anti-apoptotic gene. IAPs are characterized by the presence of one to three baculovirus IAP repeat (BIR) domains at the amino terminus and often a C3HC4 RING finger domain at the carboxy terminus (Clem, 2007). CIV is the only iridovirus known to contain putative *iap* genes in its genome and CIV ORF 193R is the only iridovirus gene so far with a BIR domain. Other iridoviruses may have other mechanisms to counteract apoptosis, such as the vertebrate grouper iridovirus (GIV), where a B-cell lymphoma *bcl-2* like gene prevents apoptosis (Lin *et al.*, 2008).

The presence of IAP genes in CIV may imply that insect iridoviruses may not use apoptosis to spread virus infection in their host. In another words, either CIV is not using apoptosis at all for virus spread or it uses the anti-apoptotic proteins within a certain time span to suppress host defence, allowing the viral DNA to replicate first. CIV late gene expression and

hence replication is observed in a large range of cultured insect cells, but progeny viruses are only efficiently produced in a limited number of hosts including the rice stem borer (*Chilo suppresaria)* and the wax moth (*Galleria mellonella*). This may imply that the mechanism of progeny virus production, and may be balancing pro-and anti-apoptotic processes, needs a tightly regulated process that needs careful interplay with the host.

All the results obtained demonstrated that the putative CIV *iap* gene studied here (193R) encodes a functional anti-apoptotic protein. The encoded protein was not present in the CIV particle and most probably only provided upon IE expression after infection. One of the future ambitions is to determine whether apoptosis plays a role in cell to cell spread of CIV in fully permissive organisms despite the presence of functional *iap* genes.

Concluding Remarks - Perspectives and Open Questions

Coupling proteomics with genomics yields important information about complex biological structures in systems involving for instance pathogens and host. This is giving us a great opportunity to unravel the biology of these pathogens within the infected host organism under various conditions. A major difficulty in functional genomics of iridoviruses is the lack of recombinant systems for producing genetic (knockout) mutants of the virus. So far, a recombinant iridoviruses cannot be generated since iridovirus DNA is not infectious by itself. To allow mutagenesis identification of the protein(s) responsible for the onset of viral gene expression is necessary to initiate infection following the introduction of the DNA into the cell. As an alternative, RNAi approaches have been applied here to study functional genomics of iridoviruses. The RNAi approach had proved to be a good strategy to study CIV gene function in a previous iridovirus study, where RNA interference effectively inhibited the expression of the major capsid protein (*mcp*) gene of tiger frog virus (Xie *et al.* 2005) and was applied here to study the inhibitor of apoptosis function of ORF 193R. However, the final goal is to create a bacmid system in order to be able to make effective gene knock outs to resolve the function of individual CIV genes and to study virus-host interactions.

The current study has successfully identified the components of the CIV virion using mass spectrometry coupled to genomics and has provided clues towards unraveling the molecular mechanisms underlying CIV virion formation and towards identifying virion proteins that allow the virus to enter cells or initiate viral gene expression. To determine which of the virion proteins are engaged in the scaffolding and assembly of virions, and which are required to initiate infection, further studies are required and a stable genetic recombination system, as pointed out above, will be very helpful. Insight in the transcriptional timing of iridovirus virion protein genes was also obtained, but how the cascade of CIV viral gene expression is regulated is still far from clear, although a DE promoter motif was identified as well as a 100 kDa protein binding to this motif. Therefore, future research may also be directed to understand the mechanisms that lead to sequential viral gene expression. Infected cell protein (ICP) profiling will assist to get an overall picture of the virus and host proteins present during the course of infection.

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Abbreviations and definitions of terms

Abbreviation	Definition
CIV	*Chilo iridescent virus*
IIV	Invertebrate iridescent virus
cryo-EM	cryo-electron microscopy
GC	Guanin-Cytosin
MCP	Major Capsid Protein
ATP	Adenosine Triphosphate
DNA	Deoxyribonucleic acid
cDNA	Complementary Deoxyribonucleic acid
RNA	Ribonucleic acid
VLTF	Viral Late Transcription Factor
EM	Electron Microscopy
PI	Glycerophosphatidylinositol
FBS	Fetal Bovine Serum
ER	Endoplasmic Reticulum
AFKM-On-H	Armand Frappier, King Mongkut Institutes, Ostrinia nubilalis, and Hemocytes
bp	base pair
AT	Adenine-Thymine
ICP	Infected Cell Protein
GEC	Grouper Embryonic Cell line
IE	Immediate Early
DE	Delayed Early
L	Late
h p.i.	hours post infection
mRNA	messenger Ribonucleic Acid
EDTA	Ethylene Diamine Tetra-acetic Acid
iap	Inhibitor of apoptosis
BIR	Baculovirus IAP Repeat
RING	Really Interesting New Gene
LC-MS/MS	liquid chromatography mass/mass spectrometry
kDa	kilo Dalton
ACN	Acetonitrile
UV	Ultraviolet
ABC	Ammonium Bicarbonate
DTT	dithiothreitol
IAA	iodoacetamide
mL	Milliliter
TFA	Triflouroacetic
MW	Molecular weight markers
min	minute
FTMS	Fourier transform mass spectra
LTQ-Orbitrap	linear trap quadrupole
NS	Nonstructural proteins
nt	Nucleotides
ppm	parts per million
ORF	Open reading frame
amu	atomic mass unit
m/z	mass/charge ratio
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
BSA	Bovine Serum Albumin
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	*Spodoptera frugiperda*
TEM	Transmission electron microscopy
sf	Bioworks Score factor
CTD	carboxy-terminal domain
NTP	nucleoside triphosphatase
vRNA	Viral RNA

Summary

Iridoviruses have been reported to cause disease in insects, fish and amphibians. Fish iridoviruses have both ecological and economic impact, and have emerged especially in marine fish farming in recent years. Iridoviruses have also been recognized as emerging pathogens in amphibians, leading to high mortality in frogs and toads. Insect iridoviruses infect for instance weevils and caterpillars, which may cause serious feeding damage in agro-ecosystems. *Chilo iridescent virus* (CIV), originally isolated from *Chilo suppressalis* (Lepidoptera; Crambidae) is the model for insect-infecting iridoviruses and a potential biocontrol agent for weevils (Coleoptera; Curculionoidea), for instance in tea and hazelnut in the Black Sea Region as well as in cotton producing areas in the South-Eastern and Mediterranean parts of Turkey. Iridoviruses have also been found in insects that transmit plant pathogens or parasites of medical importance, such as whiteflies, grasshoppers, and mosquitoes.

Iridoviruses are large, double-stranded DNA viruses, with an icosahedral capsid. The linear viral genome is terminally redundant. Viral replication initiates in the host cell nucleus, and is completed in the cytoplasm where virion assembly takes place. The complete genome sequence of CIV is known, but the function of many of the encoded proteins is poorly understood. Crucially, which of the predicted genes encode proteins that are present in the virus particle is largely unknown. This information is for instance important to determine which proteins are the main players in spreading the virus infection from cell-to-cell inside the insect body as well as how the onset of viral gene expression is regulated. Functional genomics and studies towards host-pathogen interactions in iridoviruses in general have been hampered by the lack of easy genetic recombination systems (e.g. a bacmid). A major complication is that the iridoviral DNA by itself is not infectious and that an unknown component of the virus particle is needed to initiate viral gene expression. In this thesis, the determination of virion protein composition and the regulation of the corresponding genes has received priority.

In Chapter 2, the protein content of the CIV virion was determined. In this first proteomics study of an invertebrate iridovirus, 46 CIV-encoded proteins were identified based on the detection of two or more distinct peptides in LC-MS/MS analysis. An additional 8 virion proteins were found based on a single peptide. For 36 of the identified virion protein genes homologs are present in other iridoviruses. Five of these proteins, 22L (putative helicase), 118L, 142R (putative RNaseIII), 274L (major capsid protein) and 295L, are encoded by all iridoviruses for which the genome sequence is known and, therefore, represent iridovirus core virion proteins, which likely have crucial roles in the infection process. Three proteins have homologs only in ascoviruses and the remaining 15 proteins in the proteome are so far unique to CIV. This information is not only broadening the insight in the structure and assembly of CIV virions, but is also pivotal in the unraveling the initial steps in the infection process and the role of virion proteins therein.

Knowledge on transcription regulation of the CIV genes was rather limited at the start of this PhD study. In Chapter 3, therefore, the aim was to determine the transcriptional class (immediate or delayed early, or late) of the set of virion protein genes identified in the previous chapter. In this way it was guaranteed that truly expressed ORFs were analyzed. The temporal

regulation of the CIV virion protein genes was unraveled by combining drug treatments and a novel RT-PCR strategy, especially designed to amplify the non-polyadenylated CIV mRNAs. For this analysis a *Drosophila melanogaster* cell line was used, an organism for which genomic information is available. This is relevant as we simultaneously performed an infected-cell proteomic study (see below). In contrast to the general idea that most virion protein genes would belong to the late temporal class, as the encoded proteins have to be incorporated in the virus particle, which is formed after DNA replication, they were distributed over all three temporal classes mentioned above.

In order to see how the transcriptome data fitted with virion protein presence in infected cells, an LC-MS/MS based proteomic approach was applied to follow virion protein levels during the course of infection (Chapter 4). Proteomic data were processed using a label-free quantitation approach (MaxQuant algorithm), which enabled the quantitation of peptides from samples collected at different time points post infection. The resulting virion protein profiles showed three different subsets (Groups 1 to 3). The proteins of group I were stably present during the course of infection, group 2 proteins were not found at the onset of infection, but were present as early as 1 h p.i. and were accumulating over time, and group 3 proteins were present at the start of infection, were absent for a period of time, and re-appeared at later time points after infection. The early and late transcription and probably also translation of virion protein genes indicates that several of these proteins play crucial roles in the initial stages of infection or entering to the host cells, while others may be crucial to build the structure of the virions or to enter cells. In addition, some proteins may have early and late functions.

In Chapter 5, a closer look is given to transcriptional regulation using the delayed-early CIV gene for DNA polymerase as a model. The promoter region was fine mapped by constructing a series of increasing deletions and by introducing point mutations. The effects of these mutations were examined in a luciferase reporter gene system using insect cells transfected with promoter constructs and infected with CIV. When the size of the upstream element was reduced to less than 19 nucleotides relative to the transcriptional start site, the luciferase activity was reduced to almost zero. Point mutations showed that five nucleotides located between –19 and –15 (AAAAT) were equally essential for promoter activity. This AAAAT motif was also found in DNA polymerase promoter regions of other iridoviruses and in other putative CIV delayed early genes. An intriguing finding of this study was that a protein of approx. 100 kDa interacted with this element (but not with a mutant) as was shown in South-Western analysis of CIV infected cells. This infected cell- specific viral protein may be responsible for initiating delayed-early transcription.

In Chapter 6 attention was given to the biology of the initial phase of virus infection. When cells become infected with a virus a major first line defense mechanism is programmed cell death or apoptosis. To overcome this response, many viruses encode anti-apoptotic proteins. The genome of CIV was analyzed *in silico* for genes encoding putative anti-apoptotic proteins. Three ORFs were found with homology to the baculovirus *inhibitor of apoptosis (iap)* gene family. While all three proteins contain C-terminal RING domains, only the protein encoded by ORF 193R contains a baculoviral iap repeat (BIR) domain, indicative of a functional IAP protein. CIV ORF 193R is the only gene known to exist among members of the family *Iridoviridae* that encodes a BIR domain. 193R gene is transcribed as an immediate early gene during CIV infection. When this putative CIV IAP was transiently expressed in SPC-BM-36 and Sf21 cells

using an immediate early baculovirus promoter, it significantly reduced the induction of apoptosis by the drug actinomycin-D. Silencing of the CIV *iap* gene with gene-specific double-stranded RNAs during infection resulted in apoptosis. The conclusion was that ORF 193R is the first functional *iap* gene identified in an iridovirus.

The research described in this PhD dissertation resulted in fundamental knowledge on the protein composition of CIV virions and the regulation of gene expression of these proteins. The study also revealed a promoter motif typical for delayed-early genes, which interacts with a putative trans-activator protein, and identified an anti-apoptotic protein crucial for viral infection. In Chapter 7, the data obtained are discussed, covering both fundamental findings and novel technical developments to open new venues for future research to answer remaining questions concerning the biology of the interesting iridovirus family.

Samenvatting

Iridovirussen veroorzaken ziektes in insecten, vissen en amfibieën. Iridovirussen van vissen zijn zowel ecologisch als economisch van belang, mede omdat het aantal infecties vooral in marine viskwekerijen in de laatste jaren sterk is toegenomen. Ook leiden iridovirusinfecties tot hoge sterftecijfers in kikkers en padden en in deze gastheren worden iridovirussen als opkomende pathogenen beschouwd. In insecten worden iridovirussen gevonden in o.a. snuitkevers en rupsen, die beide serieuze vraatschade aan kunnen richten in agro-ecosystemen. Het model voor iridovirussen in insecten is *Chilo iridescent virus* (CIV), dat oorspronkelijk is geïsoleerd uit *Chilo suppressalis* (Lepidoptera; Crambidae). CIV kan veel verschillende soorten gastheren infecteren en kan mogelijk ingezet worden om snuitkevers (Coleoptera; Curculionoidea) te bestrijden, bijvoorbeeld in de thee- en hazelnootcultuur in het Zwarte Zee-gebied en in de katoenproducerende regio's in het zuidoosten en in de mediterrane regio van Turkije. Insecten die pathogenen van planten of medisch belangrijke parasieten overbrengen, zoals witte vliegen, sprinkhanen en muggen, zijn ook vatbaar voor iridovirussen.

Iridovirussen hebben een groot, dubbelstrengs DNA genoom. De eiwitmantel die het DNA omgeeft heeft de symmetrie van een regelmatig twintigvlak. Het lineaire virale genoom is iets langer dan de genoomsequentie, doordat het begin van het genoom aan het einde van het DNA-molecuul herhaald wordt. Welk deel precies repeterend is verschilt per virusgenoom en zodoende is de genetische kaart circulair. De replicatie van het virale DNA begint in de kern van de cel en wordt vervolgens voltooid in het cytoplasma, waar ook de nieuwe virusdeeltjes worden samengesteld. De complete genoomsequentie van CIV is bekend, maar van de meeste gecodeerde eiwitten is niet bekend welke functie zij vervullen in het infectieproces. Zo is bijvoorbeeld slechts heel summier bekend welke genen coderen voor de eiwitten die het virusdeeltje (virion) vormen. Deze informatie is belangrijk om te kunnen bepalen welke eiwitten een hoofdrol spelen bij de verspreiding van het virus van cel naar cel en hoe de virale genexpressie wordt geïnitieerd. Studies naar de functie van de virale genen en naar de rol van de gecodeerde eiwitten bij virus-gastheer interacties worden bemoeilijkt door het feit dat er geen eenvoudige genetische recombinatiesystemen bestaan voor iridovirussen zoals een bacmidesysteem. Een complicatie bij de ontwikkeling van een dergelijk systeem is verder dat gezuiverd iridovirus-DNA niet infectieus is en dat een nog onbekende component van het virusdeeltje nodig is om de virale genexpressie te starten. In dit proefschrift ligt de nadruk op het bepalen van de eiwitsamenstelling van het CIV-virusdeeltje en de regulatie van de bijbehorende genen.

Welke eiwitten aanwezig zijn in het CIV-virion werd onderzocht in Hoofdstuk 2. CIV is het eerste insecteniridovirus waarvoor een proteoomanalyse werd uitgevoerd. Zesenveertig CIV-gecodeerde eiwitten werden geïdentificeerd op basis van detectie van twee of meer peptiden in een LC-MS/MS- analyse. Acht extra virale eiwitten werden gevonden op grond van één uniek peptide. Voor zesendertig van de gevonden CIV-virioneiwitten zijn homologe eiwitten aanwezig in andere iridovirussen. Vijf van deze eiwitten, 22L (waarschijnlijk een helicase), 118L, 142R (een mogelijk RNaseIII), 274L (het grote manteleiwit) en 295L, worden gecodeerd in alle tot nu toe bekende iridovirusgenomen. Deze gemeenschappelijke iridoviruseiwitten vervullen

hoogstwaarschijnlijk cruciale functies in het infectieproces. Drie CIV-virioneiwitten hebben alleen homologen in ascovirussen en de overige 15 zijn tot nu toe uniek voor CIV. Deze informatie heeft het inzicht in de structuur en assemblage van CIV-virions verdiept en is van essentieel belang om de rol van virioneiwitten in de beginfase van het infectieproces te kunnen vaststellen.

De kennis aangaande de transcriptie van CIV-genen was erg beperkt aan het begin van deze studie. Het doel in Hoofdstuk 3 was dan ook om van de genen, die coderen voor de virioneiwitten, de transcriptieklasse (vroeg, vertraagd of laat) te bepalen. Op deze manier werd ervoor gezorgd dat alleen echte genen werden geanalyseerd. Hoe de expressie van deze genen in loop van de infectie wordt gereguleerd, werd bepaald met behulp van eiwit- en DNA-syntheseremmers in combinatie met een nieuwe RT-PCR-strategie, die speciaal ontworpen werd om niet-gepolyadenyleerde mRNAs, zoals die van CIV, te kunnen analyseren. Hiervoor werd gebruikgemaakt van cellen van de fruitvlieg *Drosophila melanogaster*, een modelorganisme waarvan het hele genoom in kaart is gebracht. Dit is relevant, omdat tegelijkertijd een proteoomanalyse van de geïnfecteerde cel werd uitgevoerd (zie hieronder). In tegenstelling tot de algemene opvatting dat de meeste genen voor virioneiwitten tot de late klasse behoren (immers deze eiwitten moeten in het virusdeeltje worden ingebouwd, dat gevormd wordt ná DNA replicatie), waren ze vertegenwoordigd in alle drie bovengenoemde transcriptieklassen.

Om te zien of de gegevens betreffende het transcriptoom overeenkwamen met de aanwezigheid van de corresponderende eiwitten in geïnfecteerde cellen werd een proteoomanalyse uitgevoerd (LC-MS/MS), waarbij de hoeveelheid van de verschillende virioneiwitten werd bepaald gedurende de infectiecyclus (Hoofdstuk 4). Hiertoe werd een labelvrije kwantificeringsmethode gebruikt (MaxQuant algoritme), die het mogelijk maakte om peptiden te kwantificeren in monsters, genomen op verschillende tijdstippen na infectie. De hieruit voortkomende profielen lieten drie groepen van virioneiwitten zien. De eiwitten in groep 1 waren aanwezig gedurende het hele verloop van de infectie; groep 2-eiwitten waren niet aanwezig aan het begin van de infectie, maar werden reeds na 1 uur aangetroffen en accumuleerden vervolgens gedurende de infectie; groep 3 eiwitten waren in het begin aanwezig, verdwenen dan voor een bepaalde tijd om dan later in infectie weer te verschijnen. De vroege transcriptie van een aantal viriongenen (en waarschijnlijk ook de translatie van de bijbehorende transcripten) is een indicatie dat verscheidene van deze eiwitten essentiële functies vervullen in de initiële infectiestadia, terwijl andere eiwitten belangrijke bouwstenen zijn voor de structuur van de virions. Ook zijn er mogelijk eiwitten met zowel vroege als late functies.

In Hoofdstuk 5 werd de transcriptieregulatie van een vertraagd gen onder de loep genomen, waarbij het DNA-polymerase gen van CIV model stond. Het regulatiegebied, voorafgaand aan het coderende deel van het gen (= promoter), werd nauwkeurig in kaart gebracht door een serie deletiemutanten te construeren en een aantal puntmutaties aan te brengen. De gemuteerde promotersequenties werden voor een luciferase-reportergen geplaatst en getest in getransfecteerde en vervolgens met CIV geïnfecteerde insectencellen. Wanneer de lengte van het gebied voor de translatiestart werd ingekort tot minder dan 19 nucleotiden, werd de luciferase-activiteit tot vrijwel nul teruggebracht. Puntmutaties lieten zien dat 5 nucleotiden tussen positie −19 en −15 (AAAAT) essentieel waren voor het functioneren van de promoter. Zo'n AAAAT-motief werd ook gevonden in de promoterregio's van de DNA-polymerases van andere iridovirussen en in andere vertraagde CIV-genen. Een intrigerende uitkomst van deze

studie was dat een eiwit van ongeveer 100 kDa aan dit promoterelement bond, maar niet aan een mutant hiervan. Dit eiwit was alleen aanwezig in geïnfecteerde cellen en is mogelijk verantwoordelijk voor de transcriptiestart van de klasse van vertraagde genen.

In Hoofdstuk 6 wordt aandacht besteed aan de biologie van de initiële fase van CIV-infectie. Geprogrammeerde celdood of apoptose is een belangrijk eerstelijns verdedigingsmechanisme tegen virusinfectie in het algemeen. Veel virussen coderen anti-apoptotische eiwitten om deze cellulaire reactie tegen te gaan. Het genoom van CIV werd *in silico* getest op de aanwezigheid van genen met een mogelijke anti-apoptotische functie. Voor drie coderende genoomsegmenten (= open reading frames of afgekort ORF) werd homologie gevonden met leden van de baculovirus-*inhibitor of apoptosis (iap)*- genfamilie. Hoewel deze ORFs alle drie coderen voor eiwitten met een C-terminaal RING-domein, bevat alleen het eiwit gecodeerd door ORF 193R ook de repeterende sequentie, die karakteristiek is voor functionele IAP-eiwitten, het baculovirus-IAP-repeterend (BIR) domein. Voor zover bekend is ORF 193R het enige gen in de familie *Iridoviridae* dat genetische informatie bevat voor een BIR-domein. 193R behoort tot de klasse van vroege genen. Toen dit CIV-*iap*-achtige gen tot expressie werd gebracht in SPC-BM-36 en Sf21 cellen onder controle van een vroege baculoviruspromoter, werd de inductie van apoptose door actinomycine D drastisch gereduceerd. Daarentegen leidde het stilleggen van de expressie van 193R tijdens de infectie met behulp van een 193R-specifiek, dubbelstrengs RNA-molecuul tot apoptose. De conclusie uit deze experimenten was dat ORF 193R het eerste functionele *iap*-gen is, dat geïdentificeerd is in een iridovirus.

Het onderzoek, beschreven in dit proefschrift, heeft fundamentele kennis opgeleverd omtrent de eiwitsamenstelling van de virusdeeltjes van CIV en de expressieregulatie van de corresponderende genen. Ook werd een promotermotief gevonden, dat typisch is voor vertraagde CIV genen en dat interactie aangaat met een mogelijk transactivatoreiwit. Ook werd een anti-apoptotisch eiwit geïdentificeerd, dat cruciaal is voor virale infectie. De verkregen gegevens worden in Hoofdstuk 7 bediscussieerd, waarbij zowel aandacht wordt geschonken aan fundamentele resultaten als aan recente technische ontwikkelingen, die mogelijkheden bieden voor toekomstig onderzoek om de nog openstaande vragen te beantwoorden betreffende de biologie van deze boeiende virusfamilie.

Özet

Iridovirüslerin böcekler, balıklar ve amfibilerde hastalık etmeni olduğu bilinmektedir. Son yıllarda balıklarda enfeksiyona neden olan iridovirüslerin, özellikle deniz balığı yetiştiriciliği sektöründe ekolojik ve ekonomik etkilere neden olduğu belirlenmiştir. Ayrıca, iridovirusler amfibilerin önemli bir patojeni olarak değerlendirilmekte ve kurbağalarda yüksek oranlarda ölümlere yol açtığı görülmektedir. Böcek iridovirusleri tarımsal ekosistemlerde ciddi zararlara neden olan böceklere ait larvalarda enfeksiyona neden olmaktadır. *Chilo iridescent virus* (CIV), çeltik sap tırtılı *Chilo suppressalis*`den (Lepidoptera; Crambidae) izole edilemiş, böcekleri enfekte eden iridovirüsler için model teşkil etmektedir. Türkiye`de, özellikle Doğu Karadeniz Bölgesinde fındık ve çay, Güney Doğu ve Akdeniz Bölgelerinde pamuk üretim alanlarında zarara neden olan kın kanatlılar takımının (Coleoptera) hortumlu böcekgiller (Curculionoidea) ailesine (familyasına) ait böceklere karşı biyolojik kontrol ajanı olarak geliştirilebilme potensiyeline sahiptirler. Ayrıca iridovirusler, beyaz sinek, çekirge ve sivrisinek gibi tıbbi öneme sahip parazitlerde veya bitki patojenlerini taşıyan taşıyan böceklerde tespit edilmiştir.

Iridovirüsler, çift zincirli DNA içeren ikozahedral kapsid yapısına sahip büyük virüslerdir. Doğrusal yapıdaki viral genom uçlarında, tekrarlayan DNA dizileri içerir. Viral replikasyon konak hücre çekirdeğinde başlar ve virion yapının oluşturulduğu sitoplazmada son bulur. CIV genomunun tüm dizisi bilinmektedir, fakat kodlanan birçok proteinin işlevleri bilinmemektedir. En önemlisi, virus partikülünde bulunan proteinlerin öngörülen genlerin hangileri tarafından kodlandığı büyük çoğunlukla bilinmemektedir. Bu bilgi, örneğin, hangi proteinlerin viral enfeksiyonun böcek vücudunda hücreden hücreye yayılmasından sorumlu olduğunun anlaşılmasının yanında, viral gen ifadesinin başlangıcının nasıl düzenlendiğinin anlaşılması için de önem arz etmektedir. İridovirüslerde, işlevsel genomik ve konak-patojen etkileşimlerinin anlaşılmasına yönelik çalışmaların önündeki en önemli engel kolay uygulanabilir bir genetik rekombinasyon sisteminin (örn; bacmid) bulunmayışıdır. Bu konudaki başlıca zorluk, iridovirus DNA`sının kendi kendine enfeksiyon yapma yeteneğinin bulunmayışıdır. Viral gen ifadesinin başlaması için virus partikülünde bulunan henüz bilinmeyen birimlerin varlığı gereklidir. Bu tez çalışmasında, virion protein kompozisyonunun belirlenmesi ve ilgili genlerin düzenlenmesi öncelikli olarak ele alınmıştır.

İkinci bölümde, CIV virion yapısındaki proteinler belirlenmiştir. Omurgasızları enfekte eden iridoviruslere ait ilk protemik çalışması olarak gerçekleştirilen bu çalışmada, LC-MS/MS analiziyle iki veya daha fazla peptidin tespitine dayalı olarak virion yapısında 46 CIV proteini tespit edilmiştir. Ayrıca 8 virion proteini tek peptidin tanımlanmasıyla tespit edilmiştir. Tanımlanan 36 virion protein genlerinin homologları diğer iridoviruslerde tespit edilmiştir. Bu proteinlerden beşi, 22L (helikaz), 118L, 142R (RNazIII), 274L (kapsid proteini) ve 295L, genomu bilinen tüm iridoviruslerde bulunmuştur ve bu nedenle iridovirus ana virion proteinleri olarak tanımlanmakta ve Enfeksiyon prosesinde önemli rol oynadıklarına inanılmaktadır. Üç proteinin sadece ascoviruslerde homologları tespit edilmiştir. Proteomda belirlenen, geriye kalan 15 proteinin sadece CIV`ye özgün proteinler olduğu belirlenmiştir.

Bu bilgi, CIV virion yapısı ve paketlenmesiyle ilgili fikirlerimizi genişletmekle kalmayıp, viral enfeksiyonun başlangıç aşamalarının açıklığa kavuşturulması ve virion proteinlerinin bu süreçteki işlevlerinin anlaşılması için de temel teşkil etmektedir. CIV genlerinin transkripsiyonun düzenlenmesiyle ilgili bilgiler bu tez çalışmasının başlangıç aşamasında oldukça sınırlıydı. Bu nedenle, üçüncü bölümde bir önceki bölümde tespit edilen virion proteinlerinin transkripsiyonel sınıflarının (en erken, erken veya geç) tespit edilmesi hedeflenmiştir. Bu yolla, gerçekten ifade edilen açık okuma çerçevelerinin analiz edilmesi sağlanmıştır. CIV virion proteinlerinin zamana bağlı ifadelerinin tespiti inhibitor maddelerin kullanılarak, yeni bir RT-PCR stratejisinin uygulanmasıyla tespit edilmiştir. Bu yeni RT-PCR stratejisi özellikle poliadenilasyon sinyali içermeyen CIV mRNA`larının tespiti için tasarlanmıştır. Bu analiz için genom dizisi bilinen *Drosophila melanogaster* hücreleri kullanılmıştır. Bu analize eş zamanlı olarak enfekte edilmiş hücrelerden proteomik analiz çalışması gerçekleştirilmiştir (Bakınız; sonraki bölüm). Genel olarak, virion proteinlerinin virus partikülünün yapısına katılması nedeniyle geç gen sınıfına dahil olduğu düşüncesinin aksine virion proteinlerinin yukarıda bahsi geçen zamana bağlı üç sınıfa da dahil olduklara tespit edilmiştir.

Virion proteinlerinin transkriptom verileriyle, enfekte hücrelerde bu proteinlerinin bulunması arasında nasıl bir ilişki bulunduğunun anlaşılması için LC-MS/MS tabanlı protemik yaklaşımla virion proteinlerinin düzeyleri enfeksiyon sürecinde tespit edilmiştir (Bölüm 4). Proteomik verilerin analizi işaretsiz kantitatif yaklaşımla (Maxquant algoritması) gerçekleştirilmiştir. Bu yaklaşım, enfeksiyon sürecinin farklı zamanlarında toplanan örneklerden elde edilen peptidlerin kantitatif analizine imkan sağlamaktadır. Analiz sonucunda, virion proteinleri üç farklı profil göstermiştir. Grup 1 proteinleri stabil olarak enfeksiyon boyunca bulunmaktadırlar. Grup 2 proteinleri enfeksiyonun başlangıç aşamasında belirlenememelerine rağmen enfeksiyonu takip eden 1. saatten itibaren zamana bağlı olarak artmaktadırlar. Grup 3 proteinleri enfeksiyon başlangıçında tespit edilmelerine e ilerleyen zamanda kaybolmakta ve sonrasında tekrar ortaya çıkmaktadırlar. Virion proteinlerinin, erken veya geç transkripsiyonu ve muhtemelen de translasyonu, bu proteinlerden bazılarının enfeksiyonun başlangıç aşamasında veya viruslerin konak hücrelere girişinde etkin işlevlere sahipken, diğerlerinin ise viral yapının inşasında önemli işlevlere sahip olabileceğini göstermektedir.

Beşinci bölümde, DNA polimeraz geni, CIV ertelenmiş erken genlerine model olarak kullanılarak transkripsiyonel düzenlenmesine detaylı bir bakış yapılmıştır. Bir seri artan sayıda delesyon ve nokta mutasyonu içeren promotor yapılarının hazırlanmasıyla promoter bölgesi detaylı bir şekilde haritalanmıştır. Bu mutasyonların etkileri lusiferaz gen ifade sistemi kullanılarak hazırlanan promoter yapılarınının böcek hücrelerine transfeksiyonu aracılığıyla CIV enfeksiyonu varlığında test edilerek gözlenmiştir. Transkripsiyon başlangıç noktasının yukarısında bulunan bölgenin 19 nükleotiden az olduğu durumların test edildiği promoter yapılarında lusiferaz aktivitesinin neredeyse sıfır olduğu görülmüştür. Nokta mutasyonu çalışmaları, –19 ila –15 (AAAAT) bölgesinde bulunan nükleotidlerin promoter aktivitesi açısından eşit düzeyde gerekli olduklarını göstermiştir.

Bu AAAAT motifi diğer iridoviruslerin DNA polimeraz promoter bölgelerinde ve olası CIV ertelenmiş erken genlerinde de tespit edilmiştir. Bu çalışmanın en ilgi çekici bulgusu ise yaklaşık 100 kDa`lık bir proteinin bu bölge ile (fakat mutant hali ile değil) etkileşim gösterdiğinin CIV ile enfekte edilen hücrelerde South-Western analiziyle tespit edilmesidir. Enfekte hücrelere özgün bu viral proteinin ertelenmiş erken genlerin transkripsiyonundan sorumlu bir protein olabileceği düşünülmektedir.

Altıncı bölümde, virus enfeksiyonunun başlangıç fazının biolojisi üzerine yoğunlaşılmıştır. Hücreler bir virusle enfekte olduğunda ilk savunma hattı mekanizması programlanmış hücre ölümüdür (apoptosis). Bu mekanizmayla mücadele etmek için, birçok virus apoptosis inhibitor proteinleri kodlamaktadır. CIV genomu muhtemel anti apoptotic proteinleri kodlayıp kodlamadığının belirlenmesi için *in silico* olarak analiz edilmiştir. Üç açık okuma çerçevesinin baculovirus apoptosis inhibitör gen ailesiyle (*iap*) homolojileri tespit edilmiştir. Üç protein de, karboksi (C) ucundaki RING işlevsel bölgesine sahipken, sadece açık okuma çerçevesi 193R, işlevsel IAP proteininin göstergesi olan baculoviral iap tekrar (BIR) işlevsel bölgesine sahiptir. CIV 193R geni, *Iridoviridae* ailesine ait üyeler arasında BIR işlevsel bölgesi kodladığı belirlenen tek gendir. CIV enfeksiyonunda 193R geni en erken gen ifade edilen gendir. Bu olası CIV IAP proteini geçici olarak SPC-BM-36 ve Sf21 hücrelerinde en erken baculovirus promotoru kullanılarak ifade edildiğinde aktinomisin-D ile teşvik edilen apoptosisi önemli düzeyde baskıladığı tespit edilmiştir. Enfeksiyon sırasında, CIV *iap* geninin, gene özgün çift zincirli RNA`lar kullanılarak susturulması apoptosisle sonuçlanmıştır. Sonuç olarak, 193R açık okuma çerçevesinin, iridoviruslerde belirlenen ilk işlevsel *iap* geni olduğu tespit edilmiştir.
Bu tez çalışmasında yürütülen araştırmalar sonucu, CIV virion protein yapısı ve bu virion proteinlerin ifadesinin düzenlenmesi ile ilgili temel bilgiler açığa çıkarılmıştır. Mevcut çalışma ayrıca olası transaktivatör proteinle etkileşen tipik ertelenmiş erken gen promoter motifinin ve viral enfeksiyonda çok önemli işlevi olan anti-apoptotik proteinin tespitini sağlamıştır. Yedinci bölümde bu ilginç iridovirüs ailesinin biyolojisi hakkında kalan soruları yanıtlamaya yönelik yeni araştırmalara yol açan yeni tekniksel gelişmeler ile temel bulgular ve elde edilen veriler tartışılmaktadır.

摘要

虹彩病毒具有广泛的宿主域，其宿主包括昆虫，鱼类和两栖类动物。鱼虹彩病毒具有重要的生态及经济意义并在近年来在海洋渔业养殖中产生了重大影响。在两栖类动物中虹彩病毒同样是重要的新生病原体并造成了青蛙和蝾螈的大量死亡。在农业生态系统中昆虫虹彩病毒会感染象鼻虫和毛虫并造成重大影响。在二化螟幼虫（鳞翅目，草螟科）中分离得到的二化螟虹彩病毒（CIV）是昆虫虹彩病毒的模式株。二化螟虹彩病毒具有广泛的宿主域，针对象鼻虫（鞘翅目）是一种潜在的生物防治利器。这种病毒对于防治黑海地区的茶叶和榛实象鼻虫害及土耳其东南和地中海区域的棉花虫害具有防治意义。在传播植物病原物和致病性寄生虫的昆虫中，例如粉虱，蚱蜢和蚊子，也分离到了虹彩病毒。

虹彩病毒是具有二十面体衣壳结构的大双链DNA病毒。线状的病毒基因组具有末端冗余。病毒的复制起始于细胞核，在细胞质完成。病毒的组装也发生于细胞质。CIV的全基因组已得到测序，但是许多基因的功能尚未得到解析。尤其是目前尚不知道哪些基因是病毒的结构基因。鉴定病毒的结构基因对于了解病毒感染的机理具有重要的意义，包括鉴定在系统感染中的重要病毒因子和理解病毒基因表达的调控。由于缺乏一个简便的基因重组系统CIV的功能基因组学和病毒与宿主的相互作用的研究进展缓慢。一个主要的限制因素是虹彩病毒的DNA自身不具感染性，而必须同时具有一个目前未知的病毒结构组分才能起始病毒基因的表达。基于此本论文着重鉴定病毒结构蛋白及解析相关基因的调控机理。

本论文的第二章对二化螟虹彩病毒的结构蛋白组分进行了鉴定。在这项针对无脊椎动物虹彩病毒的首次蛋白质组学的研究中，利用液相级联质谱的分析方法检测到了具有两个以上的不同肽段的46种二化螟虹彩病毒结构蛋白。此外，还检测到具有单一肽段的8个病毒结构蛋白。这些蛋白的编码基因中有36个在其它的虹彩病毒中有同源序列，其中的5个在所有已测序的虹彩病毒基因组中保守，包括22L（解旋酶），118L，142R（RNA酶III），274L（主要衣壳蛋白）和295L，意味着它们是虹彩病毒的核心基因并在病毒感染过程中起重要的作用。三个蛋白仅在囊泡病毒中有同源序列，而剩下的15个蛋白是仅在二化螟虹彩病毒中存在的。这些发现不仅对于理解二化螟虹彩病毒的结构和组装机理而且对于理解病毒感染的初始阶段的机理都具有重要的意义。

在此项研究进行之初，关于二化螟虹彩病毒基因转录调控的了解非常有限。本论文的第三章致力于将鉴定出的病毒结构蛋白的基因转录进行分类，包括极早期，迟早期和晚期转录。对二化螟虹彩病毒结构蛋白转录时相的分析综合了药物处理和一种新的针对CIVmRNAs的RTPCR策略。这些分析是基于 Drosophila melanogaster细胞系，因为目前对其遗传背景有较好的了解。同时进行的被感染细胞蛋白质组学的研究使上述实验更具重要意义。研究表明CIV的结构蛋白在极早期，迟早期和晚期都有表达。这与通常的认识不同，因为一般的理论认为病毒的结构蛋白均为晚期表达。

为了分析转录谱的数据与被感染细胞内病毒蛋白表达的关系，在第四章中我们利用基于 LC MS/MS的方法来研究感染过程中病毒蛋白的表达水平。为了对不同感染时相的样品进行定量分析，我们对蛋白质谱的数据用MaxQuant算法进行了分析（一种无需标记的定量方法）。结果表明病毒蛋白可被分为三个亚组（组1至组3）。组1蛋白在感染的整个过程中稳定存在，组2蛋白在感染1小时候后出现并随着感染的持续而积累，组3蛋白在感染的起始过程中出现随后消失继而在感染的晚期重新出现。病毒蛋白的早晚期转录提示这些蛋白对于感染的起始有重要作用，而其它的蛋白则对病毒结构的组装和侵入细胞具有重要的作用。还有一些蛋白则可能具有早期和晚期的功能。

在第5章中研究的重点是CIVDNA聚合酶的转录调控。通过一系列缺失和定点突变该基因的启动子区域得到了鉴定。这一系列突变的影响是利用构建的含启动子区的质粒转染，并用CIV感染的昆虫细胞荧光素酶系统进行的检测。当缺失上游元件至转录起始点－19位时，荧光素酶的表达几乎被降至零点。点突变实验表明转录起始点－19至－15位的5个核苷酸（AAAAT）对启动子活性具有同等重要的作用。该AAAAT特征域也存在于其它虹彩病毒的DNA聚合酶和CIV的迟早期基因中。另一个有趣的发现是在感染细胞中一个约100kDa的蛋白结合于该特征域但并不结合突变的序列。该蛋白可能对于起始迟晚期基因的转录具有重要作用。

第六章的研究重点放在了病毒感染的初始阶段。细胞被病毒感染时一个重要的防御机制是细胞凋亡。为了抵御这种机制很多病毒编码了抗凋亡蛋白。我们对CIV的基因组进行了分析以鉴定出可能的抗凋亡蛋白。分析表明CIV基因组中含有三个基因与杆状病毒的凋亡抑制因子具有同源性。虽然三个蛋白均含有RING结构域，其中只有193R蛋白含有BIR结构域。CIV193R蛋白是目前已知的唯一一个虹彩病毒科中编码BIR结构域的蛋白。这RING和BIR结构域的同时存在提示该蛋白可能是一个凋亡抑制因子。在CIV感染过程中193R是一个极早期基因。这个基因在SPC-BM-
36和Sf21细胞中的瞬时表达时能显著性的抑制actinomycinD诱导的凋亡。针对CIV凋亡抑制因子的RNAi造成了被感染细胞的凋亡。该研究的结论是193R是虹彩病毒中第一个被鉴定出来的凋亡抑制因子。

本论文的研究解析了CIV病毒粒子的蛋白组分及这些蛋白表达的调控。同时我们鉴定出了一个迟早期基因的启动子序列和一个对于病毒感染非常重要的抗凋亡蛋白。论文的第七章对论文的结果进行了综合讨论，不仅针对CIV的感染机理提出了一些重要的结论，还对如何深入虹彩病毒的研究进行了展望。

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I am indebted to my colleagues, friends and students for providing a stimulating and fun environment in which to learn and enjoy. I am especially grateful to colleagues Agata Jakubowska and Jochem Verrelst, Afshin Mehraban, Anelya (Adife) Ogynyanova, Aytaç Kocabaş, Burcu Ekmekçi, Caner-Yelda Ünlü, Changyong Liang, Christina Geerts, Daniela Ribeiro, Deng Fei, Dick Peters, Bui Thi Minh Dieu, Dilek Sağlam, Fang Xu, FeiFei Yin, Gang Long, Ghulam Ali, Hakan Baykuş, Henry Kariithi, Hoa Tuyet, Liljana Georgievska, Manli Wang, Magda Biernat, Marcio Hedil, My Duyen Tran, Marianna Hallwass, Mark Zwart, Murat Yılmaztekin, Paulus den Hollander, Stineke van Houte, Xushi Xu, Tevhide Kızıldeniz and many more. I am grateful for everything I have learned from my mentors, colleagues and students. A special thanks goes to Dowty Movita, for her kind friendship and being first mentor of my daughter Tuana. She is still singing song that you teach. I am very pleased to know you as both colleague and a friend.

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I would like to acknowledge to Turkish Cultural Organization (İstanbul) for their support on the thesis book cover design and the source materials that is kindly supplied. The cover was inspired by Turkish fine art "marbling".

The take home messages are!!! I will never forget the time I have spent in Netherland and I hope it is not only the tulip that connects us, but also a long-lasting friendship☺.

List of publications

Arvind Kumar, **İkbal Agah İnce**, Ahmet Katı, Ranadhir Chakraborty and Suparna Bhowal. *Brevibacterium siligurie* sp. nov., a facultatively oligotrophic strain MB18T, isolated from River Mahananda, India. 2011. **International Journal of Systematic and Evolutionary Microbiology**, submitted.

Henry M. Kariithi, **İkbal Agah İnce**, Sjef Boeren, Adly M. M. Abd-Alla, Andrew G. Parker, Serap Aksoy, Just M. Vlak and Monique M. van Oers. 2011. The salivary secretome of the tsetse fly *Glossina pallidipes* (Diptera: Glossinidae) infected by salivary gland hypertrophy virus. **PloS Neglected Tropical Diseases**, 5: e1371. doi:10.1371/journal.pntd.0001371

Fang Xu, **İkbal Agah İnce**, Sjef Boeren, Monique van Oers and Just M. Vlak. 2011. Protein composition of the occlusion derived virus of *Chrysodeixis chalcites* nucleopolyhedrovirus. **Virus Research** 158: 1-7; doi: 10.1016/j.virusres.2011.02.014

Henry M. Kariithi, **İkbal Agah İnce**, Sjef A. Boeren, Jacques J.M. Vervoort, Max Bergoin, Monique M. van Oers, Addly M.M. Abd-Alla and Just M. Vlak. 2010. Proteomic analysis of *Glossina pallidipes* Salivary Gland Hypertrophy Virus virions for immune intervention in tsetse fly colonies. **Journal of General Virology** 91: 3065–3074; doi:10.1099/vir.0.023671-0

İkbal Agah İnce, Sjef A. Boeren, Monique M. van Oers, Jacques J.M. Vervoort and Just M. Vlak. 2010. Proteomic analysis of *Chilo iridescent virus* particles. **Virology** 405: 253-258; doi:10.1016/j.virol.2010.05.038

Hatice Katı, **İkbal Agah İnce**, İsmail Demir and Zihni Demirbağ. 2010. A novel bacterium, *Brevibacterium pityocampa* sp. nov., isolated from *Thaumetopoea pityocampa* Den. and Schiff., (Lep., Thaumetopoeidae. **International Journal of Systematic and Evolutionary Microbiology** 60: 312-316.

Remziye Nalçacıoğlu, **İkbal Agah İnce** and Zihni Demirbağ. 2009. The Biology of *Chilo iridescent virus*, **Virologica Sinica** 24: 285-294.

Hatice Katı, **İkbal Agah İnce**, Kazım Sezen, Şerife İşci and Zihni Demirbag. 2009. Characterization of two *Bacillus thuringiensis* subsp. morrisoni strains isolated from *Thaumetopoea pityocampa* Den. and Schiff., (Lep., Thaumetopoeidae). **Biocontrol Science & Technology** 19: 475-484.

İsmail Demir, Nurten Gürel, Remziye Nalçacıoğlu, **İkbal Agah İnce** and Zihni Demirbağ. 2009. Productive replication of *Malacosoma neustria* nucleopolyhedrovirus (ManeNPV) in Md203 cell line. **Turkish Journal of Biology** 33: 239-248.

İkbal Agah İnce, Hatice Katı, Hüseyin Yılmaz, İsmail Demir and Zihni Demirbağ. 2008. Isolation and identification of entomopathogenic bacteria from *Thaumetopoea pityocampa* Den. and Schiff., (Lep., Thaumetopoeidae) and determination of their biocontrol potential. **World Journal of Microbiology and Biotechnology** 24: 3005–3015.

Agata K. Jakubowska, **İkbal Agah İnce**, Salvador Herrero, Just M. Vlak and Monique M. van Oers. 2009. *Spodoptera exigua* nucleopolyhedrovirus is not infectious for *Agrotis segetum* larvae *per os*, but only after intrahemocoelic injection. **IOBC/WPRS Bulletin** 45: 99-102.

İkbal Agah İnce, Zafer Eren and İrem Gürkanlı. 2009. Effect of melatonin on the antioxidant enzyme status in the liver of Swiss albino laboratory mice in single circadian rhythm. **Journal of Applied Biological Sciences** 3: 1-6.

İkbal Agah İnce, Marcel Westenberg, Just M. Vlak and Zihni Demirbağ, Remziye Nalçacıoğlu and Monique M. van Oers. 2008. Open reading frame 193R of *Chilo iridescent virus* encodes a functional inhibitor of apoptosis (IAP). **Virology** 376: 124-131**.**

Beran Firidin, Oğuzhan Yanar, Mahmut Bilgener, Nurver Altun and **İkbal Agah İnce**. 2008. The effect of nutritional quality of some plants leaf on the feeding and development of *Hyphantria cunea* (Drury). **International Journal of Natural and Engineering Science** 2: 61-68.

Zihni Demirbağ, **İkbal Agah İnce**, Yeşim Aktürk, Just M. Vlak, Monique M. van Oers and Remziye Nalçacıoğlu. 2007. Potential promoter regions and transcriptional analysis of immediate early, delayed early and late genes of *Chilo iridescent virus* (CIV). **Genes & Genetic Systems** 82: 500.

Remziye Nalçacıoğlu, **İkbal Agah İnce**, Just M. Vlak, Zihni Demirbağ and Monique M. van Oers. 2007. The *Chilo iridescent virus* DNA polymerase promoter contains an essential AAAAT motif. **Journal of General Virology** 88: 2488-2494.

İkbal Agah İnce, İsmail Demir, Zihni Demirbağ and Remziye Nalçacıoğlu. 2007. A cytoplasmic polyhedrosis virus isolated from the pine processionary caterpillar, *Thaumetopoea pityocampa*, **Journal of Microbiology and Biotechnology** 17: 632-637.

Curriculum vitae

İkbal Agah İnce was born on the 7th of November 1978 in İstanbul in Turkey. He is a (molecular) virologist, biochemist and insect pathologist. He completed İstanbul Atatürk High School in 1995 and continued his education at Ondokuz Mayıs University, Samsun in Turkey, where he completed his Bachelor of Science, focusing on Biology and Science Education. He completed his Master of Science in Biochemistry at the Institute of Natural and Applied Science in 2003. During that period he has also served as research officer. After finishing his MSc studies, he moved to Karadeniz Technical University, Trabzon, Turkey as post graduate scientist for 3 years. In 2007 he received a PhD degree in Microbiology at the Institute of Natural and Applied Science of Karadeniz Technical University focusing on the functional roles of viral anti-apoptotic proteins. During these studies he visited the Laboratory of Virology in Wageningen two times. In 2007, he continued his studies commuting between Wageningen, Giresun and İstanbul and was supported by Tübitak, Wageningen University and Yedipete University on the functional genomics and proteomics of the invertebrate iridovirus, *Chilo iridescent virus* (CIV), the model for the Genus *Iridovirus*.

His scientific interest covers, among others, the functional genomics and proteomics of large double-stranded DNA viruses of insects and the biocontrol potential of insect RNA viruses. He is especially interested in the complexity of viral-host interactions and the dynamics of virus infections by tracking both viral RNA and proteins, and host responses applying systems biology approaches. His future ambition is to work at the interface of virology and biochemistry to provide the scientific basis for the development of novel biological insect control strategies and designing of bioprocesses of products of Pharmaceutical interest.

İkbal Agah İnce is married to Aslı Hasanoğlu and has one daughter İpek Tuana. They are currently living in İstanbul.

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 EC (=22 weeks of activities)

Review of literature (6 EC)

- Iridovirus genomics and proteomics

Writing of project proposal (4.5 EC)

- The role of apoptosis in cell to cell spread of *Chilo iridescent virus* infection

Post-graduate courses (5.2 EC)

Advanced course on proteomics (2011); Graduated School VLAG, Wageningen

- Radiation expert level 5B: Radiation Hygiene "Safe handling of radioactive materials and sources" (2011); Plant Research International and VH Larenstein, University of Applied Sciences, WUR.
- Course in Virology; Molecular Medicine Postgraduate School, Erasmus MC, Rotterdam (2008)
- Molecular phylogenies: Reconstruction & interpretation (2007); The Graduate School Experimental Plant Science, Wageningen.

Invited review of (unpublished) journal (4 EC)

- International Journal of Systematic and Evolutionary Microbiology: *Brevibacterium daeguensis* sp. nov. isolated from 4-chlorophenol enrichment culture (2011)
- Apoptosis: Involvement of the mitogen-activated protein kinase pathway in soft-shelled turtle iridovirus-induced apoptosis (2010)
- International Journal of Systematic and Evolutionary Microbiology: *Brevibacterium aquaticum* sp. nov., a novel actinobacterium isolated from well water (2009)
- International Journal of Virology: Genome sequencing, comparison and phylogenetic analysis of Citrus yellow mosaic virus isolates originating from different Citrus species in India (2009)

Deficiency, refresh, brush-up courses (12 EC)

- Cutting edge ecology (2009)
- Physical biochemistry (2007)
- Gene transfer and expression (2007)
- Advanced insect virology (2007)

Competence strengthening / skills courses (4.1 EC)

- Career assessment; Graduate Schools of Wageningen University (2011)
- Project and time management; Graduate Schools Wageningen University (2011)
- Interdisciplinary research: Crucial knowledge and skills, Wageningen School of Social Sciences (2010)
- Interpersonal communication for PhD student; Graduate Schools of Wageningen University (2010)
- Competence assessment; Graduate Schools of Wageningen University (2009)
- Scientific publishing; Graduate Schools of Wageningen University (2008)

PE&RC Annual meetings, seminars and the PE&RC weekend (5.1 EC)

- NVBMB Spring symposium: *In singulo* biochemistry: biology one molecule at a time (2011)
- Dutch annual virology symposium (2008, 2009 and 2011)
- Graduate School Experimental Plant Sciences (EPS) Career Day (2010)
- PE&RC Day : Selling science - Why and how scientists sell science (2010)
- PE&RC Day : Accelerate scientific progress - Expect the unexpected (2008)
- PE&RC Weekend (2008)
- PE&RC Day: Is our civilization able to stand the test of time? (2007)
- NVBMB Spring symposium: Mechanisms in cell specification and the pattern formation (2007)
- WSO/OWI Education day: Motivated student & committed teachers (2007)
- WIAS Seminar: Long term response to selection and QTL mapping (2007)
- NVBMB Fall symposium: Expression & maintenance of the genome; EMC, Rotterdam (2006)
- M.S.V. Alchimica symposium: Bionanotechnology (2006)
- WIAS Seminar: Invertebrate immune response in viral infections (2006)

Discussion groups / local seminars / other scientific meetings (17.8 EC)

- Experimental Evolution Discussion Group (EEDG) (2007-2011)
- Ecogenomics Discussion Group (EDG) (2008-2011)
- Insect-Plant Interaction (IPI) (2010-2011)
- Seminars in Virology Laboratory (2006-2011)

International symposia, workshops and conferences (21 EC)

- 3rd International Entomopathogens and Microbial Control Symposium, Istanbul, Turkey (2011)
- International Congress on Invertebrate Pathology and Microbial Control & 44th Annual Meeting of the Society for the Invertebrate Pathology; Halifax, Nova Scotia, Canada (2011)
- 43th Annual Meeting of the Society for the Invertebrate Pathology, 10th International Colloquium on Invertebrate Pathology and Microbial Control and The Final Meeting of COST862: Bacterial Toxins for Insect Control; Trabzon, Turkey (2010)
- 2nd Symposium on Entomopathogens & Microbial Control; Mugla. Turkey (2009)
- 42nd Annual Meeting of the Society for Invertebrate Pathology; Park City, Utah, USA (2009)
- 12th European Meeting of the IOBC/WPRS Working Group. "Insect Pathogens and Insect Parasitic Nematodes", "Subgroup Slugs and Snails" and Cost Action 862 "Bacterial Toxins for Insect Control", Pamplona, Spain (2009)
- XIV14th International Congress of Virology; Istanbul, Turkey (2008)
- 41st Annual Meeting of the Society for Invertebrate Pathology and 9th International Conference on *Bacillus thuringiensis*; University of Warwick, Coventry, United Kingdom (2008)
- Gene Expression and Genome Evolution; Okayama, Japan (2007)
- 40th Annual Meeting of the Society for Invertebrate Pathology and the 1st International Forum on Entomopathogenic Nematodes and Symbiotic Bacteria; Quebec City, Canada (2007)
- 1st International Forum on Entomopathogenic and Microbial Control; Trabzon, Turkey (2007)
- 9th International Colloquium in Invertebrate Pathology and Microbial Control, and 39th Annual Meeting of the Society of Invertebrate Pathology; Wuhan, P.R. China (2006)

Lecturing / supervision of practical's / tutorials (3.3 EC)

- Advanced course on proteomics (2011)
- Practical course on applied microbiology (2007)

Supervision of three MSc students (9 EC)

- Dengue virus inhibition of apoptosis: Identification of Dengue virus-encoded inhibitors of programmed cell death in insect cells
- Proteomic analysis of *Glossina pallidipes* salivary gland hypertrophy virus
- Development of a tetravalent Dengue virus vaccine

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