NEW INSIGHT INTO THE INTERPLAY OF

BACULOVIRUS AND SUBNUCLEAR STRUCTURES

IN HEPG2 CELLS

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Master's Thesis
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Molecular Biology
October 2007

PREFACE

This Master's thesis was carried out at the division of Molecular Biology of the Department of Biological and Environmental Science, at the University of Jyväskylä during years 2005 - 2006. First, I wish to thank Maija Vihinen-Ranta for providing me the opportunity to join the group and work in this fascinating research project. The greatest acknowledgements belong to my supervisor Johanna Laakkonen for the best advice and endless support throughout the laboratory work and the long writing process. Johanna always understood my moments of high and low, kept pushing me forward with her positive spirit and most importantly: never stopped encouraging me. Additionally, I want to express my gratitude to Irene Helkala, who gave professional assistance with the laboratory work countless times. Further important experimental and intellectual support was provided by Eila Korhonen, Arja Mansikkaviita, Juulia Jylhävä, Einari Niskanen, Teemu Ihalainen and Paavo Niutanen.

Yet more invaluable contribution during this process has come from my family, especially my mother and farther, who continuously have encouraged me with this project and supported my studies in all possible ways - thank you! Finally, a big hug to my sisters and dear friends for listening to my lab reporting and enthusiastic explanations about cell structure, viruses and gene therapy over the past years.

Jyväskylä, October 2007

Maija Häkkinen

Abstract:

Gene therapy is a fast growing research field offering more advanced and exquisite solutions contributing traditional medicine by not only alleviating the symptoms of disorders but also permanently removing their causes via gene transfer. Baculoviruses are of great interest as gene therapy vectors for they are infective only to arthropods, mainly insects, but can effectively transduce various human cells. Nevertheless, more research is needed to explore the nuclear entry process, biodistribution and properties of baculoviruses in human cells to assure the virus safe for transgene delivery. The aim of this study was to establish the active intake of *Autographa californica* multiple nucleopolyhedrovirus *(Ac*MNPV), the most studied member of *Baculoviridae* family, into the nucleus of human hepatoma cells (HepG2), to observe the possible transcription of viral genes *ie-1* and *ie-2* in human cells, in addition to exposing the subnuclear localisation of baculovirus with respect to several prominent transcription sites. Confocal microscopy studies showed baculovirus capsid accumulation into the nucleus at 4 - 24 h post transduction (p.t). The RT-PCR analysis of transduced HepG2 cells showed transcription of both immediate early viral genes *ie-1* and *ie-2* at 6 - 48 h p.t. When exploring possible transcription sites in the nucleus, baculovirus was found to be associated very close to promyelocytic leukaemia nuclear bodies at 8 - 24 h p.t. No colocalization was found between the virus capsid and nuclear speckles. Qualitative confocal microscopy experiments with H2B-EYFP histone expression plasmid and cell-permeable DNA probe DRAQ5™ revealed a change in host cell chromatin structure at 24 - 48 h p.t. Thus, this study provides more insight into baculovirus interactions with HepG2 cells: the nuclear entry, accumulation, localization and virus-induced changes in the host nuclear morphology and transcription of viral genes. Finally, these results clearly indicate that further modification of the vector is needed before more clinical trials.

Key words: gene therapy, baculovirus, HepG2 cells, nuclear speckles, promyelocytic leukaemia nuclear bodies, chromatin, *ie-1*, *ie-2*

Tiivistelmä:

Geeniterapia on nopeasti kasvava tutkimusala, minkä edistykselliset menetelmät tarjoavat perinteiselle lääketieteelle aivan uusia ulottuvuuksia. Geenisiirron avulla pystytään tulevaisuudessa paitsi hoitamaan sairauksia, myös ehkäisemään niiden puhkeaminen tai pysyvästi poistamaan niiden aiheuttajat. Bakulovirus on suosittu tutkimuskohde geeniterapian alalla, sillä poiketen muista nykyisistä humaanivirusvektoreista, bakulovirus on infektiivinen vain niveljalkaisissa, lähinnä hyönteisissä, mutta sen avulla pystytään transdusoimaan monia nisäkässoluja. Bakuloviruksen käyttäytymistä nisäkässoluissa ei kuitenkaan tunneta vielä riittävän hyvin. Tässä tutkimuksessa tarkastelimme *Baculoviridae*-perheen eniten tutkitun jäsenen *Autographa californica* multiple nucleopolyhedroviruksen (*Ac*MNPV) sisäänmenoa maksasyöpäsolujen (HepG2) tumaan, varhaisten virusgeenien *ie-1* ja *ie-2* transkriptiota sekä viruksen sijaintia transkription kannalta merkittävien tumarakenteiden suhteen. Konfokaalimikroskooppisista tutkimustuloksista voi havaita bakuloviruksen kasaantumisen tumaan 4 – 24 tuntia transduktion jälkeen. Transkriptiotutkimuksissa RT-PCR kokeet paljastivat virusgeenien *ie-1* ja *ie-2* lähetti-RNA:n tuoton 6 – 48 tuntia transduktiosta. Tumassa virus havaittiin lähellä transkriptiossa toimivia tumarakenteita 8 – 24 tuntia transduktiosta, mutta vastaavaa yhteyttä viruksen sijainnissa lähetti-RNA:n pilkkomiseen osallistuvien tumarakenteiden suhteen ei voitu osoittaa. Lisäksi havaitsimme muutoksia isäntäsolujen kromatiinirakenteessa 24 – 48 tunnin transduktion jälkeen H2B-EYFP histoniplasmidin sekä DNA:ta värjäävän DRAQ5™:n avulla. Tämä tutkimus tarjoaa uutta tietoa bakuloviruksen ja sen transdusoiman isäntäsolun välisistä vuorovaikutuksista ja perustelee lisätutkimuksen tärkeyttä turvallisemman ja tehokkaamman geeniterapiavektorin kehittämiseksi.

Avainsanat: geeniterapia, bakulovirus, HepG2 solut, tumarakenteet, kromatiini, *ie-1*, *ie-2*

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ABBREVIATIONS

*Ac*MNPV	*Autographa californica* multiple nucleopolyhedrovirus
BSA	bovine serum albumin
BV	budded virus
cDNA	complementary DNA
DIC	Differential Interference Contrast
DAPI	4'-6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
dsDNA	double-stranded DNA
EGFP	enhanced green fluorescent protein
EYFP	enhanced yellow fluorescent protein
FBS	fetal bovine serum
g	relative centrifucation force
GFP	green fluorescent protein
gp	glycoprotein
GV	granulovirus
HepG2	human hepatoma cell line
IE	immediate early
ie-1	*immediate early gene 1*
ie-2	*immediate early gene 2*
kb	kilobase
kDa	kilodalton
MEM	Minimum Essential Medium
MOI	multiplicity of infection
MW	molecular weight
NPV	nucleopolyhedrovirus
ODV	occlusion derived virus
PBS	Phosphate Buffered Saline
PFA	paraformaldehyde
pfu	plaque forming units
p.i.	post infection
p.t.	post transduction
PLP	periodate-lysine-paraformaldehyde
rpm	rounds per minute
RT	room temperature
*Sf*9	*Spodoptera frugiperda* 9
TRITC-De	Tetramethylrhodamine isothiocyanate Dextran
wt	wild type

1. INTRODUCTION

Watson and Crick discovered and published their revolutionary model of the DNA structure in 1954. However, a term *genetic engineering* had already been introduced almost twenty years before that at the Sixth International Conference of Genetics held in Ithaca, New York, in 1932. The term was used to refer to an application of genetic principles to animal and plan breeding, as the contemporary scientist had no idea of gene structure and designed engineering of gene composition was veritably impossible. Thus, the foundation for gene therapy was first laid by solving the structure of DNA and thereafter, working to escalate knowledge of the genetic bases of human disease. During the past decade, gene therapy research has emerged incredibly fast and reached a worldwide appreciated stand in the world of science.

Simply defined, gene therapy is transporting corrective genetic material into sick cells. To perform such sophisticated treatment, both the target structure which is the cell nucleus and the transport vehicle either synthetic or viral, need to be precisely pronounced. Eukaryotic nucleus is structurally composed of three main parts: nuclear membrane, nucleolus and chromatin. Nucleus holds various biologically active, dynamic domains which move within the intranuclear space, interchange proteins and become modified according to versatile cellular stress such as viral infection. Thus, the gene delivery vector needs to overcome several physical barriers to first enter the nucleus and then circumvent triggering immunological defence against foreign objects.

As a result of evolutionary host-virus competition, viruses posses very powerful machinery for DNA delivery into cells. Baculovirus has many advantageous features required for human gene delivery vector. This insect virus is able to efficiently transduce various mammalian cells but not replicate in them. However, baculovirus abilities to induce changes in human host cell have not been precisely defined yet, therefore, it is important to study to which degree baculovirus can modify its target cell. Also, studying the interplay of viral and cellular processes reveals us important information about not only the viral transduction, but also fundamental subnuclear structures and the whole cellular biology.

2. REVIEW OF THE LITERATURE

2.1 Gene Therapy – from History to Today

Gene therapy is a novel medicinal approach offering refined solutions for treating human pathological conditions in the future. The ultimate goal of gene therapy is not only to alleviate the symptoms of disorders but also permanently remove their causes, which can be achieved by conducting corrective genetic material into cells. The gene delivery vehicles, vectors, are classified into two categories according to their origin: non-viral and viral. The first category comprises all non-viral methods for gene delivery into cells, that is, a variety of synthetic approaches ranging from direct injection of DNA with a gene gun to liposome-DNA or polymer-DNA complex-based vectors, which facilitate genes to cross the cell membrane (Verma & Somia 1997). The second category includes modified viruses which have been completely or partially precluded of their pathogenic abilities. However, these viruses still poses the powerful machinery for DNA delivery into cells, the delicate product of continuous evolutionary host-virus competition. The most studied viral vectors so far have been retroviruses, lentiviruses, adenoviruses and adeno associated viruses, nevertheless, in the late 1990s, more potential vectors such as herpesvirus and baculovirus were discovered. As an insect virus, baculovirus was believed to enter only anthropods until in 1995, however, it was first discovered to efficiently express proteins in human hepatocytes (Hofmann *et al*. 1995). Today, baculovirus vectors can be used for constitutive gene expression in several mammalian cell lines (see 6.1 Study of Baculoviruses for Gene Therapy). Additionally, they are employed in a broad range of applications for example in insulin signaling studies (Andersson *et al.* 2007), transducing neural cells (Sarkis *et al.* 2000), tumor targeting in treating lung cancer (Kircheis *et al.* 2001), observing transgene expression in complement inhibition rescued mice (Hoare *et al.* 2005), producing recombinant adeno-associated viruses (Sollerbrant *et al.* 2001), regulating transduction efficiency by pegylating the virions (Kim *et al.* 2006) and many others (Abdelhamed *et al.* 1999; Delaney *et al.* 2000; Lopez *et al.* 2002; McCormic *et al.* 2002; Wang *et al.* 2004). Despite, more research is needed to learn still more about the interactions between these

insect viruses and mammalian cells to develop more delicate and safe baculoviral gene-transfer systems.

The foundation for gene therapy was first laid by solving the structure of DNA and thereafter, working to escalate knowledge of the genetic bases of human disease. In 1956, Lederberg was first to achieve permanent incorporation of viral genomes into bacterial host cell genomes, and his work was followed by similar successful approaches of stable integration of foreign DNA into mammalian cells (Bradley 1962; Kay 1961). Human genetic engineering and gene therapy were first discussed at the beginning of 1970s (Davis 1970; Friedmann & Roblin 1972) and in the following decade, foreign genes could be transferred into mammalian cells using retroviral vectors (Shimotohno & Temin 1981; Tabin *et al.* 1982; Wei *et al.* 1981). The first clinical trials to fight disease with gene therapy were carried out in 1990 by Rosenberg and colleagues. Several new gene delivery systems were developed in the late 1990s, including adenovirus, herpesvirus, adeno-associated virus and synthetic vectors (Brenner 1995; Felgner 1997; Friedmann 1997; Gao & Huang 1995; Tseng & Huang 1998). During the past 10 years, however, gene therapy has emerged rapidly and become worldwide extensively studied field of science aiming to treat various genetic and acquired diseases.

In order to understand the function of genes and their products, scientists have developed different models mimicking living organisms. The genes of interest have been introduced into targeted mammalian cells or tissues by transfection, direct injection, electroporation or viral vectors (Shoji *et al.* 1997). The further need for more efficient vectors for gene delivery is still emphasized in current gene therapical approaches. Thus, safe and efficient transfer of functional genetic material into target cells *in vitro* and *in vivo* is the critical stronghold in the success of gene therapy. Today, the vector is selected depending on the type of therapy required and the duration of gene expression needed. Verma and Somia (1997) have listed the properties of an ideal vector as follows: high concentration, convenience and reproducibility of production, site-specific integration ability or episome stability, editable regulatory elements of transcriptional unit, cell type specifity and immuneresponse free composition. In consequence, still more research is needed before these approaches can be used as routine treatment for ill patients.

2.2 Baculovirus in Gene Therapy

2.2.1 Structure and Characteristics of Baculovirus

Autographa californica multiple nucleopolyhedrovirus *(Ac*MNPV) is a large enveloped DNA virus belonging to *Baculoviridae* family (Blissard 1996). This group of anthropod pathogens is characterized by their cigar-shaped nucleocapsid 200 - 400 nm long and 40 - 50 nm in diameter (Pieroni & La Monica 2001) containing a large covalently closed circular double stranded DNA molecule (Okano *et al.* 2006). Based on the knowledge obtained from sequencing over 20 unique baculovirus genomes, baculoviruses are suggested to encode 90 - 180 genes of their total genome size raging from 82 kb to almost 180 kb (Okano *et al.* 2006). The large alteration in size is due to variation in gene content, numbers of repeated genes and the extent of non-coding sequences (Hayakawa *et al.* 2000), which well reflects the diversity of the family.

Baculovirus family comprises two *Genera* based on occlusion body morphology: the Nucleopolyhedroviruses (NPVs) and Granuloviruses (GVs) (Volkman *et al.* 1995). NPVs produce large rod-shaped occlusion bodies containing several virions produced in the nuclei of infected cells (Rohrmann, 1999), in comparison to GVs, which produce small granular occlusion body containing a single virion and the occlusion bodies are located throughout the cell (Winstanley & O'Reilly, 1999). Most research has focused on NPVs due to difficulties in developing functional cell culture systems for GV propagation (Winstanley & Crook 1993).

Both the *Genera* of baculoviruses can produce two virion genotypes which serve different functional roles in the infection cycle (Blissard 1996). Budded virions (BV) are produced in the late phase of infection cycle when nucleocapsids bud from cell surface and subsequently use components of host cell plasma membrane in their coating process. The occlusion derived virions (ODV), in contrast, are produced very late in infection when nucleocapsids become enveloped within an occlusion matrix protein in the host cell nucleus (with polyhedrin in NPVs and granulin in GVs). Subsequently, the function of

ODV is to transmit infection from insect to insect while BV passes from cell to cell within an infected insect (Lanier & Volkman. 1998). Although the two virion genotypes are structurally and functionally distinct, they are produced at different times and at different locations in the infected cells, the nucleocapsids of both of the virions are produced in the nucleus. Consequently, BV and ODV share identical DNA (Blissard, 1996).

In nature, insect larvae ingest baculoviruses with their food and the viruses are released back into the environment after the cell lysis of infected insect host (Blissard 1996). The primary infection takes place in the midgut of the host: the alkaline conditions cause solubilization of the occlusion bodies and subsequent releases of the ODVs (O'Reilly *et al.* 1994). Infection results in the production of BV, which are released and transported into other tissues via hemolymph causing the secondary infection (Volkman 1997). Before budding of BV or ODV, however, the host plasma membrane has to be modified by virally encoded envelope fusion proteins: the major 64-kDa glycoprotein gp64 or the proteins of LD130 family named `F` or Fusion proteins (Okano *et al.* 2006). Thus, due to different origin, hence a different composition of the envelope and associated structures of BV and ODV, they move through cell membranes by different mechanisms (Blissard 1996).

Baculoviruses are present in remarkable numbers in food and in the air, despite, they have never found to cause any disease in any organism outside phylum *Autographa* (Gröner 1986; Tjia *et al.* 1983; Volkman & Goldsmith 1983). Baculoviruses are used as biopesticides (Cory & Bishop 1997), and more commonly, the extensively developed insect cell-based baculovirus expression system is utilized for high-level expression of recombinant proteins (Friesen 1996; Kost & Condreay 2002). The most commonly used insect host cell lines are originally from *Spodoptera frugiperda* pupal ovarian tissue are *Sf*21 and *Sf*9, the latter being a substrain of *Sf*21 (Vaughn *et al.* 1977), and BTI-Tn-5B1-4 ("High 5 cells") derived from *Trichoplusia ni* egg cell homogenates (Granados *et al.* 1994).

2.2.2 Properties of Baculovirus Vector in Gene Delivery

Although productive infection of wild type *Ac*MNPV is limited to anthropods, however, genetically engineered baculovirus vectors changed the situation in the mid 1990s, when Hoffman and colleagues (1995) followed by Boyce & Bucher (1996) reported the first transgene expression in mammalian cells. They discovered that if the gene of interest is governed by mammalian promoter, the recombinant baculoviruses gain capacity to efficiently transfer and express genes in mammalian hepatic cells. More recently, recombinant baculoviruses have not only been successfully shown to express heterologous genes but also transduce various non-hepatic mammalian cell types, both *in vitro* and *in vivo* (Airenne *et al.* 2000; Condreau *et al.* 1999; Ma *et al.* 2000; Sarkis *et al.* 2000; Shoji *et al.* 1997; Song & Boyce 2001; Song *et al.* 2003; Wang *et al.* 2004).

Today, baculovirus is routinely used to express heterogenous proteins in insect cells, nevertheless, this virus can also efficiently enter numerous vertebrate cells *in vitro* and *in vivo* with no visible cytopathic effect (Condreay *et al* 1999; Hofmann *et al* 1995; Sarkis *et al* 2000; Shoji *et al* 1997). Genetically engineered *Ac*MNPV has shown powerful transgene delivery efficiency into several cultured mammalian cells *in vitro* including rabbit, mouse and numerous human hepatocytes such as Huh7 and HepG2 (Hofmann *et al.* 1995), chinese hamster ovary cells (CHO), african green monkey kidney fibroblasts (CV-1), transformed african green monkey kidney fibroblasts (COS-7), human epithelial carcinoma cells (HeLa), human embryonic kidney cells (HEK-293), baby hamster kidney cells (BHK) (Boyce & Bucher 1996), pancreatic islet cells (Ma *et al.* 2000), primary human fibroblasts (FFs) (Dwarakanath *et al.* 2001), human osteosarcomas (MG63), normal human osteoblasts (NHO) (Kukkonen *et al.* 2003), keratinocytes, bone marrow fibroblasts (Condreay *et al.* 1999) and neural cells (Sarkis *et al.* 2000). The minimal cytotoxicity, in addition to, the broad range of susceptible cell lines and the large insertion capacity up to 100 kb (Hartley 2006) engender baculovirus very attractive gene therapy vector for mammalian cells (Sandig *et al.* 1996; Shan *et al.* 2006). Additionally, recombinant baculoviruses are easy to construct and produce to high titres (Hofmann & Strauss 1998; Sollerbrant *et al.* 2001).

Foreign gene expression studies with insect cells show the promoter has an amble impact to the range of insect cells which can efficiently support the production of foreign protein (Morris & Miller 1992). Thus, the insertion of strong a mammalian promoter has been the prerequisite for recombinant baculovirus-mediated expression in mammalian cells respectively (Haeseleer *et al.* 2001). Efficient transduction of neural cells is essential to the development of gene therapy for neurological diseases (Sarkis *et al.* 2000). To deliver genes to central nervous system, Sarkis and colleagues (2000) designed a baculovirus-derived vector functioning under cytomegalovirus (CMV) promoter, Bac-CMV-GFP. In this study, Bac-CMV-GFP was used successfully for transducing three human primary neural cultures with an expression level of transduced cells being over 50%. Even more promising, however, was the transduction of two neuroblastomal cell lines *in vivo* by direct injection into the brain of rodents and the fact, that the vector did not become inactivated by the complement system. Thus, learning more about baculoviruses; designing and testing new baculovirus-derived vectors provides us important information for solving the current difficulties of the vectors and for developing new generation of applications of high efficacy and long-term expression.

A considerable drawback to baculovirus-mediated gene therapy is the significantly reduced gene transfer capacity when the virus is applied directly *in vivo*. This is due to first-line host defense of the complement C system (C) of native human sera designed to eliminate foreign elements (Hofmann & Strauss 1998). Baculoviral *in vivo* gene transfer has been studied in the neural, muscular and hepatic cells of rats and mice (Bilello *et al.* 2001; Pieroni *et al.* 2001). Hofmann and Strauss (1998) present two principles to protect baculovirus vectors from C inactivation: depletion or exclusion of the C system before applying baculovirus into animal models or modification of the existing baculovirus vectors. Their report demonstrates succesfull baculovirus-mediated gene transfer into hepatocytes *in vivo* achieved by simultaneous application of C blocking antibodies. Also, a complement-resistant baculovirus has been introduced (Huser *et al.* 2001) among other strategies contrieved to circumvent triggering the C cascade (Hofmann *et al.* 1999, Ojala *et al.* 2001).

Different applications of baculovirus-mediated gene transfer have greatly expanded during the past ten years. A very recently published methodology article (Andersson *et al.* 2007) introduces baculovirus as a heterologous viral gene delivery vehicle to be used for functional gene expression in cultured cells as a substitute for recombinant adenovirus vectors and lipid transfection agents. These replaced tools are utilized to study the effects of over-expression of proteins on insulin mediated events since the cationic lipid reagents give rice to insulin unresponsiveness in cell cultures and adenovirus treated cells commonly cease to respond to insulin stimulation. However, baculovirus transduced mammalian cells do allow insulin signalling studies, thus, baculovirus vector represents a more functional alternative to be employed in these examinations.

In another kind of application introduced by Sollerbrant and colleagues in 2001, baculovirus was used as a tool to produce recombinant adeno associated virus (rAAV). This baculovirus-based chimeric vector method included co-infecting the producer cells (HEK293) with three viruses: a baculovirus containing the reporter gene flanked by AAV ITRs, a baculovirus expressing the AAV *rep* gene and a helper adenovirus expressing the AAV *cap* gene, which successfully resulted in the production of infectious rAAV particles.

Due to baculovirus inability to replicate in mammalian cells, the viral genome is either lost or degraded soon after infection (Ho *et al.* 2006). This transient nature of transgene expression, which usually lasts for 7 days, has been one of the major limitations of baculoviral transduction vector (Hu *et al.* 2003). Although partial integration of the baculovirus into cellular chromosomes has been reported by Merrihew and colleagues (2001), such intregration was achieved under selective pressure and being random and infrequent, it can compromise the expression of cellular genes. Nevertheless, it is possible to enhance and prolong the expression level by superinfection (Hu *et al.* 2003). Moreover, prolonged vector maintenance up to 60 days and markedly enhanced transgene expression of recombinant baculoviruses have been reported when baculovirus-based vectors are built utilizing elements of other viruses capable of extrachromosomal maintenance, such as the episomal maintenance elements *OriP* and *EBNA-1* from Epstein-Barr virus (EBV) (Shan *et al.* 2006). What is more, without any selective pressure the improved baculovirus vector persisted in many different mammalian cells such as HEK293, monkey african green

kidney cells (Vero), Cos-7 and human epithelial tumor cells (Hone-1). In the recent years, also other approaches of enhanced and prolonged baculovirus-mediated expression systems for mammalian cells have been published including the studies of Hu *et al.* 2003; McCormick *et al.* 2002; Pieroni *et al.* 2001; Song & Boyce 2001; Song *et al.* 2003.

2.3 Baculovirus in Infected Insect Cells and Transduced Mammalian Cells

2.3.1 Entry and Propagation

Virus entry into host cell is an intricate process. After the virus has successfully passed through the cell membrane, it needs to infiltrate through the cytoplasm and pass nuclear membrane to enter the nucleus. For entering the host cell, baculoviruses encode two different envelope fusion proteins: gp64 and F (Okano *et al.* 2006). In short, the viruses are suggested to enter their host insect cells via adsorptive endocytosis (Volkman & Goldsmith 1985) and mammalian cells by macropinocytosis or clathrin-mediated endocytosis (Long *et al.* 2006; Matilainen *et al.* 2005). Once inside the cells of either type, the viral capsids are released from the endosomal vesicles and the cytoplasmic trafficking towards the nucleus is suggested to be actin-dependent (Blissard *et al.* 1992; Charlton & Volkman 1993; Lanier & Volkman. 1998; Salminen *et al.* 2005; van Loo *et al.* 2001).

Salminen and colleagues (2005) have studied the mechanisms and factors affecting the cytoplasmic trafficking of baculoviruses in a mesh of microtubules and actin networks. They suggest that endocytic modifications are not necessary for cytoplasmic trafficking, nor the nuclear import of recombinant baculovirus. However, consistent with the earlier results of Volkman and Zaal (1990), progressive reorganization and depolymerization of microtubules seems to have a major role in nucleus-oriented viral movement, consequently, MT reorganization could provide a simple method to enhance baculovirus-mediated gene delivery in mammalian cells. The intracellular mechanism of nucleus-oriented viral movement has been studied with various other viruses as well (Sodeik 2000; Stidwill & Greber 2000; Whittaker & Helenius 1998), nevertheless, more research is

needed to unveil the exact details of the events before utilization is possible in gene therapical approaches.

The dimensions of the unenveloped baculovirus nucleocapsids are appropriately adapt to allow passage through the nuclear pores without capsid deformation (Salminen *et al.* 2005; van Loo *et al.* 2001; Wilson & Price 1988). Hence, the viral capsids have been shown to localize in the nucleus at 3 - 4 h p.t. in studies carried out in human cells (Kukkonen *et al.* 2003; Matilainen *et al.* 2005; Salminen *et al.* 2005). Also, baculovirus has been shown to transduce both dividing and non-dividing mammalian cells, consequently, the nuclear entry of baculovirus is not associated with the mitotic dissociation of nuclear membrane (Laakkonen *et al.* 2007; van Loo *et al.* 2001).

2.3.2 Gene Transcription

In infected cells, baculovirus gene expression is divided into two phases; an early phase preceding viral DNA replication and a late phase at the beginning or after DNA replication begins (Blissard & Rohrmann 1990). The early phase is further divided into immediate early (IE) and delayed early (DE) stages according to the function of the gene products of each phase. Even transfected insect cells are able to transcribe *IE genes* because they require no viral gene products for their transcription in contrast to the *DE genes*, which require other viral proteins for their production. The genes of late phase are categorized into *Late genes* and *Hyperexpressed late genes*, distinguished by the levels of mRNA of the latter remaining high throughout the infection cycle (Blissard & Rohrmann 1990).

The principal transregulator of baculovirus gene expression is the *immediate early gene 1* (*ie-1*) product IE-1 transcribed from two early promoters (Blissard & Rohrmann 1990) which transactivates various early gene promoters such as *ie-1, 39K, p35, gp64, p143, dnapol, pe-38, lef-1, lef-2, lef-3* (Friesen 1996) . In addition to IE-1, three other early viral gene expression transregulator proteins have been identified: IE-0, IE-2 and PE-38. IE-0 protein is a spliced variant of IE-1 and thus proposed to be required in viral replication as well (Friesen 1996). IE-2 is suggested to stimulate reporter genes linked to the *ie-1*

promoter in addition to reinforcing IE-1-mediated transactivation of the delayed early *39K* gene (Yoo & Guarino 1994) and *pe-38* (Lu & Carstens 1993). PE-38 on its behalf has been shown to mediate transactivation of the *p143* gene promoter (Lu & Carstens 1993).

Baculovirus incompetence to replicate in mammalian cells was long considered a fact as it was proven by numerous researchers such as Volkman and Goldsmith (1983) who gained frankfurt evidence of no replication in 35 different cell lines tested. Quite recently, however, studies with HeLa14 and BHK cells have indicated that *Ac*MNPV is able to transcribe some viral genes such as *ie-0, ie-1, pe-38* and *gp64* (Fujita *et al.* 2006). Additionally, transcripts of several immediate-early viral genes were shown to accumulate in detectable levels in HEK293 and primary cultures of rat Schwann cells transduced with another member of baculovirus family, *Bombyx Mori* (Kenoutis *et al.* 2006). Thus, baculovirus genome seems to be able to act as a template for transcription in mammalian cells through common infection pathway nevertheless, no evidence for the functional expression of viral genes has yet been discovered.

2.4 Subnuclear Organization in Human Cells

The cell nucleus is a highly organized structure which can be divided into three main structures: the nucleolus, heterochromatin and euchromatin (Maul 1998). However, our understanding of the composition of the nucleus has escalated through the study of DNA viruses, which replicate in the nucleus and interact with several specific nuclear structures in nonarbitrary fashion. Thus, analyzing virus interactions with specific nuclear structure inevitably generates novel information of the biological properties of that compartment too (Maul 1998).

2.4.1. PML NBs

Promyelocytic nuclear bodies (PML NBs), also referred to as PODs, ND10 or PML bodies, are separate interchromosomal accumulations of over 60 proteins including PML and

sp100 (Maul *et al.* 2000). PML NBs have been found in most cell types and they seem have a role in almost every cellular function including cell cycle control, growth suppression, interferon-mediated cell defence, apoptosis, gene regulation, transcription, DNA repair and proteolysis (Borden 2002; Everett 2006; Maul *et al.* 2000). The morphology of PML NBs becomes altered in diseases such as hepatocellular carcinoma (Terris *et al.* 1995) or promyelocytic leukemia (Weis *et al.* 1994). The localization and structure of PML NBs have also been studied in human cells infected with different viruses such as adenovirus (Doucas *et al.* 1996; Ishov & Maul 1996), herpesvirus (Everett *et al.* 1998; Maul *et al.* 1996) and papovavirus (Guccione *et al.* 2004). In these cases, the parental genome has been proved to localize in PML NBs and the domain has been the site of early replication. Thus, the distribution and morphology of PML NBs becomes altered during viral infection; different viruses can induce various events from accumulation of PML proteins into larger cytoplasmic bodies to the degradation of the whole domain (Borden *et al.* 1998; Everett *et al.* 2004; Laakkonen *et al.* 2007; Maul *et al.* 1996).

2.4.2. Nuclear Speckles

In different literature, the nuclear speckles are referred to speckles, speckled domains, SC35 domains, splicing factor compartments, nuclear splicing speckles and interchromatin granule clusters (IGCs). The number of these domains in each cell varies from 25 - 30 as they are very dynamic structures composed of various protein- and RNA particles, mainly splicing factors and poly(A) RNA (Calado & Carmo-Fonseca 2000; Handwerger & Gall 2006; Spector 2001). Thus, nuclear speckles are reservoirs of transcription- and pre-mRNA splicing factors, which are recruited from them to the sites of transcription. An additional component of the speckles is poly(A) binding protein 2 (PABP2), which binds to the poly(A) tails of mRNAs controlling their length and extension (Calado & Carmo-Fonseca 2000). In studies of viral interactions with different nuclear domains, the nuclear speckles have been interesting as possible transcription sites of viruses: Fortes and colleagues (1995) have reported that influenza virus can alter the localization of nuclear speckles, and Hivin et al. (2005) suggest that human T-cell leukemia virus accumulates into nuclear specles. Consequently, the components of nuclear speckles are in constant move between

the nuclear speckles, nucleoplasm and the active transcription sites (Lamond & Spector 2003).

2.4.3. Cellular Chromatin

In eukaryotic cells, chromatin serves as the physiological template for gene transcription, DNA replication, and repair. O'Reilly and colleagues (1994) have studied the properties of insect cell chromatin during baculovirus infection. They reported enlargening of the host cell nuclei and dispersion of nucleolus, which they suggested to be caused by baculovirus early proteins stimulating dispersion of the host chromatin. Also an additional study (Volkman & Zaal 1990) suggests that viral interactions with tubulin can induce the host cell chromatin rearrangements.

Recently, chromatin remodeling caused by other DNA viruses has been reported in various human cells as well. Taylor and Knipe (2004) observed changes in host chromatin associated with herpes virus (HSV-1) infection in a variety of human cells. Herrera and Triezenberg (2004) studied chromatin-modifying coactivators in HeLa and Vero cells. They found HSV-1 virion protein VP16, which activates the viral immediate-early (IE) gene transcription, associated with general transcription factors and with chromatin-modifying coactivator proteins of several types. Additionally, HSV-1 viral DNA was found to be associated with histone H3 at early times of infection. Moreover, Monier *et al.* (2000) showed by live cell dynamic measurements a peripheral displacement of host chromosomes that correlated with the expansion of the viral replication compartment and increased the volume of the nucleus. Thus, studying the processes involved in viral replication, especially the use of probes to localize the virus with respect to certain subnuclear proteins or RNA-structures has revealed many functional domains in the nucleus. Consequently, in unison with developing more delicate viral vectors, increases the knowledge of nuclear design.

3. AIM OF THE STUDY

The objectives of the study were:

1. To monitor and establish the timeline of baculovirus accumulation into human hepatoma cell (HepG2) nucleus and to investigate the nuclear entry route of baculovirus.

2. To detect if the viral immediate early genes *ie-1* and *ie-2* are transcribed in HepG2 cells.

3. To study the localization of nuclear speckles and PML NBs, the possible transcription sites of baculovirus, in transduced HepG2 cells.

4. To illustrate HepG2 host chromatin morphology in baculovirus transduction.

4. MATERIALS AND METHODS

4.1 Cells

The experiments were performed in two cell lines: human hepatoma (HepG2) and *Spodoptera frugiperda (Sf*9) insect cells. HepG2 cells were grown in monolayer in Minimum Essential Medium (MEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), antibiotics (10 000 U/ml penicillin and 10 mg/ml streptomycin), L-glutamine and Non-Essential Amino Acids. The cells were maintained at +37ºC, in 5% CO_2 and cultured twice a week. *Sf*9 cells were grown in suspension at +27ºC in serum free HyQ®SFX-Insect medium without antibiotics and cultured twice a week according to standard protocols (Hyclone HyQ SFX-Insect instructions, Phelan 1998).

4.2 Viruses

This study employed two viruses: wild type (wt) *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) and recombinant baculovirus vp39EGFP (Kukkonen *et al*. 2003, kindly provided by Kari Airenne and Seppo Ylä-Herttuala). The recombinant virus displays enhanced green fluorescent protein (EGFP) attached to the C-terminus of the nucleocapsid major protein vp39. The viruses were purified and collected by sucrose gradient centrifucations (25% sucrose gradient: 7200g, 7 h, 4ºC, Sorvall RC5, HS4, continuous sucrose gradient 20 - 50%: 120 000 g , 1 h, 4ºC, LKB Bromma 2331 Ultrospin 70, SRP 28 SA). The virus titer was determined by end-point dilution method (O'Reilly *et al.* 1994), which measures the baculovirus ability to infect *Sf*9 insect cells as plaque forming units per milliliter (pfu/ml).

4.3 Transduction of Mammalian Cells

For the experiments, HepG2 cells were grown for two days on cover slips 80 000 - 120 000 cells/ 3cm^2 plate. Cell densities used varied among the demand for each experiment. Before the transduction, cells were washed with PBS (Phosphate Buffered Saline) and the transduction was performed with an appropriate virus diluted in MEM containing 1% FBS. Cells were incubated at +37ºC, in 5% CO$_2$ for 1h, followed by an addition of 10% MEM. Transfected cells (see 4.4 Electroporation of HepG2 cells) were transduced with wt using 200 pfu/ml. RT-PCR experiments were performed with wt using 1000 pfu/ml and all other experiments with vp39EGFP using 200 pfu/ml. At appropriate time point, the transduction was arrested by fixing the cells. In the fixing procedure, cells were washed once with PBS and then incubated either in 4% paraformaldehyde (PFA) RT for 20 min or in ice-cold methanol for 6 min. Cells were maintained in PBS at +4 ºC until immunofluorescence labelling (see 4.5.1 Immunofluorescence Labeling).

4.4 RT-PCR

Baculovirus transcription was studied in HepG2 cells transduced with wt *Ac*MNPV (1000 pfu/ml) at 6 - 48 h. In order to achieve controls for the transcription of *ie-1* and *ie-2* mRNA, *Sf*9 cells were infected with wt *Ac*MNPV. The *Sf*9 cell stocks contained 2 million cells / ml and the infection was performed using 5 pfu/ml and 10 pfu/ml of wt. After transduction (see 4.3 Transduction of mammalian cells), cells were collected by scrabing, dissolved in Trizol^{TM} and freezed at -70 ºC. The early gene transcription of *Ac*MNPV in transduced cells was analyzed with quantitative TaqMan RT-PCR (warm thanks to M.Sc. Minna Kaikkonen, AI Virtanen Institute, Department of Molecular Medicine, University of Kuopio, Kuopio, Finland). Total RNA was isolated using Trizol^{TM} reagent, treated with the deoxyribonuclease DNaseI and reverse-transcribed. The levels of *ie-1* and *ie-2* mRNA was quantified with SYBR® Green RT-PCR using specific primers:

IE1forw 5'-TTAACGCGTCGTACACCAGCG -3',

IE1rev 5'- TTATAATAACTTAAATAGTCGT TGGG -3',

IE2forw 5'- ATGAGTCGCCAAATCAACGC -3' ,

IE2rev 5'- GGCTTCGGGAGATGTTGTAAAG -3'.

The samples were amplified and performed as duplicates of each reaction containing 10 ng of cDNA, 12.5 µl of PCR master mix and 7.5 pmol of primers. To construct a standard curve, four serial dilutions of cDNA obtained from transduced HepG2 cells at 24 h p.t. were employed. Input amounts of cDNAs were corrected by amplification of the 18S ribosomal RNA as an endogenous control and the ratios of target gene expression and 18S expression (relative gene expression) were calculated from the amounts of cDNA using the standard curves constructed for the analysis.

4.5 Confocal Microscopy Studies

4.5.1 Immunofluorescence Labeling

As previously described (see 4.1 Cells), HepG2 cells were grown subconfluent on cover slips, transduced and fixed before labelling the samples. All of the immunofluorescence labeling procedures followed the same protocol and were performed in RT. For samples fixed with 4% PFA, the pre-washes were carried out in permeabilization buffer (0.1% TritonX/ 1% BSA/ 0.1% NaN_3 in 1xPBS) and PBS á 20 min. For the cells fixed with methanol, 1.5% PBS-BSA washing solution was used instead of permeabilization buffer. The following primary antibody incubation of 1 h was followed by another identical set of 20 min washes. The secondary antibody incubation of 30 min and the following steps were performed in dark. After the final two washes in permeabilization buffer and PBS, the cells were embedded with either Mowiol-DABCO, an anti-photobleaching mounting media (25 mg/ml) or ProLong® Gold antifade reagent with DAPI (4'-6-Diamidino-2-phenylindole). DAPI is a chemical which forms fluorescent complexes with natural double-stranded DNA without changing the ultra structure of cells (Tarnowski *et al.* 1991).

4.5.2 Confocal Microscopy and Imaging

When atoms or molecules absorb light, they become electronically excited for a very short interval, the fluorescence lifetime, after which they return to an unexcited state and emit the captured energy as light. Different atoms or molecules become excited at different wavelengths depending on their properties. In any case, however, the absorption maximum is always slightly lower than the emission maximum. Confocal microscopy enables to view the sample at different plains, which provides an excellent means to locate the labels accurately at particular cell structures. The images can then be overlaid to get an image of a very high resolution presented in xyz planes. Additionally, microscope enables to scan a 3D-like view of cell surface features when the light is transmitted, but no fluorescence channel is open, which is an imaging technique known as DIC (Differential Interference Contrast) (Zeiss Operating Manual, Olympus User's Manual). Background fluorescence can be minimized by choosing the fixatives carefully. Moreover, when using two or more fluorescent dyes in one sample, the dyes should be selected so that they do not have overlapping fluorescence wavelengths.

Imaging of the samples was accomplished with two confocal laser scanning fluorescence microscopes: LSM 510, Carl Zeiss AG, Jena, Germany (488 nm argon, 546 nm and 633 nm helium neon-lasers, 63x Plan-Neofluoar oil immersion objective (NA = 1.25), pixel resolution 100 - 110 nm/pixel, 512x512 pixels/image) and Olympus Fluo-View 1000, Olympus Optical Co., Tokyo, Japan (488 nm argon, 546 nm and 633 nm helium neon-lasers, 60x APO oil immersion objective (NA = 1.35), pixel resolution 100-110 nm/pixel, 512x512 pixels/image).

4.5.3 Electroporation

For electroporation experiments HepG2 cells were grown to semiconfluency: approx. six million cells / 75 cm^2 culture flask (4.1 Cells). In order to collect the cells, they were first trypsinized 3 min at +37ºC, in 5% CO_2, then suspended in 10% MEM, pelleted (150 g , 3 min, Heraeus Instruments Labofuge 400) and finally resuspended in 800 µl of 10% MEM.

Cell suspension was divided into two electroporation cuvettes (Bio-Rad), 400 µl each, containing three million cells. An appropriate concentration of the plasmid (50 - 100 µg/ml) was added into the cuvette and the electroporation was accomplished with Electro Cell Manipulator® 600 (Capacitance & Resistance Mode 500 V; 1700 µF Capacitance, 72 ohm Resistance, 105 V Charging Voltage, 30 msec Pulse Length). The transformed cells were grown on cover slips in 10% MEM for 24 h before transduction with wt 200 pfu/ml (see 4.3 Transduction of Mammalian Cells), immunolabeling (see 4.5.1 Immunofluorescence Labeling) and confocal microscope studies (see 4.5.2 Confocal Microscopy and Imaging). The following plasmids were used: human expression plasmid PABP2-EGFP (kindly provided by Dr. Carmo-Fonseca, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal), human histone plasmid H2B-EYFP (J. Langowski, German Cancer Research Center, Heidelberg, Germany) and human pEGFP-sp100 plasmid (Dr. G. Dellaire, The Hospital for Sick Children, Toronto, Canada).

4.5.4 Microinjection

For microinjections, HepG2 cells were grown for 48 h in densities of 20 000 - 30 000 cells / cover slip. Prior to microinjection, 5ml of 10% MEM was added on the cells to improve the contrast for injection procedure. The injection solution composed of WGA (wheat germ agglutinin) and TRITC-De (Tetramethylrhodamine isothiocyanate Dextran) by 1:4. WGA is a protein (MW 36 000) that binds to N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) residues of glycoproteins and glycolipids. In this study, WGA was used to block the nuclear pores. In order to visualize the nuclear membrane, WGA was combined with TRITC-De, a substance composed of Dextran polysaccharide (MW 10 000) conjugated with fluorescent dye TRITC. Because of the great tendency of WGA to rapidly break down, the cells were first transduced with 200 pfu/ml of vp39EGFP for 2 h (see 4.3 Transduction of Mammalian Cells) followed by an injection of WGA – TRITC-De 1:4 solution into the cytosol. The microinjection was performed by using a semiautomatic system consisting of an inverted microscope joined to a Micromanipulator 5171 and Transjector 5246 (Eppendorf, Hamburg, Germany). The injection duration was

30 min at maximum, after which the cells were incubated further at +37ºC, in 5% CO_2 for 1.5 h, or until the total viral transduction time reached 4 h (see 4.3 Transduction of Mammalian Cells). Finally, the cells were fixed with methanol and immunolabeled (see 4.5.1 Immunofluorescence Labeling).

5. RESULTS

## 5.1 Baculovirus Entry into the Nucleus

The general localization of baculoviruses in HepG2 cells was studied using confocal microscopy at 4 - 24 h p.t. The first viral capsids were detected in the nucleus at 4 h p.t., however, most capsids were still located in the cytosol. Nuclear accumulation was further observed at 6 - 24 h p.t. (Fig. 1). At 24 h p.t. the volume of capsids in the nucleus had increased, nevertheless, at all time points a proportion of the capsids remained in the cytosol. Vp39EGFP was labeled by rabbit-anti-GFP and Alexa-488-conjugated goat-anti-rabbit IgG to enhance the green fluorescence of the EGFP attached to the C-terminus of the nucleocapsid major protein vp39. The samples were embedded using ProLong® Gold antifade reagent with DAPI (4'-6-Diamidino-2-phenylindole), which labels the nucleus blue (see 4.5.1 Immunofluorescence Labeling).
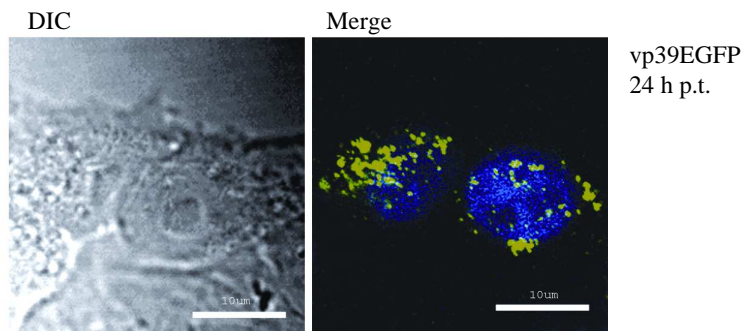
Figure 1. Baculovirus capsids accumulate into the HepG2 nucleus. Images are 3D projections from the middle parts of the cells transduced with vp39EGFP (200 pfu/ml) at 24 h p.t. The green fluorescence of the virus was enhanced with rabbit-anti-GFP and Alexa-488-conjugated goat-anti-rabbit IgG and the nucleus labeled blue using ProLong® Gold antifade reagent with DAPI. Scale bar 10μm.

To examine the previous suggestions of baculovirus entry into the nucleus of mammalian cells through the nuclear pore (VanLoo *et al.* 2001, Salminen *et al.* 2004), WGA (wheat germ agglutinin) microinjection experiment blocking the nuclear pores was performed (see 4.5.4 Microinjection). The results showed vp39EGFP (200 pfu/ml) accumulation near the nuclear membrane outside the nucleus at 4 h p.t. (Fig. 2). For better visualization the green

fluorescence of vp39EGFP is enhanced with rabbit-anti-GFP and Alexa-488-conjugated anti-rabbit IgG. The nucleus is seen red with TRITC-De, a fluorescent compound mixed with WGA in the injection solution.
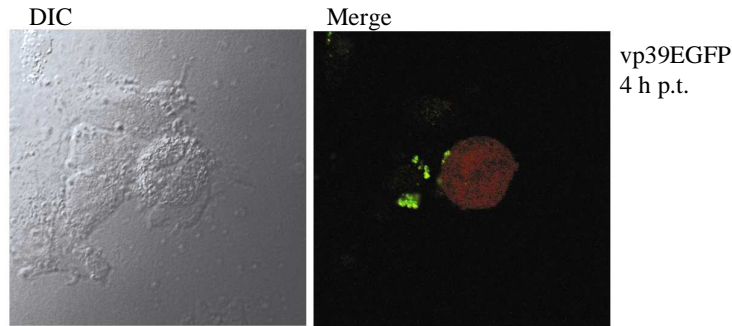
Figure 2. Baculovirus (vp39EGFP) capsids remain outside HepG2 cell nucleus at 4 h p.t. due to blocking the nuclear pores with WGA microinjection. Cell nucleus is seen red due to fluorescent TRITC-De - WGA mix. Vp39EGFP (200 pfu/ml) green fluorescence was enhanced by rabbit-anti-GFP and Alexa-488-conjugated anti-rabbit IgG. Scale bar 10µm.

5.2 Baculovirus Immediate Early Gene Transcription

Another aspect of this study was to investigate whether baculovirus produces transcripts of its immediate early genes in human cells. HepG2 cells were transduced with wt (1000 pfu/ml) at 6 - 48 h and the levels of *ie-1* and *ie-2* mRNA were analyzed with quantitative RT-PCR analysis (kindly performed by M.Sc. Minna Kaikkonen). Transcription of both the early viral genes *ie-1* and *ie-2* was detected in HepG2 cells at 6 - 48 h p.t. (Fig. 3). At 6 h p.t. the immediate early gene transcription was diminutive; no *ie-1* mRNA was detected and the quantity of *ie-2* transcripts was very scarce. However, the relative gene expression level of both mRNA transcripts became prominent at 24 h p.t. and increased further up to 48 h p.t.
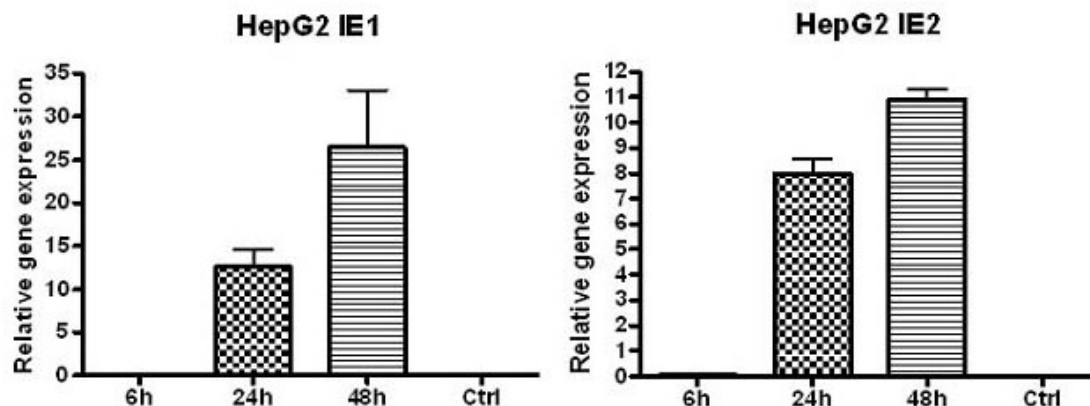
Figure 3. Baculovirus immediate early genes *ie-1* and *ie-2* are transcribed in HepG2 cells at 6 - 48 h p.t.
HepG2 cells were transduced with wt 1000 pfu/ml.

5.3 Localization of Baculovirus and Subnuclear Structures

In order to monitor the localization of baculovirus with certain subnuclear structures, two distinct methods were used: either antibodies designed against a selected protein in the structure of interest, or plasmids coding for fluorescent proteins known to localize in those structures. After introducing the appropriate agent into HepG2 cells, the cells were transduced with either vp39EGFP or wt at selected time points.

5.3.1 PML NBs

Promyelocytic leukemia nuclear bodies (PML NBs) were studied by transfecting HepG2 with pEGFP-sp100 expression plasmid (Muratani *et al.* 2002), which codes for sp100 protein constituent of PML NBs. The transfection was followed by wt transduction (200 pfu/ml). Confocal microscopy studies assigned that wt capsids labeled with mouse anti-vp39 and Alexa-633-conjugated mouse IgG were located with or within the PML NBs already at 8 h p.t. Similar localization pattern was more evident at 24 h p.t. (Fig. 4). Due to very low transfection efficiency of the plasmid no colocalization kvantification was performed.

wt 8 h p.t.   wt 24 h p.t.
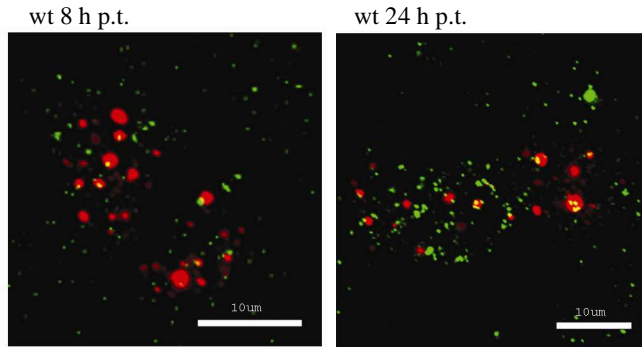
Figure 4. Close association of wt and sp100 protein found in PML NBs is detected at 8 h p.t. and becomes more prominent at 24 h p.t. HepG2 cells were transfected with pEGFP-sp100 expression plasmid, the fluorescent EGFP-tag is shown in red in this image. The wt capsids were labeled with mouse anti-vp39 and Alexa-633-conjugated mouse IgG shown in green. The colors have been modified for better visualization of the results. Scale bar 10µm.

5.3.2 Nuclear Speckles

To study if the virus had any interaction with nuclear speckles involved in pre-mRNA processing (Borden 2002), HepG2 cells were transfected with an expression plasmid PABP2-EGFP (Calapez *et al.* 2006) coding for a poly(A) binding protein 2, which is a component of nuclear speckle structure. The cells were then transduced with wt (200 pfu/ml) for 24 h. The viral capsids were labeled with mouse an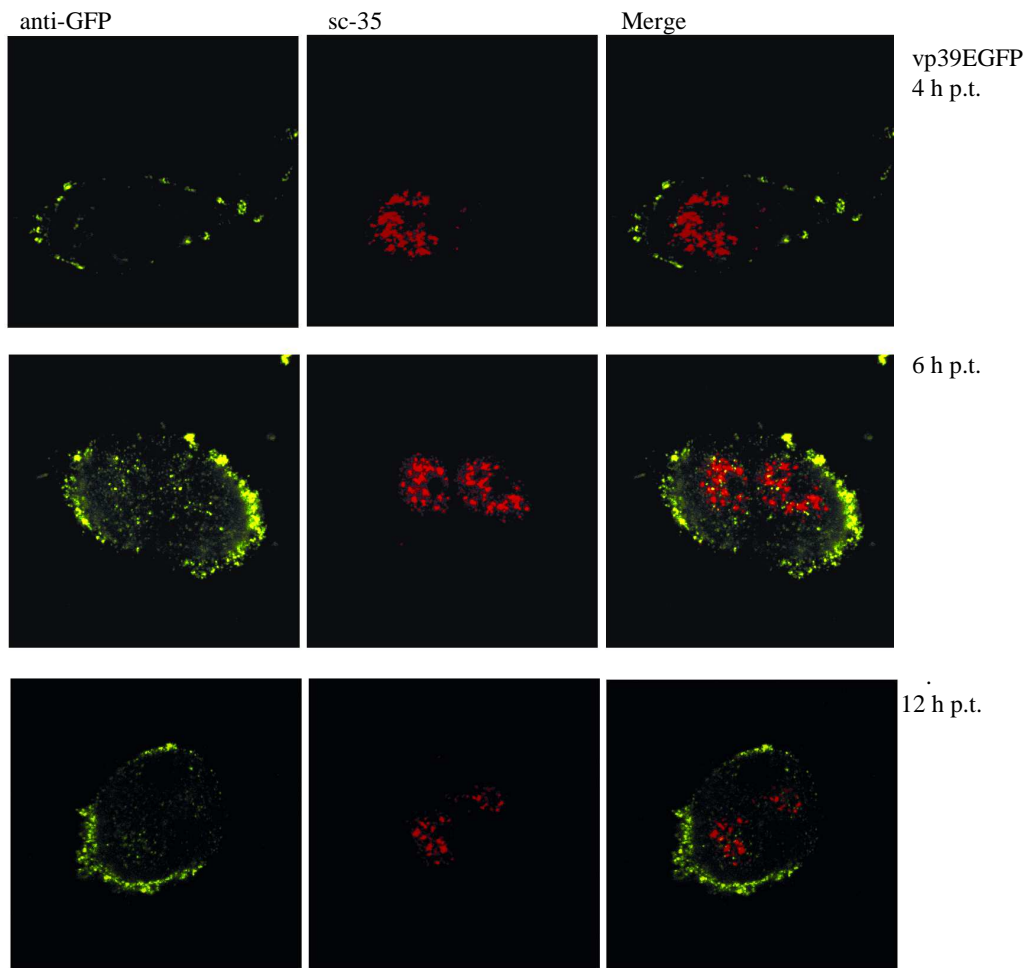ti-vp39 and Alexa-633-conjugated mouse IgG. As the results show, the virus localizes near the nuclear speckles, but no colocalization is observed (Fig. 5A). To examine the interplay of baculovirus and nuclear speckles further, a time series of vp39EGFP (200 pfu/ml) transductions at 4 - 48 h was performed. In this experiment, nuclear speckles were labeled using another nuclear speckle marker sc-35 antibody and Alexa-555-conjugated mouse IgG. The green fluorescence of the virus was enhanced with rabbit-anti-GFP and Alexa-488-conjugated rabbit IgG. Supporting the results obtained with PABP2-EGFP plasmid experiments, the virus was in close proximity to nuclear speckle structure (Fig. 5B) being most prominent at 6 - 12 h p.t., nevertheless, baculovirus did not colocalize with nuclear speckles at any timepoint.
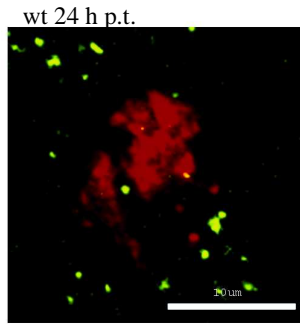
wt 24 h p.t.

Figure 5A. Baculovirus (wt) capsids localize close to nuclear speckles, but no colocalization is observed at 24 h p.t.. HepG2 cells were transfected with an expression plasmid PABP2-EGFP shown in red and viral capsids were labeled green using mouse anti-vp39 and Alexa-633-conjugated mouse IgG. The colors have been modified for better visualization of the results. Scale bar 10µm.

anti-GFP	sc-35	Merge	
			vp39EGFP 4 h p.t.
			6 h p.t.
			12 h p.t.

anti-GFP   sc-35   Merge

vp39EGFP
16 h p.t.

24 h p.t.
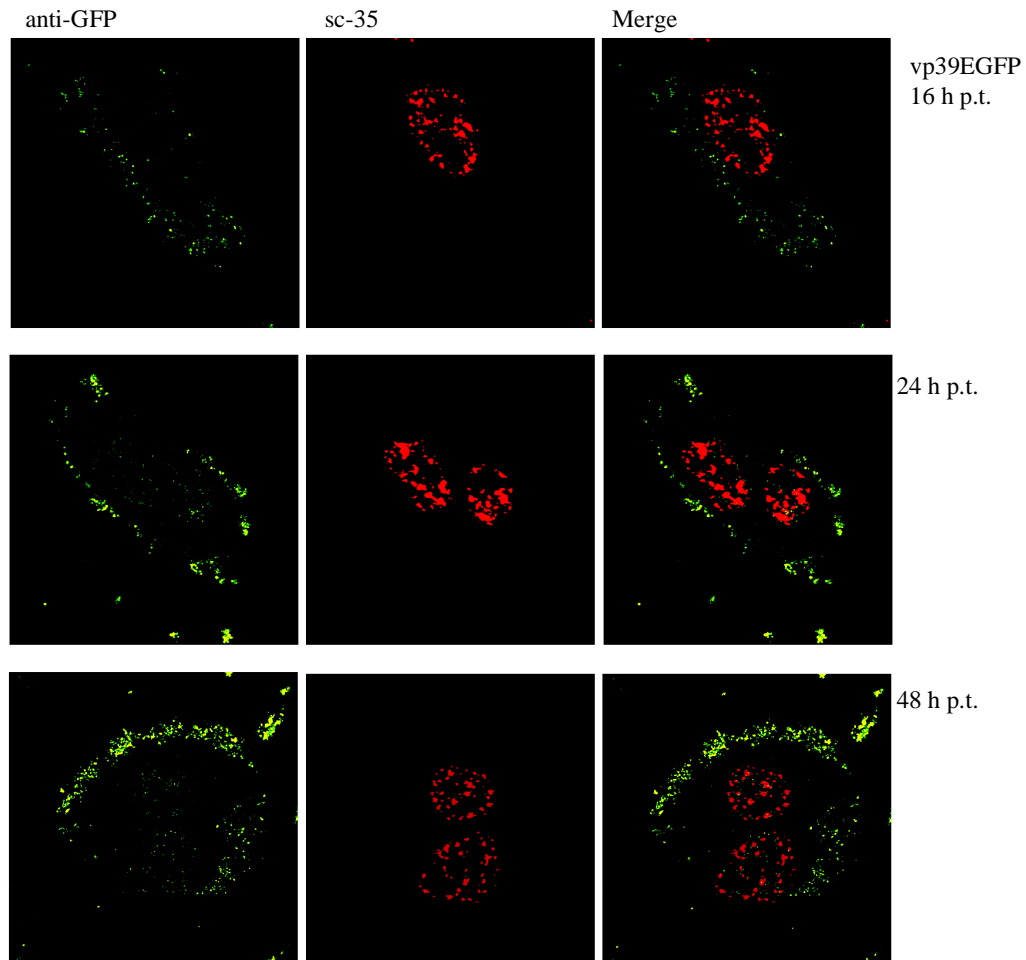
48 h p.t.

Figure 5B. Baculovirus (vp39EGFP) does not colocalize with nuclear speckles at 4 – 48 h p.t. The green fluorescence of the virus was enhanced with rabbit-anti-GFP and Alexa-488-conjugated rabbit IgG. Nuclear speckles were labeled red with mouse sc-35 and Alexa-555-conjugated mouse IgG. Scale bar 10µm

5.3.3 Cellular Chromatin

Baculovirus is known to stimulate host chromatin rearrangements in *Sf9* cells (O'Reilly *et al.* 1994; Volkman & Zaal 1990). Due to detecting the transcription of viral genes *ie-1* and *ie-2* in HepG2 cells, it was most interesting to study if baculovirus transduction would effect the nuclear organization of chromatin structure also in human cells. In the first experiment, HepG2 cells were transduced with vp39EGFP (200 pfu/ml) at 24 h p.t. Chromatin was dyed using DRAQ5™, which is a synthetic fluorescent compound with

high affinity for DNA, and the green fluorescence of the virus was enhanced using rabbit-anti-GFP and Alexa-488-conjugated rabbit IgG. As can be seen from the control cells, host heterochromatin seems to line the nuclear lamina and nucleoli. After 24 h of transduction, chromatin ceases to have this pattern and is spread randomly all around the nucleus (Fig.6).

vp39EGFP 24 h p.t.

Anti-GFP   DRAQ5™   Merge

Control DRAQ5™

Figure 6. Morphology of HepG2 chromatin is altered by baculovirus (vp39EGFP) transduction at 24 h p.t. In control cells, chromatin seems to line around the nuclear membrane and nucleoli, however, the chromatin in vp39EGFP (200 pfu/ml) transduced HepG2 is randomly dispersed around the nucleus. Chromatin was dyed red using DRAQ5™ and the green fluorescence of the virus was enhanced with rabbit-anti-GFP and Alexa-488-conjugated rabbit IgG. Scale bar 10μm.

To study the interactions between baculovirus and host chromatin further, the plasmid H2B-EYFP (Weidemann *et al.* 2003) was used to monitor the virus localization with respect to H2 protein component of histone core complex. The distribution of histone protein was clearly altered in the HepG2 cells transduced with wt (1000 pfu/ml) at 24 h p.t. (Fig. 7). Thus, the observed change in histone location strengthens the preceding result of virus-induced alterations in the morphology of HepG2 chromatin.

wt 24 h p.t.
Anti-vp39   H2B-EYFP   Merge

Control H2B-EYFP

Figure 7. The chromatin location is altered due to wt transduction in HepG2 cells at 24 h p.t. The cells were transfected with H2B-EYFP plasmid and transduced with wt (1000 pfu/ml) at 24 h p.t. The colors in the images were modified to accomplish better image visualization. Thus, in all figures, the H2B-protein fluorescence is shown in red and the wt capsids labeled with mouse anti-vp39 and Alexa633 conjugated mouse IgG shown in green. Scale bar 10µm.

6. DISCUSSION

6.1 Study of Baculoviruses for Gene Therapy

Baculoviridae virus family comprise a number of common features and share a unique life cycle, nevertheless, the spectrum of viruses in the family is truly large (Okano *et.al.* 2006). Most research on *Baculoviridae* has been carried out in *Autographa californica* multiple nucleopolyhedrovirus *(Ac*MNPV) due to its prominent manipulation possibilities and ease of growth in cell culture. Following preceding experiments, the viruses chosen for this study included the wild-type (wt) *Ac*MNPV and vp39EGFP (Kukkonen *et al*. 2003, kindly provided by Kari Airenne and Seppo Ylä-Herttuala, AI Virtanen Institute). The recombinant virus was constructed to allow better visualization of the viral capsids, however, the enhanced green fluorescent protein (EGFP) attached to the C-terminus of the nucleocapsid major protein vp39, was not sufficiently detectable in confocal imaging. Consequently, in this study the capsid fluorescence was enhanced with anti-GFP and Alexa-conjugated secondary IgG.

Gene delivery efficiency is much dependent on the virus dosage, hence, in each experiment the infectious titer and multiplicity of infection (MOI) indicating the baculovirus transducing ability were chosen carefully. The virus titers were determined by end-point dilution method in *Sf*9 cells (O'Reilly *et al.* 1994) and expressed as plaque forming units per milliliter (pfu/ml). Nevertheless, although these parameters are commonly used in calculating the virus dosage in vast majority of published gene therapy articles up to date, new data concerning the preciseness of these methods has emerged. Chan *et al.* (2005) have developed a titration protocol which directly measures the biological activity of baculovirus in transduced mammalian cells. Additionally, they suggest that as the infectious titer and the associated MOI are based on baculovirus ability to infect insect cells, consequently, these parameters are not precise in defining transducing titers in mammalian cells. Therefore, since the infectious titer in this study was obtained in *Sf*9, it might not be as accurate as if calculated using the new titration method introduced by Chan

and colleagues (2005). Thus, there might be slight variation in the reported infectious titers of different virus stocks and their real efficiencies in transducing HepG2 cells. Nevertheless, the O'Reilly's method is widely used and accepted in giving very good direction of the virus dosage. However, the accuracy of the new titration protocol will certainly be helpful when designing future experiments with baculovirus and other viral vectors.

Baculoviruses are pathogenic only for their natural hosts, insects, predominantly of the orders *Ledioptera, Hymoenoptera* and *Diptera*. However, wt and recombinant baculoviruses have been shown to effectively transduce various mammalian cells without cytopathic effects even at very high virus titers up to 5000 pfu/ml (Duisit *et al.* 1999; Kost & Condreay 2002; Airenne *et al.* 2000). The human hepatoma (HepG2) cell line used in this study was chosen due to previous research, which assigns that *Ac*MNPV enters human hepatocytes in preference to other mammalian cells (Bilello *et al.* 2001; Boyce & Bucher 1996; Hoffman *et al.* 1995) and the transgene delivery efficiency of recombinant baculoviruses is well approaching 100 % in hepatocytes (Boyce & Bucher 1996; Hoffman *et al.* 1995; Shoji *et al.* 1997). Additionally, baculovirus-mediated gene expression was achieved for the very first time in human hepatocytes in 1995 (Hoffman *et al.* 1995) which was soon followed by successful results in HepG2 respectively (Boyce & Bucher 1996). Since then, different studies have demonstrated recombinant baculovirus vectors capable of high-level transgene expression in hepatic cells (Gao *et al.* 2002; Sollerbrant *et al.* 2001, McCormick *et al.* 2002, Wang *et al.* 2004).

In addition to several hepatic cells, baculoviruses are capable of delivering transgenes into non-hepatic mammalian cells, such as HeLa, COS7 (Shoji *et al.* 1997), neuroblastoma cells, human primary neural cell cultures (Condreau *et al.* 1999; Sarkis *et al.* 2000), human pancreatic islet cells (Ma *et al.* 2000), human p53-null tumor cells (Song & Boyce 2001), human osteogenic sarcoma cell line Saos-2 (Song *et al.* 2003) and a variety of other cell lines studied (Airenne *et al.* 2000; Wang *et al.* 2004). Furthermore, baculovirus vectors have not only shown to transfer genes with a credible function efficiently into various cell types *in vitro* (Boyce & Bucher 1996; Condreay *et al.* 1999; Dwarakanath *et al.* 2001; Hofmann *et al.* 1995; Kukkonen *et al.* 2003; Ma *et al.* 2000; Pieroni *et al.* 2001; Sarkis *et*

al. 2000), but have also accomplished to achieve foreign gene expression *in vivo* (Bilello *et al.* 2001; Hofmann *et al.* 1999; Hofmann & Strauss 1998; Huser *et al.* 2001; Ojala *et al.* 2001; Pieroni *et al.* 2001; Shoji *et.al.* 1997; Song & Boyce 2001; Song *et al.* 2003). For these reasons, baculoviruses are of great interest as nonhuman viral DNA vectors in gene therapy.

Nevertheless, more research is needed to assure the virus safe and functional for transgene delivery. The major matters of interest are the exact nuclear entry process, the biodistribution *in vivo* and the properties of baculoviruses in human cells. Therefore, the aim of this study was to establish the active intake of baculovirus into the nucleus of human hepatoma cells (HepG2), observing whether baculovirus is able to transcribe viral mRNA of its immediate early genes *ie-1* and *ie-2*, in addition to, exposing the subnuclear localisation of baculovirus in HepG2 cells.

6.2 Nuclear Entry and Accumulation of Baculoviruses

In order to study general localization of baculoviruses in HepG2 cells, the cells were transduced with vp39EGFP within a time period of 4 – 24 h and the events were observed by confocal microscopy. This experiment was arranged to show that baculovirus does enter the cell nucleus, in addition to, witnessing the minimum time of the nuclear entry. In each experiment, the nuclei of the cells were labeled for distinguishable sorting of the viral capsids localized in the nucleus from the capsids still in cell cytoplasm. As a result, the virus was seen in HepG2 cytoplasm in all time points studied (4 – 24 h p.t.) and the accumulation of the viral capsids into the cell nucleus was observed starting at 4 h p.t., which was in agreement with previous knowledge (Kukkonen *et al.* 2003; Matilainen *et al.* 2005; Salminen *et al.* 2005). Accumulation into the nucleus increased further in later time points and was most prominent at 24 h p.t. (Fig 1). Previously, the localization of baculovirus nucleocapsids in inoculated human cells has been studied by Salminen and colleagues (2005) who utilized immuno electron microscopy technique to show virus capsids close to the nuclear pores in the nucleus at 3 – 4 h p.t.. In insect cells, the accumulation of viral capsids close to nuclear pores is prominent already at 1 – 4 h post

infection (p.i.) (Granados *et al.* 1981; Knudson & Harrap 1975; Wilson & Price 1988). Thereafter, the accumulation has been described more precisely by quantification of capsid localization in the nucleus (Laakkonen *et al.* 2007).

Baculovirus entry into the nucleus of mammalian cells is suggested to function through the nuclear pore (VanLoo *et.al.* 2001, Salminen *et.al.* 2005) and the results of this study (see 5.1 Baculovirus Entry into the Nucleus) were in agreement with the previous ones. In the experiment, HepG2 cells were first transduced with the virus and the WGA microinjection used to block the nuclear pores was performed at 2 h p.t., which is well before vp39EGFP could have entered the nucleus, but late enough for the WGA substance to stay stable for the rest of the transduction time. The schedule of transductions and injections was built on the knowledge obtained from the publications of Kukkonen *et al.* (2003) and Matilainen *et al.* (2005), which both showed baculovirus in HepG2 cell nucleus at 3 - 4 h p.t. Also, according to studies with other mammalian cell lines including human endothelial cells (EaHy), human osteocarcomas (MG63) and normal human osteoblasts (NHO) cells (Kukkonen *et al.* 2003), the viral capsids have already escaped from early endosomes at 4 h p.t. Thus, the experiment was arranged carefully considering two important facts: the virus will reach the nuclear membrane at the latest at 4 h p.t. and WGA has a very short operating time before breaking down. Additional challenge to the procedure was brought by ensuring the cell survival as the cells experience a rough trial when subjected to microinjection: They become shocked by a needle and have to survive unsheltered in poor conditions in RT for 30 min. Moreover, injecting into the cytoplasm of a hepatic cancer cell with greatly enlarged nucleus and a very small cytoplasmic area is challenging. In the end, however, the experiment was successful and the results show the virus remains outside the nucleus due to the blocked nuclear pores (Fig. 2). However, further studied concerning the nuclear entry of baculovirus is needed to reveal the actual elements that operate in the transport machinery of the host cell. In addition, revealing the baculoviral proteins involved in nuclear entry is essential in order to develop a functional gene transport system.

6.3 Baculovirus Immediate Early Gene Transcription

Twenty five years ago in 1983, Volkman and Goldsmith proved that baculovirus is incompetent to replicate in mammalian cells. Thereafter, no reports of the exact course of baculovirus gene transcription process in mammalian cells, nor the machinery of it have yet been published, although that should be of outermost interest in the field of baculovirus gene therapy. Thus, another objective of this study, therefore, was to investigate whether baculovirus transcribes its immediate early genes in HepG2 cells. For this experiment, a long time series (6 - 48 h) of wt transductions was performed and the levels of *ie-1* and *ie-2* mRNA were analyzed with quantitative RT-PCR analysis. A recent study (Fujita *et al.* 2006) has revealed that apparently *Ac*MNPV is able to transcribe some of its viral genes (*ie-0*, *ie-1*, *pe-38*, *gp64*) in HeLa and BHK cells. The results of our study in HepG2 cells further promoted baculovirus ability to exploit mammalian host machinery in its viral gene transcription: HepG2 cells showed transcription of both the early viral genes examined, *ie-1* and *ie-2* respectively, already at 6 h p.t. and increasing up to 48 h p.t. (Fig. 3). As the viral capsids were seen in the nucleus of HepG2 cells starting at 4 h p.t. (see 5.1 Baculovirus Entry into the Nucleus), hence, the time point of baculovirus entry into the nucleus well corresponds to the detected time point of the beginning of transcription, that is, the results support each other.

Laakkonen and colleagues (2007) have expanded the study of *ie-1* and *ie-2* gene transcription into human embryonic kidney cell line (293) at 24 h p.t. and as a result, found viral mRNA in transduced cells although the amounts of it were much lower than in HepG2 cells. Additionally, for the first time, they addressed expression of viral IE-2 protein in human cells at 4 - 48 h p.t. In insect cells, baculovirus is suggested to transcribe its immediate early genes using the host RNA polymerase II (Friesen 1996). Accordingly, Laakkonen *et al.* (2007) suggest that baculovirus transcription process in human cells resembles that of the insect cells, and the machinery probably includes the host RNA II polymerase and associated transcription factors. As viral proteins are not wanted by-products in human gene therapy, it would be highly interesting to study how deletion of the *ie-2* gene would affect the vector properties - Could it perhaps in part attenuate the host

defence against foreign elements (Hofmann & Strauss 1998) occuring during baculovirus transduction in human cells.

6.4 Localization of Baculovirus and Subnuclear Structures

Another objective in this study was to monitor the localization of baculovirus with respect to specialized domains or subnuclear organelles within the nucleus: PML NBs (promyelocytic leukemia nuclear bodies), nuclear speckles and chromatin. Previously, the initiation of transcription and the early replication process of many viruses including adenovirus (Carvalho *et al.* 1995; Doucas *et al.* 1996), herpesvirus (Everett 2001; Everett & Murray 2005; Maul *et al.* 1996) and papovavirus (Guccione *et al.* 2004) have been shown localize in PML NBs, moreover, these viruses have been shown to induce alterations in the whole nuclear organization. Thus, PML NBs, nuclear speckles and chromatin are interesting in now solving the possible transcription site of baculoviruses in mammalian hepatocytic cells, in addition to, observing the possible effects the transduction might cause to the organization of the cell nucleus.

PML NBs have a role in almost every function cells perform i.e. gene regulation, apoptosis, p53 function, DNA repair, chromatin dynamics, growth suppression, proteolysis, protein modification, interferon response and cell senescence (Borden 2002; Everett 2006; Maul *et al.* 2000). The experiments of this study were performed using two different methods: antibodies designed against a selected protein and/or plasmids coding for fluorescent protein products known to localize in this structure. The localization of a virus with respect to any cellular structure is more complicated to detect using a plasmid than an antibody due to the variations in the plasmid transfection efficiency. Therefore, the experiments were repeated various times to ensure the reliability of the results.

In the results obtained in HepG2 cells at early time points of transduction, the viral capsids (wt and vp39EGFP) localized in close proximity to the sp100EGFP-marker recognizing a sp100 protein found in PML NBs (Fig. 4). Nevertheless, in the subsequent experiments and colocalization analysis by Laakkonen and colleagues (2007), no statistically significant colocalization of viral capsids and PML proteins was found at 6 – 24 h p.t. An interesting finding, however, was that during viral transduction, the number of PML NBs per cell

decreased and their size increased (Laakkonen *et al.* 2007). Previously, Borden and colleagues (1998) have shown the accumulation of PML proteins into cytoplasm due to choriomengitis virus infection, which is contrary to the observed degradation of PML NBs during HSV-1 infection (Everett & Maul 1994; Everett & Murray 2005). The increased level of PML NBs during Epstein-Barr virus (Bowling & Adamson 2006) and cytomegalovirus (Kelly *et al.* 1995) have also been reported before. Moreover, the increase in the size of PML NBs has previously been linked to cellular stress and virus-induced interferon response (Buonamici *et al.* 2005), however, no cellular stress signals appeared during baculoviral transduction experiments reported by Laakkonen *et al.* (2007). Furthermore, no cytopathic effects of baculovirus have been observed in transduced HepG2 cells (Hofmann *et al.* 1995), nor in other mammalian cell lines studied *in vitro* (Duisit *et al.* 1999), even at very high virus titers (up to 5000 pfu / ml). Consequently, Laakkonen *et al.* (2007) suggest that the alteration of PML NBs might be induced by the virus to rearrange the structures better suitable for virus transcription or disassembly sites. Nevertheless, the exact function of the PML NBs still remains uncertain in baculovirus transduction. Perhaps more precise information of baculovirus location in the nucleus of transduced cells could be obtained by examining the placement of the viral genome more carefully. Thus, in future experiments it might be useful to track the viral genome rather than the capsid, since there is no data of how long the genome persists in the capsid during the transduction.

Nuclear speckles are dynamic structures containing factors which are recruited from them to sites of transcription (Spector 2001) and are suggested to be involved in pre-mRNA processing (Borden 2002; Spector 2001). Studying the interaction between baculovirus and nuclear speckles, the HepG2 cells were transfected with an expression plasmid PABP2-EGFP coding for a poly(A) binding protein 2, a component of nuclear speckle. The resulting confocal images revealed no specific association between the plasmid and the virus (wt) (Fig. 5A). The position of nuclear speckles with respect to viral capsids was also studied in another set of experiments utilizing a nuclear speckle marker sc-35 and a recombinant virus vp39EGFP. Still, supporting the previous results, no specific association between the virus and nuclear speckle structure at 6 - 24 h p.t. could be detected (Fig. 5B).

Another object of examination was the possible impacts of *Ac*MNPV transduction on the morphology of chromatin of HepG2 cells. Previous studies of the morphogenetic nuclear events late in *Ac*MNPV infection (about 22 - 24 h p.i.) in *Sf*9 cells have shown baculovirus induced changes in the intranuclear ring zone (Stoltz *et al.* 1973; Volkman *et al.* 1976). The lack of continuity with the nuclear membrane, and the patches and vesicles that develop within the ring zone seem to be connected to baculovirus infection switchover to occluded virus production (OV), where the nucleocapsids become enveloped to form preoccluded virions (POV). Additionally, the analysis of Williams and Faulkner (1997) shows strong evidence to support nuclear membrane involvement in the process of POV morphogenesis. Further, nuclear membrane disintegration that grossly resembles the events of GV morphogenesis has been suggested to be caused by phosphorylation of nuclear lamins by a cell-cycle-dependent nuclear-membrane associated kinase (Dessev *et al.* 1988). Still, the NPV and GV replication induced changes in the nuclear membrane have not been determined in detail. In this study, we wanted to extend the research to the morphology of mammalian host cell chromatin and the possible baculovirus induced changes in this structure. In the infection of insect host cells, baculovirus early proteins cause nuclear expansion and scattering of chromatin (O'Reilly *et al.* 1994). Likewise, peripheral displacement of host chromosomes has been reported to take place in human cells due to infection with another DNA virus, herpes simplex virus-1 (HSV-1) (Monier *et al.* 2000). As a part of HSV-1 replication process, the virus radically alters the nuclear architecture: host chromatin first becomes marginalized and later dispersed. In our experiments, the host chromatin structure in transduced (vp39EGFP) HepG2 cells was detected using a synthetic cell-permeable DNA probe DRAQ5™. The confocal images of untransduced control cells showed the condensed host chromatin neatly lining the nuclear envelope and nucleoli, however, this pattern of package was clearly broken when the cells were transduced with vp39EGFP (Fig. 6). This finding was later authorisized by Laakkonen *et al.* (2007), who demonstrated the remodeling pattern by performing a quantitative analysis of chromatin content in these cells. Additionally, they proved that using high viral titers (up to 1000 pfu / ml) does not induce interferon responce.

Subsequently, it was interesting to examine if the virus induced alterations in HepG2 nucleosome core complex of chromatin consisting of H2A, H2B, H3 and H4 protein

components. In this experiment, human H2B-EYFP expression plasmid was used to monitor the virus localization with respect to H2 component of the nucleosome core. Supporting the previous results, the host chromatin in transduced cells no longer clearly lined the nuclear lamina or nucleolus, but in stead, was randomly dispersed around the nucleus at 24 h p.t. (Fig. 7). Laakkonen and colleagues (2007) have further analyzed the virus-induced changes in the host chromatin by quantifying the relative deviation of histone protein H2B distribution in untransduced and baculovirus transduced cells and the results show the differences are statistically significant in HepG2 cells. Consequently, this data suggests that baculovirus does remodel the ultrastructure of the nucleus and thus, certainly needs modification before further clinical trials.

6.5 Conclusions

1. Baculovirus accumulates into HepG2 cell nucleus starting at 4 h p.t. and increases further up to 24 h p.t. The virus seems to enter the nucleus via nuclear pores.

2. Baculoviral immediate early genes *ie-1* and *ie-2* are transcribed at 6 - 48 h p.t. in HepG2 cells.

3. Close association of baculovirus and promyelocytic leukemia nuclear bodies (PML NBs), which are possible transcription sites of the virus, was detected at 8 - 24 h p.t. Baculovirus does not localize near nuclear speckles at 4 - 48 h p.t.

4. Host chromatin structure of HepG2 cells seems to be remodeled due to baculovirus transduction. Compared to untransduced control cells, the chromatin pattern in transduced cells evidently changes at 24 - 48 h p.t.

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APPENDICES

APPENDIX 1

Antibodies

All antibodies were tested before use to obtain the best possible dilutions for confocal microscopy studies. Testing was performed in untransduced HepG2 cells, which were grown to subconfluency and fixed according to the antibody used (see 4.3 Transduction of Mammalian Cells).

Primary Antibodies:

anti-GFP, rabbit IgG fraction (Molecular Probes^{TM}), dilution 1:300
anti-vp39, mouse IgG fraction (Molecular Probes^{TM}), dilution 1:70
anti-sc-35, mouse IgG fraction (Abcam Ltd.), dilution 1:100

Secondary Antibodies:

Alexa Fluor 488 goat anti-rabbit (Molecular Probes^{TM}), dilution 1:200
Alexa Fluor 555 goat anti-mouse (Molecular Probes^{TM}), dilution 1:200
Alexa Fluor 633 goat anti-mouse (Molecular Probes^{TM}), dilution 1:200

Plasmids

PABP2-EGFP
(Calapez et al. 2006) from Dr. Carmo-Fonseca, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal.

H2B-EYFP
(Weidemann et al. 2003) from J. Langowski, German Cancer Research Center, Heidelberg, Germany

pEGFP-sp100
(Muratani et al. 2002) from Dr. G. Dellaire, The Hospital for Sick Children, Toronto, Canada

Solutions and Reagents

BSA
Albumin bovine serum, Fraktion V
1g BSA/100 ml PBS (1%)
(Merck)

DABCO
1.4 Diazbicyclo [2.2.2]-octan 98%
25 mg/ml in Mowiol-DABCO embedding solution
(Sigma-Aldrich)

DAPI
4'-6-Diamidino-2-phenylindole
(ProLong® Gold, Molecular Probes^{TM})

Draq5^{TM}
Red fluorescent cell-permeable DNA probe
(Biostatus Ltd.)

FBS
Foetal Bovine Serum, 10% heat inactivated
 (PAA Laboratories GmbH)

HyQ®SFX-Insect medium
(HyClone Inc., Logan)

Immersol®, 518N
(Zeiss)

L-glutamine 100x
(GibcoBRL, Life Technologies)

MEM
Minimum Essential Medium, Eagle's Supplement, L-glutamine
(GibcoBRL, Life Technologies)

Added before use (in 500 ml MEM):
5ml L-glutamine
5 ml Penicillin-Streptomycin
5 ml Non-Essential Amino Acids
5 ml Na-pyryvate (100 mM)
50 ml inactivated FBS (10%)

Methanol
(Baker)

Mowiol
(EMD Biosciences Inc. Calbiochem)

NaN$_3$
(Baker)

Na-pyryvate, 100 mM (sterile filtered)
11 g $C_3H_3NaO_3$
1 l dH_2O
(Merck)

Non-Essential Amino Acids
(GibcoBRL, Life Technologies)

PBS 25x, pH 6.4
400 g NaCl
10 g KH_2PO_4
72.4 g Na_2HPO_4
10 g KCl
2 l dH_2O

PFA
paraformaldehyde
$HO(CH_2O)nH$
(Merck)

Penicillin (G sodium 10.000 U/ml) - Streptomycin
Sulfate in 0.85 % saline 10.000 µg/ml
(GibcoBRL, Life Technologies)

Permeabilization buffer (250 ml)
250 µl Triton®-X 100
1.25 ml BSA
2.5 ml NaN_3 (10 %)
dilute in 1xPBS

TRITC-De
Tetramethylrhodamine isothiocyanate-Dextran
(Molecular Probes^{TM})

Triton®-X 100
(Sigma)

WGA
wheat germ agglutinin
(Molecular Probes^{TM})

APPENDIX 2

Baculovirus-mediated Immediate Early Gene Expression and Nuclear Reorganization in Human Cells

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ABSTRACT

Baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), has the ability to transduce mammalian cell lines without replication. The general objective of this study was to detect the transcription and expression of viral immediate early genes in human cells and to examine the interactions between viral components and subnuclear structures. Viral capsids were seen in large, discrete foci in nuclei of both dividing and nondividing human cells. Concurrently, the transcription of viral immediate early transregulator genes (*ie-1, ie-2*) and translation of IE-2 protein were detected. Quantitative microscopy imaging and analysis showed that virus transduction altered the size of promyelocytic leukaemia nuclear bodies, which are suggested to be involved in replication and transcription of various viruses. Furthermore, altered distribution of the chromatin marker Draq5™ and histone core protein (H2B) in transduced cells indicated that the virus was able to induce remodelling of the host cell chromatin. To conclude, this study shows that the non-replicative insect virus, baculovirus and its proteins can induce multiple changes in the cellular machinery of human cells.