Molecular mechanisms in baculovirus defective interference: keys for improved recombinant protein expression

G. P. Pijlman

Promotoren: prof. dr. J. M. Vlak
Persoonlijk Hoogleraar bij het Laboratorium voor Virologie

prof. dr. R.W. Goldbach
Hoogleraar in de Virologie

Co-promotor: dr. D. E. Martens
Universitair Docent bij de Leerstoelgroep Bioprocestechnologie

Samenstelling promotiecommissie:

prof. dr. W.M. de Vos (Wageningen Universiteit)
dr. ir. C. D. de Gooijer (Wageningen Universiteit)
dr. D. A. Theilmann (Pacific Agri-Food Research Centre, Summerland, Canada)
dr. P. J. A. Sondermeijer (Intervet International B.V., Boxmeer)

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1. Het baculovirus 'non-*hr*' startpunt van DNA replicatie (non-*hr ori*) speelt een belangrijke rol in het ontstaan en de accumulatie van 'defective interfering particles'.
   Dit proefschrift.

2. Baculovirus Bacterial Artificial Chromosomes (BACs) kunnen de huidige virusopslag voor grootschalige eiwitproductie in insectencellen vervangen, mits de intrinsieke genetische instabiliteit van de BAC vector zelf wordt verholpen.
   Dit proefschrift.

3. De baculovirus non-*hr ori* is een typisch voorbeeld van een replicator.
   Richard Dawkins (1976), The Selfish Gene.

4. Het gastheerbereik van een virus wordt niet alleen bepaald door binnenkomst en replicatie, maar is tevens sterk afhankelijk van de mogelijkheid om de interferonrespons van de gastheercel te kunnen omzeilen.
   Parisien *et al.* (2002) J Virol 76:6435-6441; Park *et al.* (2003) J Virol 77:9522-9532.

5. Het bestaan van een adaptieve immuunrespons in ongewervelde dieren brengt niet alleen de ontwikkeling van vaccins tegen pathogenen in deze organismen nader bij, maar kan potentieel de basis vormen voor een nieuwe immunologische theorie.
   Kurtz and Franz (2003) Nature 425: 37-38; Witteveldt *et al.* (2004) J Virol, in press.

6. Flavivirussen zijn d.m.v. 'reverse-genetics' moeilijk te bestuderen, omdat virale eiwitsynthese, genoomreplicatie en virion assemblage direct aan elkaar gekoppeld zijn.
   Khromykh *et al.* (2001) J Virol 75:4633-4640.

7. Het bepalen van de volledige nucleotidenvolgorde van niet-commercieel verkregen plasmiden betekent uiteindelijk een tijds- en kostenbesparing.

8. Voor het ophelderen van de nucleotidenvolgorde van het kangaroegenoom moet diep in de buidel worden getast.

Stellingen behorende bij het proefschrift:

Molecular mechanisms in baculovirus defective interference: keys for improved recombinant protein expression

Gorben P. Pijlman

Wageningen, 17 december 2003

Foar myn Heit en Mem

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CHAPTER 1

Introduction

INTRODUCTION TO THE BACULOVIRUSES

Structure

Baculoviruses (family *Baculoviridae*) infect the larval stages of arthropods, mainly insects of the orders Lepidoptera, Hymenoptera, and Diptera (Murphy *et al.*, 1994), and comprise two genera, the genus *Nucleopolyhedrovirus* (NPV) and the genus *Granulovirus* (GVs). The circular, covalently closed double stranded DNA genome ranges in size from 80 to 180 kb (Büchen-Osmond, 2003). As a result of a biphasic virus replication cycle, the rod-shaped nucleocapsids appear in two phenotypically distinct forms (Fig. 1.1.) (Funk *et al.*, 1997). In case of the budded virus (BV), newly formed nucleocapsids become enveloped by budding from the membrane of the infected cell. Upon release the BVs spread the infection through the

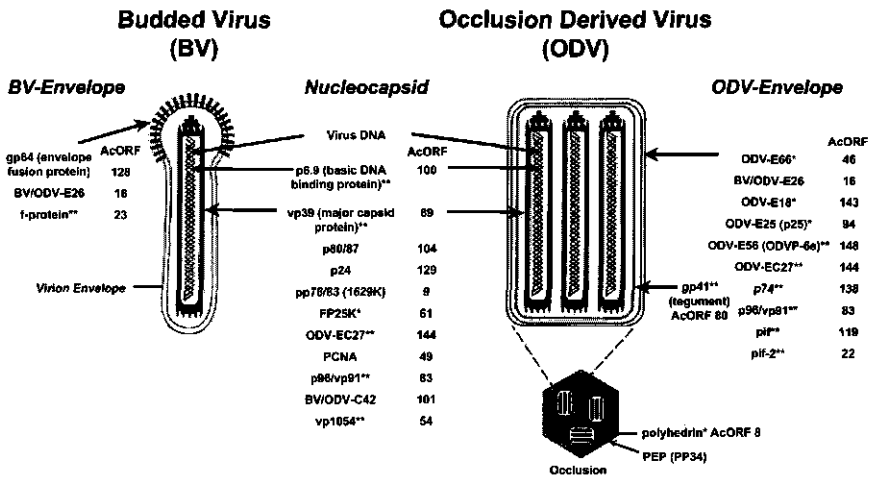

Figure 1.1. Structural composition of the budded virus (BV) and the occlusion derived virus (ODV), adapted from Funk *et al.* (1997) and Braunagel *et al.* (2003). Nucleocapsid proteins common to both virion types are indicated in the middle. Proteins specific to BV and ODV envelopes are indicated on the left and right, respectively. Corresponding open reading frames (ORFs) in *Autographa californica* multicapsid nucleopolyhedrovirus are indicated. Conserved lepidopteran baculovirus genes are indicated (*), as well as structural baculovirus core genes (**) (according to Herniou *et al.*, 2003).

insect (Blissard, 1996). In cultured insect cells, the BVs are released into the culture medium and spread the infection to other cells. Occlusion derived viruses (ODVs) of the NPVs contain multiple (MNPV) or single (SNPV) nucleocapsids wrapped in a *de novo* formed envelope (Blissard, 1996). The ODVs are immobilized in a paracrystalline matrix mainly constituted of the virus-encoded polyhedrin protein. The so-called polyhedral occlusion bodies or polyhedra are involved in the horizontal virus transfer from one insect to another and protect the virions from degradation in the environment.

Infection cycle

In cultured cells the baculoviral infection cycle (Fig. 1.2A) starts by attachment of a BV to the cell surface (1). The BV enters the cell by endocytosis (2) and fuses with the endosomal envelope achieving the nucleocapsids (NCs) to be released in the cytoplasm (4). The NC is transported to the nucleus (5) and the DNA is released (6) to be replicated in association with the virogenic stroma (8). The newly formed NCs are transported to the cell membrane (9,10) where they leave the cell by budding (11). One round of replication is referred to as one virus passage.

The infection cycle in a midgut cell (Fig. 1.2B) starts when polyhedra are ingested by the insect. In the alkaline environment of the midgut the polyhedrin matrix dissolves (1) and the ODVs are released into the lumen of the midgut (2). The ODVs are first transported through the peritrophic membrane and subsequently fuse to the microvilli of the midgut epithelial cells (3). The NCs are transported to the nucleus of the midgut cells (4,5), where the initial rounds of virus replication take place (6,7,8). In the late phase of infection, newly formed NCs are transported to the cell membrane (9,10) where they leave the cell by budding (11) into or through the basal membrane. The BVs subsequently spread the infection to neighboring cells and via the hemocoel further through the insect to other tissues and organs such as fat body and epithelial cells. In the very late phase of infection, the NCs are assembled in a *de novo* formed envelope to become ODVs (12). The ODVs are subsequently embedded in a matrix of the polyhedrin protein (13), which is produced in large quantities in the very late phase of infection. At the end of the infection the polyhedra are released upon cell rupture.

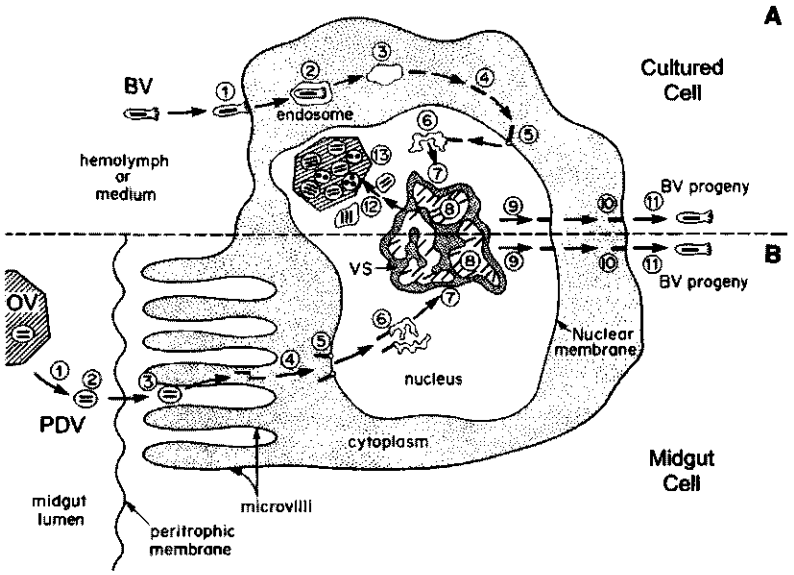

Figure 1.2. Infection cycle of a multicapsid nucleopolyhedrovirus (MNPV) in cultured (*in vitro*) cells and in larval midgut (*in vivo*) cells.

BACULOVIRUS DNA REPLICATION

Origins of DNA replication: hrs

Baculovirus DNA replication takes place in the nucleus of the infected insect cell, while the virus encodes its own genes for this process. The *cis*-acting elements required for replication (*ori*s) have been identified by the analysis of defective genomes obtained after serial virus passage in cell culture (Kool *et al.*, 1991; Lee and Krell, 1992; Kool *et al.*, 1993a) and by transient replication assays (Pearson *et al.*, 1992; Kool *et al.*, 1993b; Leisy and Rohrmann, 1993). *Ori* activity in baculoviruses was found to be associated with the homologous regions (*hr*s) (Lu *et al.*, 1997). *Hr*s are homologous repeated regions containing palindromic sequences interspersed with short direct repeats, and are found scattered along the baculovirus genome. *Hr*s can also act as transcriptional enhancers (see Friesen, 1997, for review). The presence of *hr*s is a common feature of baculoviruses, although not all have clearly defined *hr*s but rather short repeat units dispersed throughout the genome (Luque *et al.*, 2002). The contribution of *hr*s to the genetic heterogeneity of virus isolates was shown by the duplication/reiteration of *hr*s in AcMNPV, AgMNPV, and SeMNPV (Burand and Summers, 1982; Garcia-Maruniak *et al.*, 1996; Muñoz *et al.*, 1999).

Non-hr oris

Besides the *hr*-type *oris*, non-*hr* sequences with *ori* activity have been identified by transient replication assays in the genomes of OpMNPV (Pearson *et al.*, 1993), AcMNPV (Kool *et al.*, 1994), SeMNPV (Heldens *et al.*, 1997a) and SpliMNPV (Huang and Levin, 1999). These so-called non-*hr oris* consist of sequences lacking typical *hr*-type palindromes or repeats, and may in some cases even be located within an open reading frame (AcMNPV *p94*; Kool *et al.*, 1994). The non-*hr oris* of different baculoviruses have no overall structural homology to each other or to *hr*-type *oris* in the same genome. Nevertheless, non-*hr oris* contain basic structures that are found in the consensus eukaryotic *ori* (DePamphilis, 1993), such as multiple direct and inverted repeats, palindromes and AT-rich stretches. Unlike *hr*-type *oris*, the presence and distribution of structural motifs within non-*hr ori* sequences is most likely more important for *ori* activity than the primary sequence itself.

Besides the fact that *hrs* and non-*hr oris* are active as *ori* in transient replication assays, both *ori* types were also enriched in defective interfering (DI) baculoviruses upon serial passage in cell culture (Kool *et al.*, 1993a; Lee and Krell, 1992, 1994), providing further evidence for a genuine role of these sequences in DNA replication. Additional evidence that in particular non-*hr oris* are active during baculovirus DNA replication came from quantitative PCR studies with AcMNPV (Habib and Hasnain, 2000), the interaction of a SpliMNPV non-*hr ori* with the viral DNA polymerase (Huang and Levin, 2001), and the *in vivo* expansion of a non-*hr ori* in the CrleGV genome (Jehle, 2002). An overview of all known non-*hr oris* or non-*hr ori*-like sequences is shown in Figure 1.3.

	Baculovirus	structural properties	replication assay	enrichment in DIs	expansion *in vivo*	Reference
Group I NPV	**AcMNPV**	✓	✓	✓		Lee and Krell, 1992; 1994. Kool *et al.*, 1994
	BmNPV	✓				Kool *et al.*, 1994
	OpMNPV	✓	✓			Pearson *et al.*, 1993
Group II NPV	**SeMNPV**	✓	✓	✓		Heldens *et al.*, 1997. Pijlman *et al.*, 2002
	SpliMNPV	✓	✓			Huang and Levin, 1999; 2001
	BusuNPV	✓				Hu *et al.*, 1998
GranuloVirus	**CpGV**	✓				Luque *et al.*, 2002
	CrleGV	✓			✓	Jehle, 2002
	AdorGV	✓				Wormleaton *et al.*, 2003

Figure 1.3. Occurrence of non-*hr oris* and non-*hr ori*-like sequences in baculovirus genomes.

Trans-acting sequences and DNA replication mechanism

The *trans*-acting elements required for baculoviral DNA replication include among others DNA polymerase (*dnapol*), *p143* (*helicase*), *lef-1* (*DNA primase*) and *lef-2*, four genes which are found in all sequenced baculovirus genomes at present (Herniou *et al.*, 2003). The minimal set of genes required for *ori*-dependent DNA replication in transient replication assays are *ie-1*, *lef-1*, *lef-2*, *lef-3*, and *p143*. However, dependent on the conditions, *ie-2*, *lef-7*, *dnapol*, *p35*, and *pe38* are required for or stimulated DNA replication (Crouch and Passarelli, 2002).

In transient replication assays, *hr*-containing plasmids replicated as high molecular mass concatemers (Leisy and Rohrmann, 1993). In another study, pUC-based plasmids without any viral *ori* sequence replicated in infected insect cells as concatemers and/or integrated in the viral genome (Wu *et al.*, 1999). These findings suggested a "rolling circle"-like DNA replication mechanism (Kornberg and Baker, 1992), whereas Kool *et al.* (1995) suggested a possible combination of a "theta-like" and a "rolling circle" mechanism. Oppenheimer and Volkman (1997) were able to detect multiple unit-length genome fragments from replicating viral DNA, suggesting that AcMNPV replicates in a head-to-tail manner via a rolling circle mechanism.

BACULOVIRUS APPLICATIONS

Bioinsecticide

The earliest application of baculoviruses is as biological pest control agent in forestry, agriculture and horticulture (Cunningham, 1995). Many baculoviruses have been registered as biological pesticide, but their relatively slow speed of kill has limited practical use to date (Black *et al.*, 1997). Therefore, baculoviruses have been genetically engineered to accelerate their speed of action (Inceoglu *et al.*, 2001). Insertion of genes encoding insect juvenile hormone esterase (Eldridge *et al.*, 1992) or insect neurotoxins such as AaIT (Stewart *et al.*, 1991) have generated recombinant viruses with improved speed of kill, resulting in decreased feeding damage. Deletion of the viral gene encoding ecdysteroid UDP-glucosyltransferase (*egt*) has also resulted in increased speed of kill (O'Reilly and Miller, 1989), although the results are ambiguous (Bianchi *et al.*, 2000) and the mechanism is not yet completely understood. Recombinant baculoviruses for pest control can be produced *in vivo* by village industries, giving it potential for developing countries (Inceoglu *et al.*, 2001). Industrial *in vitro* scale-up systems may provide standardization and can potentially be more cost-effective, although some baculoviruses may lose their *in vivo* infectivity as a consequence of *in vitro* replication (Heldens *et al.*, 1996).

Expression vector

Another major application of baculoviruses is the use as protein expression vectors in insect cells. The baculovirus-insect cell expression system was initially developed for the production of biologically active (glyco)proteins in a well-established and safe eukaryotic environment (Summers and Smith, 1987). Expression of proteins in insect cells permits proper folding, post-translational modifications (e.g. glycosylation, phosphorylation, internal cleavage) and oligomerization like their authentic counterparts (Vialard, 1995). Cells of lower organisms like bacteria, yeast and fungi lack most of these abilities (Kitts, 1996), while mammalian cells can generate more complex glycosylation patterns. Furthermore, insect cell lines are relatively easy to maintain as the cells grow equally well in suspension as on surfaces. They also do not support growth of viruses or expression of oncogenes affecting humans (Agathos *et al.*, 1990) and they can be cultivated in serumfree media. Biological activity is especially valuable in the production of antigenic proteins for *in vitro* diagnostics (e.g. parvovirus B19; Brown *et*

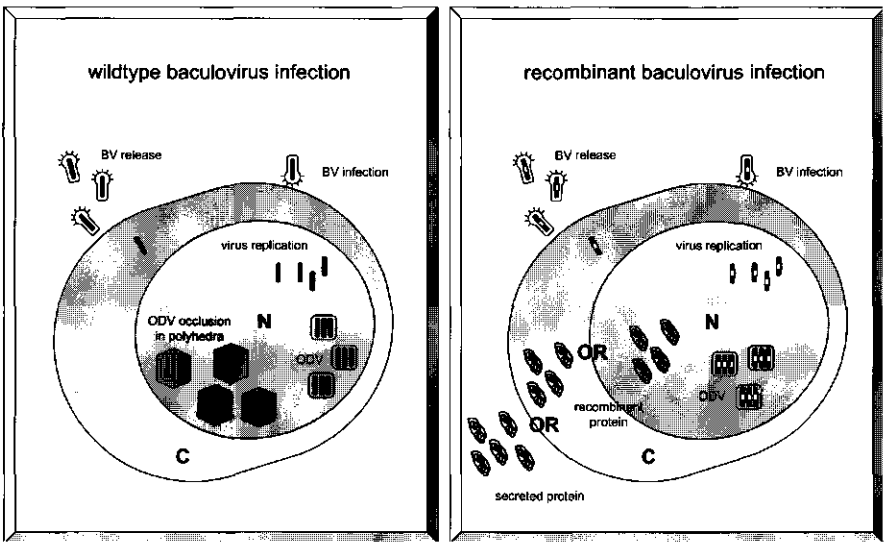

Figure 1.4. Baculovirus expression system. A schematic representation of a wildtype baculovirus infection is indicated on the left. Virus replication takes place in the nucleus of the infected cell, where ODVs are embedded in the polyhedrin matrix to form polyhedra, which are released upon cell lysis. On the right, a recombinant baculovirus infection is depicted, where the polyhedrin gene is replaced by a foreign gene. ODVs are still being produced inside the nucleus, and the heterologous protein is abundantly expressed from the very late polyhedrin promoter. Depending on the signal sequence of the recombinant protein, it can reside in the cytoplasm (C), be transported to the nucleus (N), or be secreted in the cell culture fluid.

al., 1991), while safety is important for therapeuticals and recombinant subunit vaccines (e.g. marker vaccine against *Classical swine fever virus*; Van Rijn *et al.*, 1999).

Historically, the baculovirus expression system is based upon the allelic exchange of two highly expressed baculovirus genes that are not essential for replication in cell culture, polyhedrin and p10 (Fig. 1.4). The polyhedrin gene is the major occlusion protein and is expressed at a very high level in the very late phase of infection but is not essential for virus replication *in vitro* (Smith *et al.*, 1983). Also baculovirus expression vectors based upon the (very late) p10 gene, which is associated with fibrillar structures in the nucleus and cytoplasm (Van Oers, 1994), were developed (Vlak *et al.*, 1990). Because the polyhedrin gene remains intact, these p10 expression vectors may be exploited for the expression of heterologous proteins in insects infected *per os* and for the enhancement of baculovirus pathogenicity for insect control.

The conventional method for allelic exchange uses a two-step approach. A segment of the viral DNA is cloned into a plasmid vector, and modified to obtain a so-called transfer vector (Smith *et al.*, 1983). A transfer vector usually contains the recombinant gene to be expressed under control of a (strong) baculovirus promoter, flanked by viral sequences used for recombination with the viral genome. Insect cells are subsequently (co)transfected with the transfer vector and the wild-type viral genome. After homologous recombination inside the insect cells has generated a mutant virus, several rounds of plaque purification are required to isolate the recombinant virus. Numerous strategies to enhance the recombination efficiency and/or to reduce the background of wild type virus have been developed (reviewed by Kitts, 1996). However, these methods still rely on homologous recombination in insect cells and are relatively time-consuming. Also, difficulties appear when sequences or genes need to be knocked out, which give the recombinants a replicative disadvantage. Due to the large size of baculovirus genomes, sensitivity to shearing and to the paucity of unique restriction sites, direct manipulation of the viral DNA remains difficult (Kitts, 1996).

Besides the development of a few yeast-based systems (Patel *et al.*, 1992; Heldens *et al.*, 1997b), a protocol based on maintenance and recombination of full-length baculovirus genomes in *E. coli* has gained most attention, and has become the method of choice in many laboratories. Luckow *et al.* (1993) developed an efficient and rapid method to generate recombinant baculoviruses, which is based on transposition of an expression cassette from a donor plasmid into a bacterial artificial chromosome (BAC) containing the entire baculovirus genome and an 8.5 kb bacterial

element with a mini-F replicon, Tn7 transposon acceptor sites and an antibiotic selection marker (bacmid). The bacmid is maintained as a single-copy plasmid in *E. coli* and infectious virus can be generated upon reconstitution of bacmid DNA in insect cells. The advantage of this system (Fig. 1.5) is that the entire process of constructing the recombinant bacmid DNA takes place in *E. coli*; only the virus reconstitution step requires insect cells. Recombinant bacmids are isolated from *E. coli* colonies after selection with appropriate antibiotics. The other advantage of the bacmid system is that a pure preparation of recombinant bacmid DNA is obtained before transfection of insect cells occurs, eliminating the need for multiple rounds of plaque purification. This method has reduced the time to identify, purify and amplify a recombinant virus from 4 to 6 weeks (typical for the conventional method) to 7 to 10 days.

Gene therapy

Baculoviruses may also find a future application in gene therapy. A particular advantage is the ease of construction (in *E. coli*) and the possibility to insert large segments of DNA or multiple genes. However, the successful use of baculoviruses as gene delivery vehicles will depend on several factors that determine whether a viral vector is suitable or not (Lundstrom, 2003).

In the first place, the viral vector must have efficient gene delivery capabilities. For baculoviruses, gene delivery in cell culture can be high (Merrihew *et al.*, 2001; Lehtolainen *et al.*, 2002), but it remains to be seen whether similar efficacies can be obtained *in vivo*.

Secondly, with respect to gene delivery, it is important that the vector is specifically directed to the target cells or tissue. Proper delivery may be improved by the utilization of the baculovirus surface display technology with scFv (Mottershead *et al.*, 2000). This technology utilizes the baculovirus GP64 glycoprotein as a backbone for membrane anchoring of foreign (eukaryotic) proteins at the cell surface (Grabherr *et al.*, 2001).

Thirdly, the duration of expression is important and is related to stability of the vector, integration in the host genome and persistent vector replication. Baculovirus DNA was shown to be partially integrated in the genome of cultured mammalian cells, while stably expressing a reporter gene (Condreay *et al.*, 1999; Merrihew *et al.*, 2001).

Finally, probably the most important aspect is the safety of the viral vector. At present, gene therapy with baculovirus vectors has not been targeted to defined locations in the mammalian genome and thus contains the risk of chromosomal

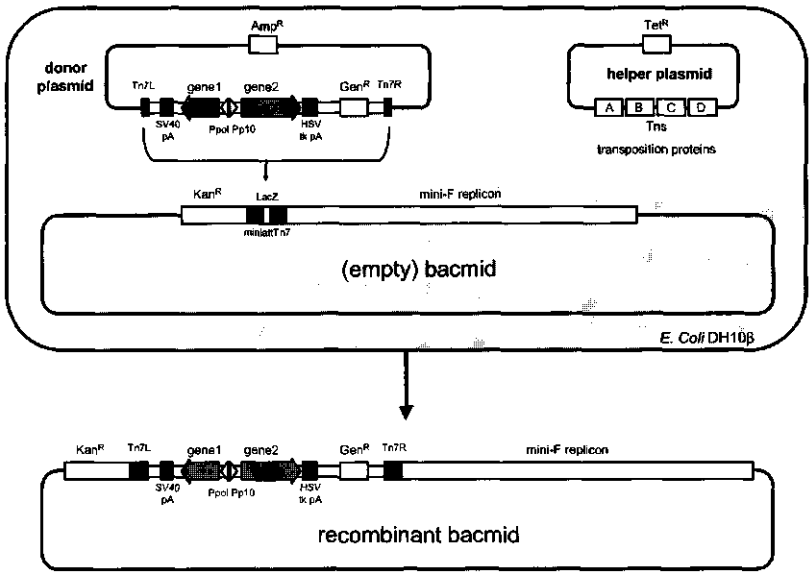

Figure 1.5. Bacmid expression system (adapted from the Bac-to-Bac Baculovirus Expression System manual (Invitrogen) and from Luckow *et al.*, 1993). *E. coli* DH10β cells harbour a sinlge-copy bacterial artificial chromosome (BAC) vector containing the entire AcMNPV genome. The BAC vector contains a kanamycin resistance gene (Kan^R), a mini-F replicon for maintenace in *E. coli*, and a LacZ cassette with a transposon integration site (attTn7) for bacterial transposon Tn7. The donor plasmid contains the Tn7 tranposon (Tn7L and Tn7R), an ampicillin resitance gene (Amp^R), and a cassette for baculovirus expression of recombinant proteins (gene 1 and 2) in insect cells, under control of baculovirus polyhedrin and p10 promoters. The expression cassette is transposed with the aid of transposition proteins TnsABCD, located on tetracyclin resistant (Tet^R) helper plasmid.

integration at undesired positions, which could be potentially dangerous. Moreover, cytotoxic and immunogenic effects of baculoviruses are extremely important (Lundstrom, 2003), but unknown at present. With the availability of more mammalian genomes and further trials with targeted gene integration in cultured mammalian cells, baculoviruses may have potential to be used as gene delivery vector to treat diseases such as cancer (Stanbridge *et al.*, 2003).

LARGE-SCALE PROTEIN PRODUCTION IN INSECT-CELL BIOREACTORS

Monolayer cultures of insect cells in roller bottles and tissue culture flasks, or suspension cultures in shaker flasks, are well suited for protein production on a small

scale. As these systems are labor-intensive and costly in terms of scale-up, large-scale production usually requires the use of suspension cultures in a bioreactor (Tramper and Vlak, 1988). Insect cells are cultured in different bioreactor systems, such as spinner flasks, bubble columns, airlift reactors, packed-bed reactors and (continuous) stirred tank reactors (Tramper *et al.*, 1993; review Agathos, 1996). More recent developments in this area include rotating wall vessels (Klaus, 2001), wave bioreactors (Singh, 1999), and perfusion cultures (Chu and Robinson, 2001). Due to the lytic infection of baculoviruses the production time of single (fed) batch and perfusion reactors is limited in contrast to continuous reactors in series. The first continuous insect-cell bioreactor system was developed by Kompier *et al.* (1988). Two headspace-aerated stirred reactors were connected in series. Medium was continuously fed to the first cell-growth reactor, and cell suspension was pumped into the second reactor, where baculovirus infection took place. A continuous set-up separates cell growth and virus production in place, which favors this system relative to (fed) batch-operated systems.

However, the main disadvantage of a continuous system is the large distribution in residence times of the cells and virus in the infection reactor. Consequently, the outflow from the infection reactor may contain a significant fraction of cells not yet containing recombinant protein. This problem was overcome to a large extent by replacing the infection reactor by two or more infection reactors in series maintaining the same overall residence time, thereby creating a more plug-flow like infection system (Van Lier *et al.*, 1990). In this way an increase in the fraction of infected cells and higher (initial) protein yields could be obtained. However, an earlier decrease in production was observed than with a single infection reactor. This indicated the occurrence of a faster passage effect (reviewed by Krell, 1996) caused by the accumulation of DI baculoviruses (Kool *et al.*, 1991). De Gooijer *et al.* (1992) developed a structured dynamic model to describe the process of baculovirus infections in insect-cell bioreactors. With this model the observed decrease in infection (and thus production) level due to the passage effect could be well described. This model suggested a repeated batch operation for optimal production levels, which was experimentally confirmed by Van Lier *et al.* (1995). These studies further indicated that the generation and subsequent accumulation of DI baculoviruses pose a major limitation to large-scale (bioreactor) productions where many virus passages are required.

BACULOVIRUS GENOME INSTABILITY

Baculovirus replication is prone to the generation of genetic alterations (reviewed by Krell, 1996), leading to a range of different genotypes and phenotypes. This may provide baculoviruses genetic flexibility to adapt under adverse conditions. Firstly, the phenotype of the mutant is dependent on the nature of the alteration(s). Genotypic alterations include deletions, insertions of viral or foreign origin, inversions, point mutations, frameshifts and reiterations. In some cases, the imprints of homologous and heterologous recombination steps, between different viruses or within virus families, are found in baculovirus genomes. Secondly, the alteration can generate a defective or nondefective virus, with the first requiring a helper virus for replication. Thirdly, the mutant virus can be interfering or non-interfering, independent of whether the virus is defective or not. A few examples of baculoviruses with genetic alterations reported in literature are given below:

1. *Infectious mutants with no apparent different phenotype.* The occurrence of this type of mutants relates to (common) genotypic differences within a natural baculovirus isolate, and includes for instance single nucleotide polymorphisms (Chen *et al.*, 2001). It should be noted, however, that minor events like point mutations/frameshifts could have major phenotypical effects. Another example is the insertion of transposon TED (Friesen and Nissen, 1990) in the AcMNPV p94 gene, which did not result in an apparent phenotypical change. Other alterations with no direct phenotypical effect are *hr* duplications as mentioned before (Muñoz *et al.*, 1999), and non-*hr ori* expansions in a natural CrleGV isolate, although the biological activity of the latter mutant has not yet been investigated in detail (Jehle, 2002).
2. *Infectious (deletion) mutants with different biological properties.* Upon replication of SeMNPV in cell culture, large deletions in the *Xba*I-A restriction fragments are generated. These deletions result in (non-defective) mutants that sometimes have lost the ability to liquefy infected larvae (Dai *et al.*, 2000) or entirely lack oral infectivity *in vivo* (Heldens *et al.*, 1996). Such mutants were also shown to be present in the mixture of genotypes present in the natural SeMNPV isolate (Dai *et al.*, 2000).
3. *Infectious interfering deletion/insertion mutants.* Kumar and Miller (1987) conducted the first detailed investigation of the effect of serial passage of AcMNPV in cell culture. Spontaneous deletions were repeatedly generated in the *Pst*I-G and *Pst*I-I fragments. These deletion mutants became predominant upon

serial passage, indicating that they had somehow obtained a replication advantage over the parental virus. Insertional mutations were found repeatedly in the *Pst*I-E region, which is the FP-locus containing the 25K gene (Beames and Summers, 1989). FP25K mutants produce a higher amount of BVs and this causes their predominance over time and passage (Kumar and Miller, 1987; Bischoff and Slavicek, 1997).

4. *Defective viruses without a replicative advantage.* These mutants, which require a helper virus for replication, are likely to be repeatedly generated during virus replication, but simply disappear upon virus passage or are retained at submolar levels.
5. *Defective viruses with a replicative advantage.* Due to their smaller size and/or an increased density of *ori*s as a result of sequence deletions in between adjacent *ori*s, these defective viruses interfere with the replication of the parental virus and can eventually become predominant in the virus population. An example of is the AcMNPV M5 mutant described by Carstens (1982; 1987), and the AcMNPV DIs generated in a bioreactor (Kool *et al.*, 1991). Another striking example of the occurrence of these mutants *in vivo* has been reported for natural SeMNPV isolates (Muñoz *et al.*, 1998). This study aimed to describe the most apparent genotypes in a natural SeMNPV isolate using *in vivo* cloning methods. However, some apparent genotypes could not be cloned and were named parasitic genotypes, which co-replicated with the helper virus.
6. *Defective interfering viruses with reiterated oris.* During baculovirus replication, *ori*s may be reiterated, as exemplified by the duplication of *hr*s (Kool *et al.*, 1993a) and non-*hr*s (Lee and Krell, 1992; 1994). These defective interfering viruses may obtain a strong replicative advantage upon virus passage, thereby decreasing the amount of helper virus dramatically.

DEFECTIVE INTERFERING VIRUSES

Defective interfering viruses (DIs; Huang and Baltimore, 1970) were first discovered by Von Magnus (1954) during serial passage of Influenza virus in chicken. DIs have since been recognized in virtually all virus families, and their effects on virus replication and pathogenesis *in vitro* and *in vivo* were reviewed by Roux *et al.* (1991). DIs may affect the outcome of vaccination campaigns with live vaccines. DIs are replication-defective viral deletion mutants which arise spontaneously during virus replication and interfere specifically with the growth of the

parental virus (Bangham and Kirkwood, 1990). The ratio of DI:parental virus increases upon serial passage with high multiplicity of infection (MOI), and this ratio and the extent of interference depend on the virus species as well as the host cell. The striking observation of a cyclical variation in the titer of parental virus and DI upon repeated passage is known as the Von Magnus phenomenon, which can be mathematically explained by dynamic models (Bangham and Kirkwood, 1990; De Gooijer *et al.*, 1992; Sanderson *et al.*, 1999). Interference of DIs with the replication of the (intact) parental virus mainly occurs at the replication level, by competition for replication factors such as DNA polymerases and helicases. DIs are often enriched in *ori*s and have a smaller size (Fig. 1.6. and Lee and Krell, 1992), providing them with a strong replicative advantage over the parental virus. However, interference can also act at the packaging level, but in this case DI molecules are often less efficiently packaged into mature virus particles than the parental virus (Roux *et al.*, 1991).

Figure 1.6. Electron micrograph of bioreactor sample (high passage number), taken from Kool *et al.* (1991). N: normal, intact virus particle. D: defective interfering virus particle.

Baculoviruses with defective interfering properties (Fig. 1.6) are readily generated upon serial passage of AcMNPV in insect cells (Kool *et al.*, 1991; Wickam *et al.*, 1991; Lee and Krell, 1992; Kool *et al.*, 1993a) and in baculovirus-infected insect-cell bioreactors (Kool *et al.*, 1991; Van Lier *et al.*, 1992). For a given virus-cell-bioreactor combination, the MOI determines the ratio DI:parental baculovirus and consequently the level of recombinant protein production (Wickam *et al.*, 1991). First, it was hypothesized that the accumulation of baculovirus DIs with a deletion of the recombinant gene was the major cause of the drop in production in the bioreactor (Van Lier *et al.*, 1992). In a following study, however, the recombinant gene was placed in the p10 locus, and was retained in the DIs upon passage (Van Lier *et al.*, 1994). Nevertheless, also in this case the protein production declined to low levels within 2 weeks. This indicated that, although the recombinant

gene was retained, DIs may lack one or more virus encoded factors that are involved in late and very late gene expression (Todd *et al.*, 1995).

The generation of AcMNPV DIs upon serial passage in cell culture was studied in detail by Lee and Krell (1992), who found that at passage 65 (P65) the majority of the (presumably defective) genomes had a mean size of around 50 kb, which is around 40% of the parental virus. At P81 the majority of defective genomes had retained only a small sequence of 2.8 kb, while the amount of helper virus was very low. This 2.8 kb DNA segment was later shown to contain sequences sufficient for viral DNA replication in AcMNPV-infected cells (Lee and Krell, 1994). Kool *et al.* (1994) showed that this non-*hr* sequence was active in transient replication assays, and hence was named non-*hr* *ori*. The role of non-*hr* *ori*s in the generation/maintenance of defective interfering baculoviruses is therefore of special interest.

OUTLINE OF THE THESIS

Baculovirus defective interference is a phenomenon of major concern with respect to large-scale or continuous protein production in insect cell bioreactors, because a considerable number of viral passages is required to obtain seed virus or to have maximum production. As a result of the passage effect, the current baculovirus technology requires the establishment of low-passage virus seed stocks to be able to guarantee the yield and quality of a production run. This thesis focuses on the origin and genetic content of baculovirus DIs, and the underlying (molecular) mechanisms of baculovirus defective interference. In addition, it investigates a number of strategies to overcome the problems associated with this phenomenon.

At the onset of this thesis research, the question was whether AcMNPV DIs with large genomic deletions were detectable in low-passage virus inocula used for large-scale production. If so, these DIs could have been generated as an artifact of *in vitro* virus replication. Alternatively, these DIs could have been derived from pre-existing DIs present in the authentic AcMNPV isolate, which was never passaged in cell culture. A sensitive, (nested) PCR-based test is developed in **Chapter 2** to investigate the origin and nature of defective interfering baculoviruses derived from AcMNPV. A full-length, genetically homogeneous clone of AcMNPV (bacmid) is used to address whether or not DIs with major deletions are generated within a single round of replication.

Compared to AcMNPV infections in cultured insect cells, major deletions in the genome of SeMNPV occur much earlier during passage in cell culture. (Defective) mutants with large deletions, which often comprise genes essential for virus replication *in vivo*, usually predominate early passages. In **Chapter 3,** SeMNPV is used as a reproducible and rapid model virus to study the defective interference phenomenon in cell culture and in particular the role of non-*hr ori*s therein. In order to obtain a genetically homogenous inoculum, SeMNPV is cloned as a bacterial artificial chromosome in *E. coli*. In addition, the generation of the SeMNPV bacmid will facilitate the previously troublesome generation of SeMNPV (deletion) mutants aimed to investigate the effect of a non-*hr ori* deletion on DI formation.

The nature of the passage effect and the prevalence of DIs may not only vary between different species of baculoviruses, the respective cell line may be a major determinant as well. In order to gain more insight in differences between cell lines with regard to baculovirus DI replication, the cell-line specificity of SeMNPV infection and concomitant accumulation of DIs with reiterated non-*hr ori*s is investigated in **Chapter 4**.

SeMNPV replication in cell culture is prone to the deletion of major parts (up to 25 kb) of the viral genome, which are dispensable for *in vitro* replication. However, the resulting deletion mutants often lack the ability to infect insects by the oral route, suggesting that one or more genes essential for *in vivo* infectivity are spontaneously deleted in cell culture. This characteristic of SeMNPV complicates the *in vitro* scale-up of SeMNPV as biopesticide and prevents the efficient generation of biologically active recombinants. The focus of **Chapter 5** is the identification of the SeMNPV encoded gene(s) responsible for the observed loss of oral infectivity in insects using bacmid mutagenesis strategies.

While in Chapter 3 and 4 the non-*hr ori* in the SeMNPV model system is subject of investigation, in **Chapter 6** the role of non-*hr ori*s in the generation of DIs is studied for AcMNPV, which is the most widely applied and best-studied baculovirus to date. Although AcMNPV is relatively stable in cell culture, serial passage in cell culture eventually leads to the generation of DIs enriched in non-*hr ori*s. In contrast to non-*hr ori*s from other baculoviruses, the AcMNPV non-*hr ori* is located within an open reading frame, encoding the (non-essential) p94 gene. Since non-*hr ori*s accumulate upon serial passage it is hypothesized that deletion of this sequence would improve the virus stability.

The genetic instability of the frequently deleted region in the SeMNPV genome (Chapter 5) appears to coincide with a low density of *ori*s (or *hr*s), as the distance between adjacent *ori*s (or hrs) is the largest in this particular genomic region. In

Chapter 7 the hypothesis that deletions are more likely to occur in regions with a low *ori* (or *hr*) density is investigated. In contrast to AcMNPV (Chapter 6) the passage effect in case of SeMNPV is notable after a few passages. Therefore, recombinant SeMNPV bacmids are equipped with an extra *hr* sequence in the non-essential BAC vector insertion, and the maintenance of its flanking sequences is studied in a continuous bioreactor set-up consisting of a cell-growth reactor in a cascade with two parallel infection reactors. If this hypothesis appears to be correct, the distribution of *hr*s may regulate the generation of deletion mutants (including DIs), and thus may supply a tool to enhance baculovirus genome stability by genetic engineering.

In **Chapter 8**, the overall results from the previous chapters are discussed in the context of origin and role of DIs in baculovirus replication in cell culture. Furthermore, the implications of the various findings for the construction of stabilized baculovirus vectors is discussed. Finally, a novel approach towards long-term protein production via positive selection of recombinant baculoviruses upon infection is described and a proposal for an improved baculovirus expression vector is presented.

CHAPTER 2

Autographa californica baculoviruses with large genomic deletions are rapidly generated in infected insect cells

Defective interfering baculoviruses (DIs) lack considerable portions of the genome, interfere with the replication of helper virus and cause the so-called 'passage-effect' during serial passaging in insect cells and in bioreactor configurations. We investigated their origin by (nested) PCR and demonstrated that DIs lacking approximately 43% (d43) of their DNA are present in low-passage AcMNPV-E2 virus stocks and in polyhedra, but not in the authentic AcMNPV isolate obtained prior to passage in cell culture. To investigate whether DIs are rapidly generated de novo in Sf21 insect cells, a genetically homogeneous AcMNPV bacmid was serially passaged, resulting in the generation of d43 DIs within 2 passages. AT-rich sequences of up to 66 nucleotides of partly unknown origin were found at the deletion junctions in the d43 DI genomes. These data suggest that the rapid generation of DIs is an intrinsic property of baculovirus infection in insect cell culture and involves several recombination steps.

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INTRODUCTION

The baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) is widely exploited as a safe eukaryotic expression vector in insect cells (King and Possee, 1992). The baculovirus insect-cell expression system usually gives high yields of heterologous proteins, which in many cases are immunologically and biologically similar to their authentic counterpart (Vialard *et al.*, 1995). Engineered baculoviruses are further envisaged as improved bioinsecticides (Cunningham, 1995; Black *et al.*, 1997) and may have potential as vectors for gene therapy (Hofmann *et al.*, 1995).

Baculovirus and recombinant protein production following infection of insect cells is accompanied by the occurrence and accumulation of defective interfering (DI: Huang and Baltimore, 1970) particles (Kool *et al.*, 1991; Wickham *et al.*, 1991). These DIs are generated upon multiple passaging at high multiplicity in insect cells and are responsible for the so-called 'passage-effect' observed, among others, in bioreactor configurations (Van Lier *et al.*, 1992). This phenomenon prevents the development and exploitation of stably operated continuous insect cell bioreactor production systems and complicates the scale-up of baculovirus and recombinant proteins *in vitro*. The nature and origin of these DIs are not well understood.

DIs most likely have a replication advantage as a result of their smaller size and the increased density of origins of DNA replication successfully competing for essential trans-acting factors (Krell, 1996). After 81 passages of AcMNPV-E2 in Sf21 insect cells DI genomes were generated and they were largely composed of reiterations of a short genomic sequence (Lee and Krell, 1992, 1994), which was shown to contain a putative origin of DNA replication (Kool *et al.*, 1994). *In vivo* activity of this non-*hr* origin of DNA replication has now been demonstrated (Habib and Hasnain, 2000).

Kool *et al.* (1991) observed the accumulation of DIs containing a major genomic deletion of approximately 43% (d43) upon serial passage of AcMNPV-E2 in Sf21 insect cells. In several independent studies the generation of AcMNPV DIs with a similar deletion was demonstrated (Carstens, 1982; Lee and Krell, 1992; Van Lier *et al.*, 1994; Wickham *et al.*, 1991). The deleted region of the genome contains genes which are considered to be involved in baculovirus DNA replication, such as *lef-1, lef-2, lef-3* and *DNA polymerase* (Kool *et al.*, 1995; Lu and Miller, 1995). DIs of Bombyx mori NPV (BmNPV), a baculovirus closely related to AcMNPV with respect to gene order and overall gene homology, were also found to contain deletions spanning this

region (Hashimoto *et al.*, 1993; Yanase *et al.*, 1998). Thus, the accumulation of baculovirus DIs with major deletions is a general phenomenon.

It is not clear whether the accumulation of AcMNPV d43 DIs is the result of a generation mechanism that involves progressive deletions over time or, alternatively, by specific selection of DIs from a mixed population in the virus inoculum. We used a sensitive (nested) PCR-based method to detect minor amounts of AcMNPV DIs in low-passage virus stocks and insect-derived polyhedra. DIs may be part of a natural baculovirus population that subsequently predominates in insect cell culture. Alternatively, DIs may be generated solely as an artifact of *in vitro* cell culture. To answer this question, the original isolate of AcMNPV (Vail *et al.*, 1971), which had never been replicated in cell culture, was investigated for the presence of DIs. Furthermore a recombinant baculovirus, which was generated from a 'bacmid', was serially passaged in insect cells. Bacmids are baculovirus shuttle vectors that replicate in *E. coli* as a low-copy plasmid and are genetically homogeneous (Luckow *et al.*, 1993). These bacterial artificial chromosomes (BACs) are stably maintained in well characterized recombination deficient *E. coli* host strains (e.g. DH10β). No rearrangements have been observed in BACs after 100 generations of growth (Shizuya *et al.*, 1992). The use of this bacmid resulted in a starting material composed only of intact viral genomic DNA. Our results indicate that DIs are rapidly generated in cell culture and can persist in insects.

RESULTS

AcMNPV DI detection by PCR

A haemolymph preparation derived from fourth instar *Spodoptera exigua* larvae orally infected with a lethal dose of AcMNPV-E2 was defined as passage zero (P0) budded virus (BV) stock. The first round of infection in Sf21 insect cells was initiated with a multiplicity of infection (MOI) of 10. Polyhedra, virus containing haemolymph (P0) and virus serially passaged through Sf21 cells (P1 to P4) were investigated for the presence of DIs by (nested) PCR (Fig. 2.1). This PCR was specifically designed to amplify junction fragments resulting from deletions of approximately 43% (d43) of the genome on a defined location (Kool *et al.*, 1991). For the BV and intracellular virus (ICV) preparations of P4 only, a PCR product of 1.1 kbp was amplified (Fig. 2.1B, panel I). A nested PCR using nested primers resulted in the generation of single PCR products of 0.8 kbp for all samples except the sample in lane 1 (Fig. 2.1B, panel II). PCR amplification products from AcMNPV-E2 (polyhedra P0, haemolymph P0, BV P1 and P4 and ICV P1 and P4) were cloned. To investigate the

presence of d43 DIs in a natural baculovirus population, a DNA preparation of the original AcMNPV isolate from the alfalfa looper *Autographa californica* (Vail *et al.*, 1971) was subjected to PCR. The one-step or nested PCR (Fig. 2.1B, panels I and II) generated no products.

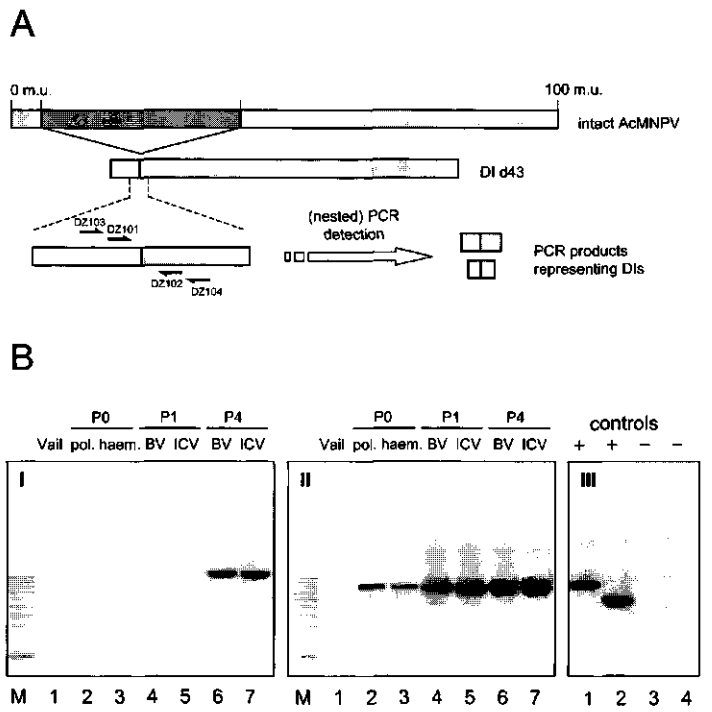


Figure 2.1.

A) PCR detection of d43 deletions with (nested) primers flanking the AcMNPV deletion junction site. B) PCR detection of d43 DIs in DNA preparations of AcMNPV virus stocks. (panel I) One-step PCR on DNA from polyhedra of AcMNPV-Vail (lanes 1) and on AcMNPV-E2 DNA preparations; insect-derived polyhedra (lanes 2), haemolymph derived budded viruses (lanes 3), serially passaged BV and intracellular (ICV) viral DNA of the first (P1, lanes 4 and 5, resp.) and the fourth passage (P4, lanes 6 and 7, resp.). (panel II) Nested PCR on amplification products from the one-step PCR. (panel III) Plasmid pMK1 used as a positive control template for one-step and nested PCR, giving products of 894 bp (lane 1) and 630 bp (lane 2), respectively. For the negative controls no template DNA was used for the one-step (lane 3) and nested (lane 4) PCR. Lane M contains a 100-bp DNA size marker.

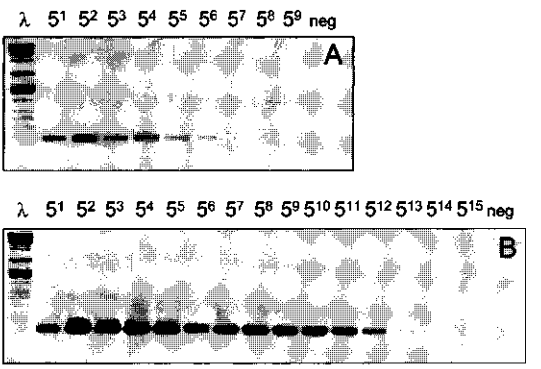
Plasmid pMK1, containing the *Sst*II-BE deletion junction fragment of AcMNPV (Kool *et al.*, 1991), served as positive control template, giving products of 894 and 630 bp in the one-step and the nested PCR, respectively (Fig. 2.1B, panel III). The sensitivity of the one-step and nested PCR was demonstrated by testing a dilution series of pMK1. With the one-step PCR only, the lower detection limit was dilution 5^7,

corresponding with approximately $0.6*10^6$ molecules (Fig. 2.2A), whereas the detection limit of the nested PCR was dilution 5^{13}, giving an improvement of at least 10^4-fold, resulting in a detection level between 10 and 100 molecules per 50 µl PCR reaction volume (Fig. 2.2B).

The quality and authenticity of the DNA templates used for the DI PCR experiments described above was demonstrated by a PCR, specifically amplifying a genomic region of 819 bp in the PstI-E fragment of AcMNPV. This region is located within the major deletion of d43 DIs and is indicative of the presence of intact viral DNA. This PCR proved to be positive for all investigated AcMNPV viral DNA samples, including the authentic AcMNPV isolate (data not shown).

Figure 2.2.

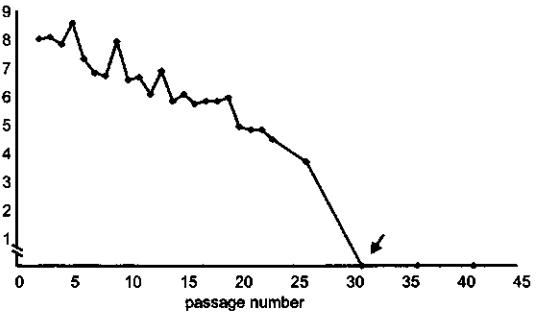
Sensitivity of one-step (A) and nested (B) PCR for detection of DIs. Five-fold dilution series from 5^1 to 5^{15} of pMK1 (containing an AcMNPV-E2 *Sst*II-BE junction fragment) were tested using primersets DZ103/104 and DZ101/102, respectively. One µl reaction product of the one-step PCR was used as template for nested PCR.



Passage effect upon serial passage of AcMNPV bacmid bGFP

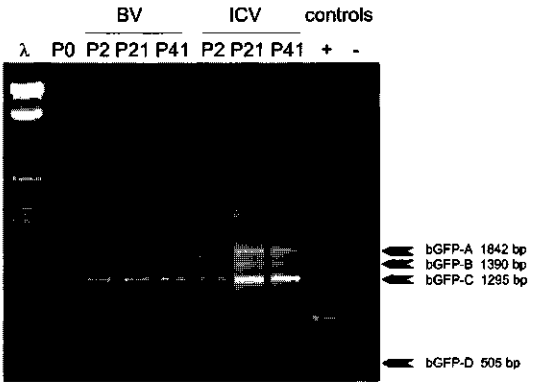
To investigate whether DIs are rapidly generated de novo in insect cells, we serially passaged a recombinant baculovirus that was generated from a genetically homogeneous source (bacmid). In order to facilitate virus titration and to observe changes in recombinant protein production during passaging, the bacmid was equipped with the S65T variant of the green fluorescent protein (GFP) and was designated bGFP. Sf21 insect cells were transfected with bGFP DNA and the transfection supernatant containing BV was defined as the first passage (P1) virus stock and was used to initiate serial passaging with a MOI of 20. Throughout passaging a gradual but definite decrease in virus titre was observed from approximately 10^8 $TCID_{50}$ units/ml in the early passages to a complete loss of GFP expression from P31 and further on (Fig. 2.3).

Figure 2.3.
Titration bGFP virus stocks during serial passage in Sf21 insect cells as measured by GFP expression. The arrow indicates the earliest passage number with loss of GFP expression.



DNA preparations from the serially passaged bGFP-derived virus productions were analyzed for the presence of DIs by PCR. For P2, P21 and P41 of both BV and ICV DNA preparations a similar set of amplification products was generated with sizes from about 0.5 up to 2.0 kbp (Fig. 2.4). The dominant 4 PCR products of bGFP (BV P2) with sizes of 1.8, 1.4, 1.3 and 0.5 kbp were designated bGFP-A, -B, -C and -D, respectively, and subsequently cloned.

Figure 2.4.
PCR detection of major deletions in DNA preparations from bacmid bGFP (P0), serial passaged bGFP-derived budded virus (BV) and intracellular virus (ICV). Passage numbers are indicated above the lanes. Cloned amplification products are designated bGFP-A to -D. Positive (+) and negative (-) controls were plasmid pMK1 and no template DNA, respectively.



Sequence analysis of DI deletion junction sites

The PCR amplification products from AcMNPV-E2 (polyhedra P0, haemolymph P0, BV P1 and P4 and ICV P1 and P4: Fig. 2.1B) and the four abundant PCR products of bGFP (Fig. 2.4) were cloned and sequenced, and the DNA organization at the junction sites was determined (Fig. 2.5). The sequences of the six (nested) PCR products from wildtype AcMNPV-E2 (Fig. 2.1B, panel II) proved to be identical and revealed the presence of an insertion of 66 nucleotides (AT 67%) at the junction site. The precise origin of this sequence could not be identified, but 21 contiguous nucleotides were identical to a part of ie-1, a gene located at nt positions 127198 to 128944 (Ayres *et al.*, 1994).

The four bGFP amplification products were cloned and sequenced and revealed differences in the organization at the junction site. The largest fragment (bGFP-A), representing the smallest deletion, contained an additional 11 bp (AT 100%) at the junction site. The second (bGFP-B) and the third (bGFP-C) product both contained an extended insertion of 48 bp (AT 75%) and 66 bp (AT 82%), respectively. Interestingly, 27 nucleotides of the bGFP-C insertion sequence were identical to a region in the mini-F replicon, which is part of the bacmid vector. Fragment bGFP-D did not have an insertion, but an overlap of 2 nucleotides (TG) on either side of the junction instead.

fragment	left	junction sequence	right
Kool *et al.* (1991)	(2459) CGGC	*TGTT*	GAGT (59268)
AcMNPV-E2 wt	(2627) TCAT	*ATGACGATTGGTTTGCGCGTAATACATATAATGCAACGTCCGATATTATTTGTATTGCATGTTAAT*	AGAT (59305)
bGFP-A.	(3343) TATG	*TTTAATAATTA*	AGTT (59211)
bGFP-B.	(2711) GCGC	*TTTATAGTTTGTGTACATATTTACTGTGTTCAATTTAAAACTCACGTA*	ACGC (59068)
bGFP-C.	(2536) GGCA	*ATATTTTCTGACTAAGTATTTAAAATTTTAGATTGTCACACTAAATAAAAAAAACTTAAATTCACA*	CGCT (59006)
bGFP-D.	(2290) TATG	**TG**	TGCC (59482)

Figure 2.5. Sequence of DI deletion junction sites. Viral sequences on the junction site borders are indicated on the left and right. Insertion sequences are in italics. Numbers are referring to positions of viral nucleotides at the border of the junction based on Ayres *et al.* (1994). Overlapping viral sequences are indicated in bold. Underlined sequences indicate continuous stretches of nucleotides identical to parts of the ie-1 gene (AcMNPV-E2 wt) and the mini-F replicon (bGFP-C), respectively.

DISCUSSION

We investigated whether DIs are present in a natural virus isolate and subsequently accumulate in cell culture or, alternatively, whether their generation is solely an artifact of cell culture. For AcMNPV, the origin of the predominant DI d43 as described by Kool *et al.* (1991) has never been further investigated, due to the lack of sensitive detection methods. DI detection by nested PCR generated identical amplification products of 0.8 kbp in all the virus stocks of AcMNPV-E2 (Fig. 2.1B) indicating that this technique is robust and sensitive (Fig. 2.2). For the virus samples of P4, a product was already generated by one-step PCR only, indicating an increased proportion of d43 DIs as compared to the samples of lower passages. Since polyhedra and haemolymph-derived virus preparations were found positive, we concluded that d43 DIs were present in the starting material (polyhedra,

haemolymph) isolated from infected larvae, and subsequently accumulated in cell culture to reach a visible stage by restriction enzyme analysis (Kool *et al.*, 1991). The detection of d43 DIs in AcMNPV-E2 polyhedra from insects indicates that DIs can persist in insects for more than one passage.

The presence of DIs in polyhedra reflects the occurrence of DIs *in vivo*, as was also shown by Muñoz *et al.* (1998). The latter authors demonstrated that in a natural baculovirus population of *Spodoptera exigua* MNPV (SeMNPV) deletion mutants, which could not be purely isolated by *in vivo* cloning, acted as parasitic genotypes. In an independent study, Dai *et al.* (2000) showed the presence of deletion mutants with maintained infectivity in the wildtype US1-isolate of SeMNPV by PCR. Serial passage of Spodoptera exigua MNPV in SeUCR cells resulted in the prevalence of mutants with a 25 kb deletion, lacking virulence *in vivo* (Heldens *et al.*, 1996). These observations suggest that genetic heterogeneity including the presence of DIs is not uncommon in natural baculovirus isolates.

It is not understood why DIs eventually do not predominate in insects, despite the fact that baculovirus recombination readily occurs *in vivo* and deletions are frequently observed (Muñoz *et al.*, 1997; Hajós *et al.*, 1998; Dai *et al.*, 2000). The preferential maintenance of parental virus *in vivo* might be due to a local low MOI in infected larval tissues or a specific selection barrier against the encapsulation of DIs.

In contrast to these reports, investigation of the authentic wild type AcMNPV (Vail *et al.*, 1971) by nested PCR did not reveal the presence of d43 DIs (Fig. 2.1B). However, it cannot be excluded that their presence is below the detection level of this technique (approx. 100 DI molecules per 100 ng DNA). This implies that d43 DIs in the AcMNPV-E2 isolate may have found their origin in the original plaque purification procedure of the several AcMNPV variants (including the E2-strain) in cultured TN-368-10 insect cells (Smith and Summers, 1978) or in subsequent amplifications. PCR detection of major deletions upon transfection and subsequent infection of insect cells with genetically homogeneous bacmid-derived AcMNPV DNA (Fig. 2.4) supports the suggested rapid generation of d43 DIs upon infecting in insect cells. However, possible enhanced instability resulting from the presence of the bacterial expression cassette of the bacmid causing defective genomes cannot be excluded.

Assuming that large genomic deletions are the result of recombination, sequences at the deletion junction sites may contain the remains or imprints of possible intermediate deletion mutants. For AcMNPV-E2, only one PCR product was generated, suggesting that a particular d43 DI had become predominant in the virus inoculum. At least 4 different PCR products were generated from bGFP, indicating that a variety of DIs with deletions in the same genomic region was generated and

maintained upon passaging. Sequence data of the amplification products generated by (nested) PCR revealed in 4 out of 5 cases the presence of short AT-rich (67-100%) sequences of (largely) unknown origin (Fig. 2.5). For bGFP-C, 27 bp out of a total insertion sequence of 66 bp was found identical to a region in the mini-F replicon of the bacmid, which is physically mapped within the d43 deletion and therefore supports a progressive deletion mechanism. In contrast, 21 bp of the deletion insertion sequence of AcMNPV-E2 of 66 bp was identical to the AcMNPV ie-1 gene, which is not located within the d43 deletion and therefore suggests that several recombination mechanisms be involved in the generation of deletions. We were unable to determine a consensus insertion sequence or evident homology between viral sequences flanking the junction sites. Although, in two cases we found an overlap of 2 bp (bGFP-D) and 8 bp (from a recombinant AcMNPV DI, unpublished results) from sequences flanking the deletion junction site, likely to be the result of homologous recombination.

The presence of insertion sequences at the deletion junction sites of DIs are consistent with the findings of Kool *et al.*, (1991), who determined an insertion of 4 nucleotides (TGTT) in a cloned junction fragment of an AcMNPV DI (Fig. 2.5). More recently, Wu *et al.* (1999) described the random integration of plasmid DNA into the AcMNPV genome due to co-replication in Sf21 insect cells. Analysis of integration junction sites revealed large genomic deletions and several insertions of short, AT-rich sequences of unknown origin. Analogous to our DI sequences, comparison of the junction insertion sequences did not reveal any consensus sequence or obvious homology between pUC19 and AcMNPV DNA. Although this study (Wu *et al.*, 1999) focused on the integration of foreign sequences into the baculovirus genome, our data show complementary evidence, suggesting that the same recombination mechanisms may be involved. Homologous recombination between a pair of integrated inverted repeat IS50 elements *in vitro* readily occurred during AcMNPV replication in Sf9 cells (Martin and Weber, 1997). Moreover, homologous recombination at high frequency (Hajós *et al.*, 2000) is an important feature of baculovirus replication. Non-homologous recombination *in vitro* was shown to occur between foreign and AcMNPV DNA in Sf9 cells (Xiong *et al.*, 1991; Schorr and Doerfler, 1993). These studies enforce the view that the process of baculovirus DNA replication in insect cells involves several mechanisms of hetero- and homologous recombination that may result in the rapid generation of defective genomes.

From this study it was concluded that the rapid generation of DIs is an intrinsic property of baculovirus infection in insect cell culture. We assume that continuous hetero- and homologous recombination of baculovirus DNA in infected insect cells

occurs in combination with a selection for mutants which have a replication advantage and are able to multiply faster at the expense of the helper virus. With the development of a specific (nested) PCR strategy for the detection of DIs a sensitive tool was obtained, which we will further use in experiments to demonstrate whether the rate of DI generation differs among cell lines from various origins, and whether the genetic stability of baculovirus in cell culture can be enhanced by genetic engineering.

MATERIALS & METHODS

Cells, insects, and virus

Spodoptera frugiperda (Sf-AE-21) cells (Vaughn *et al.*, 1977) were maintained at 27°C in Grace's supplemented insect medium (Gibco BRL) with 10% fetal calf serum (FCS). Routine cell culture maintenance was performed according to established procedures (Summers and Smith, 1987; King and Possee, 1992). Polyhedra of the E2 strain of wildtype (wt) AcMNPV (Smith and Summers, 1978) were propagated in fourth instar larvae of *Spodoptera exigua*, which were raised on artificial diet (Smits *et al.*, 1988). Haemolymph was taken from infected larvae by cutting a proleg. The preparation was defined as the passage zero (P0) virus stock and was used for a first round of infection (passage) in Sf21 cells at a multiplicity of infection (MOI) of 10 ($TCID_{50}$ units per cell). Budded virus (BV) solutions were titrated using the endpoint dilution assay (Vlak, 1979). Serial undiluted passaging was performed in 25 cm^2 tissue culture flasks (Nunc) by incubation for 2 h of $2.5*10^6$ cells with 1 ml of the virus inoculum of the previous passage. The cells were washed with fresh medium and were further incubated in 4 ml of medium for 72 h.

Bacmid-derived virus

The S65T GFP gene from pGreenlantern (Gibco BRL) was cloned into the MCS of pFastBac1 (Gibco BRL) as a *Not*I fragment to generate bGFP by transposition according to the Bac-to-Bac baculovirus expression systems manual (Gibco BRL). Bacmid bGFP was kindly provided by Dr. Ray Harris (Life Technologies) as a glycerolstock, which was subsequently plated and amplified from a single colony. Isolation and transfection of bacmid DNA to Sf21 cells was done according to the Bac-to-Bac baculovirus expression systems manual (Gibco BRL). The bacmid-derived BV inoculum was defined as bGFP P1 and was used to initiate undiluted serial passaging in Sf21 cells at an initial MOI of 20 $TCID_{50}$ units/cell.

Isolation of viral DNA

Budded virus was purified from infectious supernatant by centrifugation. BV viral DNA and total intracellular (ICV) DNA was isolated as described by Summers and Smith (1987), but viral DNA was purified by ethanol precipitation instead of dialysis.

PCR, cloning and sequencing

PCR was performed using custom designed (DNAstar Primerselect) and synthesized (Gibco BRL) primers. The forward primer VW293 for amplification of a part of AcMNPV *Pst*I-E (from position 44013 to 44832, according to Ayres *et al.* (1994)) was 5' AGTTACAGAGTTTTCCGTGGTTCAG 3' and the reverse primer VW294 was 5' CCCCGTTATCGTCAATTTTCTCAAG 3'. Forward DZ103 and nested-forward DZ101 primers for detection of DIs were 5' GTACCGCAGGTTGAACGTATCTTC 3' and 5' CGCTACAACACTCGTCGTTATG 3', located on the AcMNPV complete genome (Ayres *et al.*, 1994) at positions 1968-1991 and 2125-2146, respectively. Reverse DZ104 and nested-reverse DZ102 primers were 5' CCACACTTGATGCTAATCTCAAATAC 3' and 5' GTACACGCACACCGAGTTGTTTGTC 3', located at positions 59667-59642 and 59560-59536, respectively. PCR was carried out for 30 amplification rounds in a reaction volume of 50 µl using approx. 100 ng viral DNA template per reaction. For nested PCR, 1 µl of the amplification product from the first round was used as a template for 30 additional amplification rounds. Plasmid pMK1 (6.45 kbp) containing the AcMNPV *Sst*II-BE deletion junction fragment in PJD118 as described by Kool *et al.* (1991) was used as a positive control template for the PCR detection of major deletions. Five-fold dilution series until 5^{15} of pMK1 (stock was 166 ng/µl) were made to test the sensitivity of the (nested) PCR method.

PCR products were run in 0.8% agarosegels, purified with Glassmax (Gibco BRL), and cloned into the pGEM-T vector (Promega Inc.) according to the manufacturer's protocol and using standard techniques (Sambrook *et al.*, 1989). As a DNA size marker, λ-DNA digested with *Eco*RI/*Hind*III/*Bam*HI was used. Plasmid DNA was purified using the High Pure Plasmid Isolation Kit (Roche). Automatic sequencing was performed using an ABI prism 310 genetic analyzer (Perkin Elmer) at the department of Molecular Biology, Wageningen University. Sequence analyses were performed using FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1997) from the UWGCG computer programs (release 10.0). All plasmids were maintained in DH5α *E. coli* cells.

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CHAPTER 3

Pivotal role of the non-*hr* origin of DNA replication in the genesis of defective interfering baculoviruses

The generation of deletion mutants, including defective interfering viruses, upon serial passage of SeMNPV in insect cell culture has been studied. Sequences containing the non-*hr* origin of DNA replication (non-*hr ori*) became hypermolar in intracellular viral (ICV) DNA within 10 passages in Se301 insect cells, concurrent with a dramatic drop in budded virus (BV) and polyhedra production. These predominant non-*hr ori*-containing sequences accumulated in larger concatenated forms and were generated *de novo* as demonstrated by their appearance and accumulation upon infection with a genetically homogenous bacterial clone of SeMNPV (bacmid). Sequences were identified at the junctions of the non-*hr ori* units within the concatemers, which may be potentially involved in recombination events. Deletion of the SeMNPV non-*hr ori* using RecE/RecT-mediated homologous ET-recombination in *E. coli* resulted in a recombinant bacmid with strongly enhanced stability of virus and polyhedra production upon serial passage in insect cells. This suggests that the accumulation of non-*hr oris* upon passage is due to the replication advantage of these sequences. The non-*hr ori* deletion mutant SeMNPV bacmid can be exploited as a stable eukaryotic heterologous protein expression vector in insect cells.

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INTRODUCTION

Baculoviruses are large enveloped, circular dsDNA insect viruses which are widely used as bioinsecticides in agriculture and forestry and can be genetically engineered to improve their effectiveness (Black *et al.,* 1997; Inceoglu *et al.,* 2001). More recently, baculoviruses were shown to have potential as gene delivery vectors for gene therapy (Hofmann *et al.,* 1995; Merrihew *et al.,* 2001; Van Loo *et al.,* 2001) or as vectors for surface display of complex eukaryotic proteins (Grabherr *et al.,* 2001). Yet, their major application to date is as viral vector for the expression of heterologous proteins in insect cells (King and Possee, 1992; O'Reilly *et al.,* 1992). The prototypic and most intensively studied baculovirus *Autographa californica* MNPV (AcMNPV) has been primarily used as expression vector, while other baculoviruses may become exploited as well, especially when appropriate cell lines are available.

A major drawback in the large-scale production of baculoviruses as bio-insecticide or for heterologous protein production is the so-called passage effect. This effect is notable as a significant drop in production by prolonged virus passaging in insect cell culture (reviewed by Krell, 1996) and is a result of the accumulation of defective interfering particles (DIs) (Kool *et al.,* 1991). These DIs are rapidly generated in cell culture (Pijlman *et al.,* 2001) and become predominant after prolonged passaging, meanwhile interfering with the replication of intact helper virus. However, the mechanism of the generation of DIs is still enigmatic and the sequences involved are unknown.

DIs have retained cis-acting elements essential for baculovirus DNA synthesis, such as origins of replication (*ori*) (Krell, 1996). Transient virus-mediated plasmid replication assays demonstrated that baculovirus homologous regions (*hr*s) have a putative *ori* function (Kool *et al.,* 1993a; Kool *et al.,* 1993b; Leisy and Rohrmann, 1993; Pearson *et al.,* 1992), as well as baculovirus early promoter regions not containing *hr* sequences (Wu and Carstens, 1996). In addition, these assays showed that also other non-*hr* regions, with structural similarities to eukaryotic *ori*s, may have an *ori* function (Heldens *et al.,* 1997a; Huang and Levin, 1999; Kool *et al.,* 1994; Pearson *et al.,* 1993). Their *ori* activity *in vivo* was recently demonstrated, but was unfortunately not compared to the *ori* activity of *hr*s (Habib and Hasnain, 2000). Strikingly, AcMNPV DIs were enriched in such a non-*hr ori* (Lee and Krell, 1992; Lee and Krell, 1994). This suggests a prominent role of baculovirus non-*hr ori*s in the generation of DIs.

For large-scale or continuous production of heterologous eukaryotic proteins using the baculovirus expression system in insect cell bioreactors, the passage effect is a major obstacle. For prevention of the negative consequences of the passage effect, a genetically stable viral genotype is highly demanded. This may be achieved by selection for viruses with enhanced stability or higher polyhedra (or recombinant protein) production (Slavicek *et al.*, 1996) or, alternatively, by site-directed mutagenesis of viral sequences putatively involved in the generation and/or maintenance of DIs. Therefore, we chose the non-*hr ori* sequence as a target for mutagenesis studies.

Compared to AcMNPV infections in widely used cell lines such as Sf21 and Sf9, the generation and predominance of *Spodoptera exigua* MNPV (SeMNPV) deletion mutants (including DIs) in various *S. exigua* cell lines occurs significantly faster (Dai *et al.*, 2000; Heldens *et al.*, 1996). This virus-cell system thus provides a better model system than AcMNPV to study the passage effect, the mechanism of DI generation and the pivotal role of non-*hr ori* sequences therein.

In this paper we have studied the rapid passage effect during serial passaging of wildtype SeMNPV in the established *S. exigua* cell-line Se301 (Hara *et al.*, 1995). To follow the generation of DI genomes over passage and to study the role of the non-*hr ori* sequences in this process a full-length infectious clone of SeMNPV propagated in *E. coli* (bacmid) was constructed and used in serial passage experiments. This revealed the pivotal role of the non-*hr ori* in the genesis of DIs and led to the generation of a recombinant SeMNPV bacmid with enhanced stability in cultured insect cells.

RESULTS

Serial passage SeMNPV in Se301 insect cells

SeMNPV-US1 was serially-passaged 25 times in the *S. exigua* cell line Se301 using budded virus (BV) from infectious hemolymph, defined as passage 0 (P0) inoculum. A decrease of polyhedra production was observed after less than five passages, indicating a dramatic passage effect. Intracellular viral (ICV) DNA was purified and subjected to *Xba*I (Fig. 3.1A) digestion. A rapid reduction of the major genomic *Xba*I-A fragment was observed (Fig. 3.1A). At the same time, a novel *Xba*I-fragment of about 9 kb became more abundant and was cloned and sequenced. This fragment was already present in the P1 DNA and appeared to consist of the remnants of the *Xba*I-A fragment as a result of a 26.5 kb deletion (from nt 15301 to 41759), according to the complete genome sequence of SeMNPV (IJkel *et al.*, 1999).

The occurrence of mutants with deletions in this particular genomic region is a common phenomenon of SeMNPV infection in cell culture, but these deletions do not compromize BV or protein production (Dai *et al.*, 2000; Heldens *et al.*, 1996). *In vivo*, such deletion mutants also exist and can act as parasitic genotypes (Muñoz *et al.*, 1998).

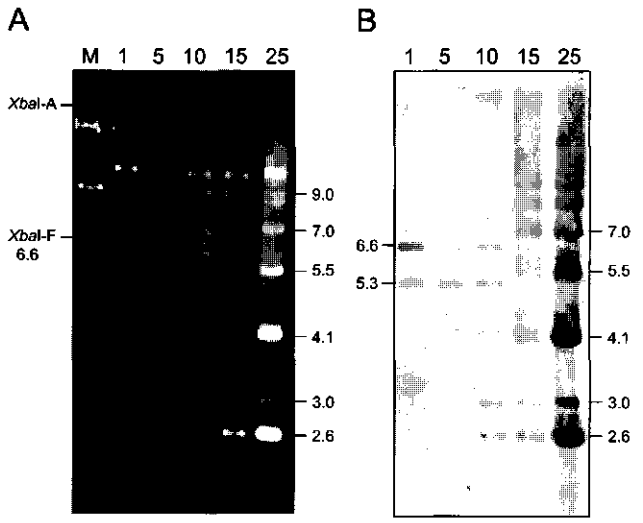

Figure 3.1. Restriction profile of intracellular DNA of wildtype SeMNPV-US1 upon passaging (P1-25) in Se301 insect cells. (A) DNA digested with *Xba*I and run in a 0.6 % agarose gel. Passage numbers are indicated above the lanes and the viral genomic *Xba*I-A and -F fragments on the left. Lane M contains a λ/*Eco*RI/*Bam*HI/*Hin*dIII DNA size marker. Sizes (kb) of the hypermolar novel bands (2.6 to 7.0) and the novel 9 kb fragment are indicated on the right. (B) Southern blot using the SeMNPV non-*hr ori* (nt 83122 – 84048) as a probe. The viral genomic 6.6 kb *Xba*I-F (containing the non-*hr ori*) and an additional hybridizing 5.3 kb band are indicated on the left.

Analysis hypermolar bands

Hypermolar fragments accumulated in Se301 cells from P10 onwards and they were visualized as *Xba*I restriction fragments of 2.6 and 3.0 kb in agarose gels (Fig. 3.1A). From P15 onwards also bands of 4.1, 5.5, 7.0 kb and higher became hypermolar. The abundant 2.6, 3.0, and 4.1 kb *Xba*I bands were cloned and sequenced and it was found that the *Xba*I sites on either side of the cloned inserts corresponded to the SeMNPV *Xba*I restriction site at position 82132, according to the complete sequence of SeMNPV (IJkel *et al.*, 1999). Most interestingly, both the 2.6 and 3.0 kb fragments contained the entire SeMNPV non-*hr* origin of DNA replication (nt 83286 – 83932 (Heldens *et al.*, 1997a)) and a junction of sequences flanking this

non-*hr ori* (Figure 2). The borders and the junction of the 4.1 kb fragment appeared to be identical to that of the 2.6 kb fragment (Fig. 3.2B). The difference in size is a consequence of a duplicated non-*hr ori* present in this 4.1 kb fragment. Noteworthy is the presence of an overlapping stretch of 9 bp at the junction site in the 2.6 and 4.1 kb fragment (Fig. 3.2B), which in the complete SeMNPV genome is present on either side of the non-*hr ori*, leaving 2.6 kb in between.

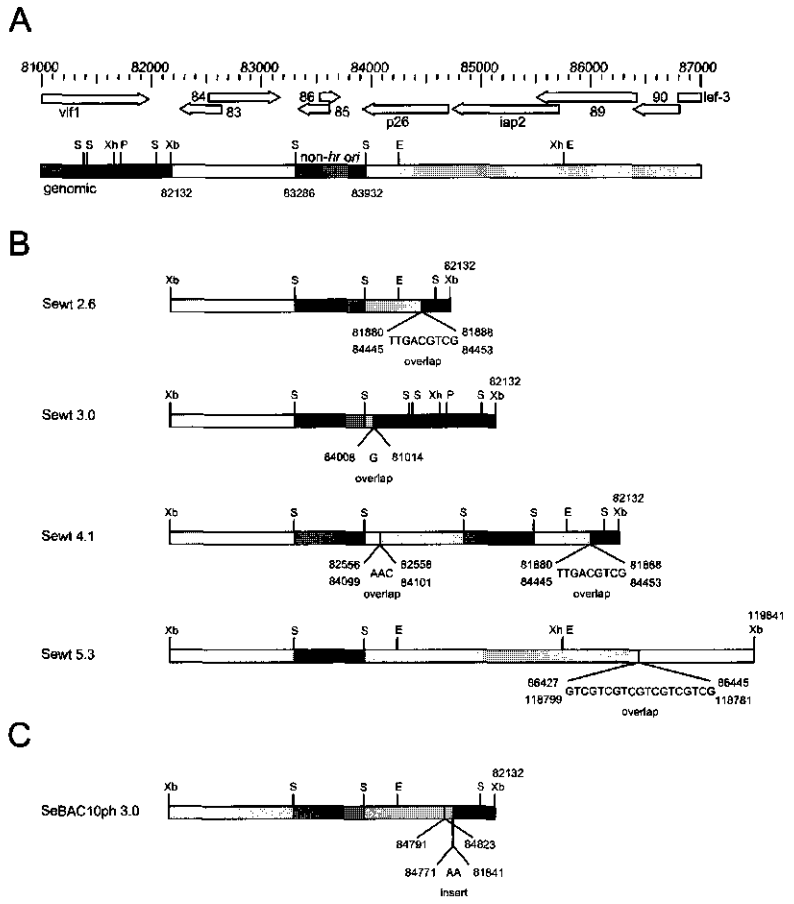

Figure 3.2. Schematic overview of the genetic organization of hypermolar and other non-*hr ori* hybridizing bands compared to the complete SeMNPV genome. (A) Genetic organization of the genomic DNA with nucleotide positions according to the complete SeMNPV genome (16). Block arrows represent the respective ORFs. Grey and light-grey boxes refer to sequences on either side of *Xba*I (Xb) 83132, containing *Ssp*I (S), *Pst*I (P), *Eco*RI (E), and *Xho*I (Xh) sites. The non-*hr ori* is presented as a cross-hatched box between the two *Ssp*I sites (11). (B) Genetic arrangement hypermolar 2.6, 3.0 and 4.1 kb fragments of SeMNPV-US1 (Sewt) and non-hypermolar cohybridizing 5.3 kb fragment (genomic fragment of a SeMNPV deletion mutant) in the Southern blots. Nucleotide positions and sequence overlaps/insertions are indicated at the junction sites. (C) Genetic arrangement hypermolar 3.0 kb fragment of SeBAC10ph, containing two junctions.

Because of the presence of a junction site and the same *Xba*I (position 82132) on either side of the fragments, it was concluded that the hypermolar fragments must exist in the ICV DNA preparation either as DNA minicircles or as tandem repeats in a larger concatenated form. To investigate whether the other hypermolar bands of 5.5, 7.0 and higher also contained the non-*hr ori*, a Southern blot was made with a non-*hr ori* probe. The result (Fig. 3.1B) showed that these fragments hybridized strongly to the probe and therefore it was concluded that a range of molecules of different sizes containing the SeMNPV non-*hr ori* predominated upon serial passage.

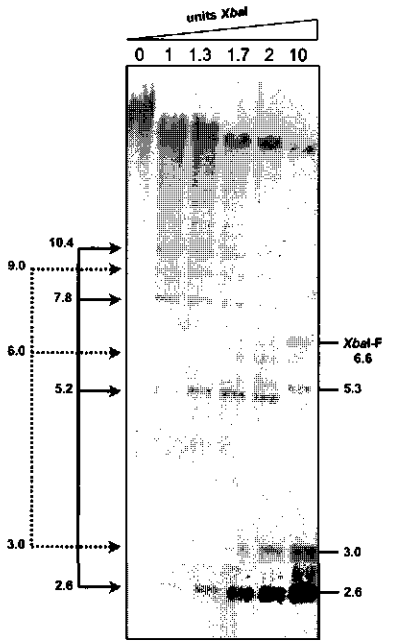

Figure 3.3. Replicative form of the hypermolar 2.6 and 3.0 kb *Xba*I fragments by partial digestion of ICV SeMNPV-US1 DNA of P10, using increasing amounts of *Xba*I. On the right the genomic 6.6 kb *Xba*I-F and the additional 5.3 kb band as well as the hypermolar *Xba*I bands of 2.6 and 3.0 kb are indicated. On the left the multimers of the 2.6 and 3.0 kb *Xba*I fragments are indicated by arrows.

In addition to the non-*hr ori* containing genomic *Xba*I-F fragment of 6.6 kb, an unexpected additional band of 5.3 kb hybridizing to the non-*hr ori* probe (see Figs. 1B and 3) became submolar from P15 onwards. Sequencing revealed that the 5.3 kb fragment consisted of two joined, but distantly located sequences from the SeMNPV genome (Fig. 3.2B). The ends of the fragment corresponded to *Xba*I sites at positions 82132 and 119846, respectively. The junction between the two fragments was formed by an overlapping sequence stretch of 19 bp, containing multiple GTC-repeats, located at positions 86426-86446 and 118807-118780. The presence of this 5.3 kb band in the wildtype SeMNPV DNA was confirmed by Southern hybridization.

Replicative form hypermolar 2.6 and 3.0 kb *Xba*I-fragments

To investigate whether the abundant *Xba*I fragments of 2.6 and 3.0 kb exist as minicircles or as tandem repeats in a larger concatenated form, ICV DNA of P10 (at a stage that only the 2.6 and 3.0 bands were abundant) was subjected to partial digestion with *Xba*I, using increasing amounts of restriction endonuclease during

digestion for 20 min. Hybridization was performed with the same non-*hr ori* probe as described above (Fig. 3.3). The partial *Xba*I digests of P10 viral DNA showed a "step-ladder" of multimers of the 2.6 and 3.0 kb bands. This suggests that the accumulation of the SeMNPV non-*hr ori* occurs via high molecular weight concatemers of tandem repeats of different sizes. Not only is this likely to be the case for the 2.6 and 3.0 kb fragments, but also for the 4.1, 5.5, 7.0 kb and larger fragments from P15 onwards.

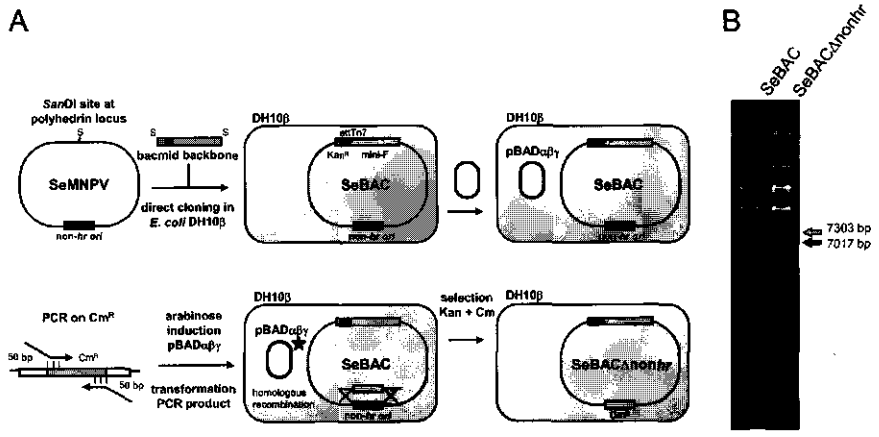

Figure 3.4. A) Construction of SeMNPV bacmid by direct cloning and subsequent deletion of the non-*hr ori* by ET-recombination. B) Restriction profile (*Pst*I) of parental SeMNPV bacmid SeBAC10 and the non-*hr ori* deletion mutant SeBAC10Δnonhr. The genomic non-*hr ori* containing fragment *Pst*I-I (7017 bp) and with Cm^R gene insertion (7303 bp) are indicated.

Serial passage SeMNPV bacmids in Se301 insect cells

A genetically homogeneous SeMNPV bacmid (SeBAC10) and a derived non-*hr ori* deletion mutant (SeBAC10Δnonhr) were constructed (Fig. 3.4) to determine whether non-*hr ori* concatemers are generated *de novo* in cell culture or pre-exist and become selectively amplified, and whether virus stability might be enhanced by deletion of this non-*hr ori*.

Prior to serially passaging the bacmid-derived BVs in cell culture, the polyhedrin gene was reintroduced. After transfection of Se301 cells, the BV containing supernatant was defined as the passage 1 (P1) virus stock and was used to initiate serial undiluted passage. ICV DNA was purified and digested with *Xba*I. Similar to SeMNPV-US1 (this study, Dai *et al.*, 2000; Heldens *et al.*, 1996), deletions in *Xba*I-A occurred for both bacmids SeBAC10ph and SeBAC10phΔnonhr (Fig 5).

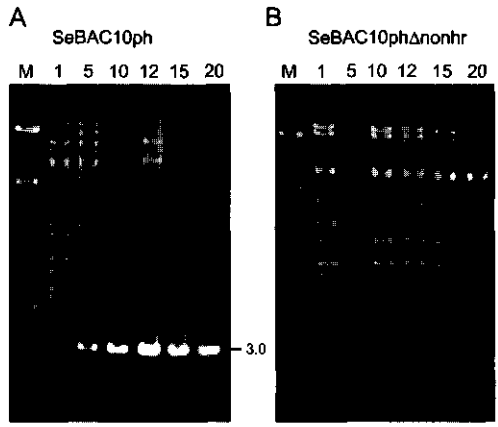

Figure 3.5. Restriction profile (*XbaI*) of ICV DNA of serially passaged SeMNPV bacmids SeBAC10ph (A) and SeBAC10phΔnonhr (B) in Se301 insect cells. Passage numbers (top) and the hypermolar band of 3.0 kb (SeBAC10ph) are indicated.

The deletion in SeBAC10phΔnonhr was mapped as a junction overlap of 3 nt (AAC) from 20162(4) to 36396(8), spanning ORF17-35. From P6 onwards a small hypermolar *XbaI* fragment of 3.0 kb was visible in DNA preparations of SeBAC10ph (Fig. 3.5A). This fragment was cloned and sequenced and appeared to contain the non-*hr* origin of DNA replication and a junction sequence (Fig. 3.2C) also observed with SeMNPV-US1 wildtype (Fig. 3.2B). In contrast, the analysis of ICV DNA from SeBAC10phΔnonhr-infected cells did not reveal any accumulation of hypermolar fragments (Fig. 3.5B).

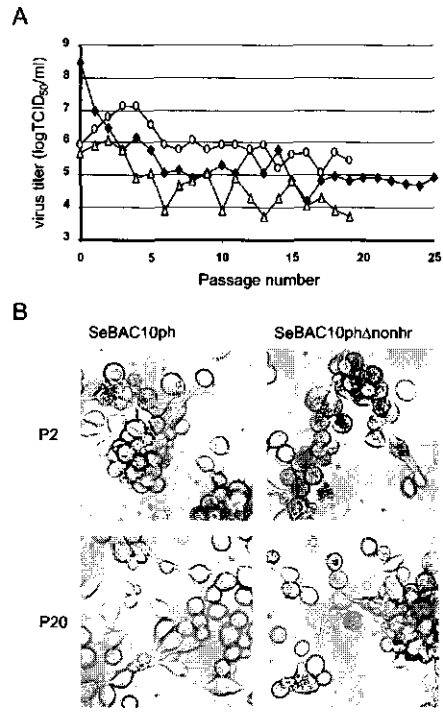

Figure 3.6. A) Titers of serially passaged BV of SeMNPV-US1 (♦), SeBAC10ph (Δ), and SeBAC10phΔnonhr (o). B) Pictures of infected Se301 insect cells with SeBAC10ph and SeBAC10phΔnonhr at P2 and P20, respectively.

SeBAC10phΔnonhr BV titers remained at higher levels throughout the entire period of serial passaging than those of SeBAC10ph and SeMNPV-US1 wildtype (Fig. 3.6A). Polyhedra production of SeBAC10phΔnonhr levels remained constant for at least 20 passages, in contrast to SeBAC10ph (Fig. 3.6B). These results demonstrated that absence of the non-*hr ori* strongly increased the stability of the SeMNPV genome in Se301 insect cells.

DISCUSSION

The rapid accumulation of DI particles with DNA containing hypermolar non-*hr oris* appears to be an artifact of serial passage of SeMNPV in Se301 cells and the major cause of the decrease of virus and polyhedra production. Ultimately, these hypermolar molecules form the majority of the viral DNA. By partial digestion we showed that these molecules exist as high molecular mass concatemers, in agreement with a supposed rolling circle mechanism for baculovirus DNA replication (Kool *et al.*, 1995; Leisy and Rohrmann, 1993; Oppenheimer and Volkmann, 1997; Wu *et al.*, 1999). Their rapid multiplication, together with previous data from *in vitro* replication assays (Heldens *et al.*, 1997a) provide support for the view that the SeMNPV non-*hr ori* might be a genuine origin of DNA replication.

In order to elucidate whether these non-*hr ori* concatemers were newly formed in Se301 insect cells or, alternatively, only accumulated from a genetically heterogeneous wildtype isolate, a full-length infectious clone (bacmid) of SeMNPV was constructed. Such a bacmid could be stably maintained as a single copy bacterial artificial chromosome (BAC) in *E. coli* DH10β and used as a starting material in passage experiments in insect cells (Luckow *et al.*, 1993; Pijlman *et al.*, 2001). Transfection of Se301 insect cells with SeBAC10ph (with a re-introduced intact polyhedrin gene) showed normal polyhedra production, but serial passage again resulted in a decrease of viral titers and polyhedra production and the rapid accumulation of non-*hr ori* containing molecules.

Since the non-*hr ori* could be removed from the viral DNA without affecting virus replication, we must conclude that this non-*hr ori* is not essential. The two minor open reading frames (ORFs) 85 and 86 within the non-*hr* region are also non-essential. These ORFs do not have known baculovirus or other homologues and it is unknown whether they are transcriptionally active. Furthermore, deletion of this non-*hr ori* strongly enhanced the genomic stability in cell culture. Transfection of Se301 cells with the non-*hr ori* deletion mutant bacmid SeBAC10phΔnonhr gave normal polyhedra production and high viral titers (Fig. 3.6), which were maintained up to at least 20 passages. Together with an unchanged restriction profile from P1 to P20 these results indicate that this recombinant has an enhanced stability in cell culture as compared to both wildtype SeMNPV and the parental bacmid SeBAC10ph. The increased overall stability is probably not due to an increase in intrinsic stability of the SeMNPV genome by itself, but rather a consequence of the lack of a cis-acting

element (non-*hr ori*) that has a strong replicative advantage during baculovirus DNA replication.

Our findings with SeMNPV are in line with the results from Lee and Krell (1992, 1994), who demonstrated that AcMNPV DIs at P81 largely consisted of reiterations of about 2.8 kb of the standard genome, which was later shown to contain an active non-*hr* origin of DNA replication (Kool *et al.*, 1994; Habib and Hasnain, 2000). In addition, previous work on AcMNPV showed that DIs could also be enriched in *hr*s (Kool *et al.*, 1993a). These observations suggest that reiteration and predominance of baculovirus *hr*s but particularly non-*hr oris*, which are complex structures comprising multiple direct and inverted repeats, is a more common phenomenon upon multiple passage and may contribute to a rapid passage effect. Single copies of non-*hr oris*, which resemble eukaryotic *ori*s based on structural similarities (DePamphilis, 1993), have been identified in many other baculovirus genomes by transient replication assays (OpMNPV (Pearson *et al.*, 1993), SpliMNPV (Huang and Levin, 1999; 2001)) or based on sequence and structural similarity only (BmMNPV (Kool *et al.*, 1994), BusuNPV (Hu *et al.*, 1998), CpGV (Luque *et al.*, 2001)). The conservation of non-*hr oris* in baculovirus genomes implies an important (biological) role in virus replication and may be related to viral latency and persistence in insect populations. The resemblance to eukaryotic *oris* suggests that baculoviruses may have obtained these sequences from the host genomes to be able to replicate in the insect without the requirement for viral encoded replication factors.

At the junctions of non-*hr ori* concatenated molecules and junctions of major genomic deletions, sequence overlaps of 1 up to 19 bp were found, potentially involved in the causative recombination mechanism. The sequence of the additional 5.3 kb fragment in the Southernblots showed a 19 bp overlap at the deletion junction, consisting of multiple GTC repeats (Fig. 3.2B). These GTC repeats of up to 27 bp were found scattered throughout the SeMNPV genome on both strands and were more frequent than expected on a random basis. For the 2.6 kb *Xba*I-fragment concatemers, a 9 bp TTGACGTCG overlap from flanking sequences was found at the junction site (Fig. 3.2B). Also this repeat was found more frequent (12 times) in the genome than expected on a random basis (<1). The 9 bp overlap implies that the concatemers of the 2.6 kb fragment were generated during serial passage by looping and subsequent excision (homologous recombination) of non-*hr ori* containing genomic DNA, followed by continued replication and consequent concatenation of this intervening region. The same 9 bp overlap was found at the junction of the 4.1 kb fragment, which contains a duplicated non-*hr ori* (Fig. 3.2). This suggests that the 4.1 kb fragment was generated from the 2.6 kb molecule by another recombination event

and became rapidly hypermolar because of the presence of two copies of the non-*hr ori*. This hypothesis is consolidated by the appearance of the 4.1 kb fragment in later passages than the 2.6 kb fragment (Fig. 3.1A). For the other junctions, smaller overlaps (1 or 3 bp) or even insertions were found, which was also demonstrated for AcMNPV DIs in an earlier study (Pijlman *et al.*, 2001), suggesting that the same recombination mechanisms are involved.

The strategy of deletion of sequences that have a replicative advantage, accumulate upon serial passage, and interfere with virus and protein production (e.g. non-*hr oris*), may now be applied for other baculoviruses as well and will contribute to the solution of problems associated with large-scale applications for protein production in insect cells using baculovirus expression vectors. In further studies we plan to map the sequences in the SeMNPV non-*hr ori* involved in the generation of DIs in more detail by reintroducing mutant SeMNPV non-*hr oris* in SeMNPV bacmids. In addition, we want to investigate whether the deletion of the non-*hr ori* affects baculovirus persistence *in vivo*.

MATERIALS AND METHODS

Cells, insects and virus

The *Spodoptera exigua* cell line Se301 (Hara *et al.*, 1993; 1995) was donated by Dr. T. Kawarabata (Institute of Biological Control, Kyushu University, Japan) and was propagated at 27°C in Grace's supplemented medium (Gibco BRL) containing 10% foetal calf serum (FCS; Gibco BRL). Fourth instar *S. exigua* larvae were infected by contamination of artificial diet with $4x10^5$ SeMNPV-US1 (Gelernter and Federici, 1986b) polyhedra per larva (Smits and Vlak, 1988). Haemolymph was collected as previously described (IJkel *et al.*, 2000) and was defined as the passage zero (P0) budded virus (BV) inoculum to initiate serial passage in cultured Se301 cells. Serial undiluted passaging was carried out as previously described (Pijlman *et al.*, 2001). Infectious budded virus titers were determined using the endpoint dilution assay (Vlak, 1979).

DNA isolation, Southern hybridization, colony lift, molecular cloning and sequencing

Intracellular viral DNA and budded virus DNA was isolated as previously described (Summers and Smith, 1987). Digested viral DNA was run overnight in ethidium bromide stained 0.6% agarose gels, and Southern blotting was performed by standard capillary upward blotting (Sambrook *et al.*, 1989) using Hybond-N (Amersham Pharmacia) filters. As a DNA size marker, λ-DNA digested with *Eco*RI/*Hin*dIII/*Bam*HI was used. Randomly primed DNA probes for Southern hybridization were made using the DIG non-radioactive nucleic acid

labeling and detection system (Roche). PCR products (927 bp) of the SeMNPV non-*hr ori* were made with reverse primer DZ127 5'-CATCGATGCGTACGTGACTTTC-3' (nt 84027 – 84048 (IJkel *et al.*, 1999)) and forward primer DZ128 5'-CCTTGCGTTCCTTTGGTG-3' (nt 83122 – 83139), purified using the High pure PCR purification kit (Roche), and DIG-labeled overnight. Hybridization and colorimetric detection with NBT-BCIP (Gibco BRL) were performed according to the manufacturer's recommendations. Hypermolar viral *Xba*I bands were cut from the gel, purified with Glassmax (Gibco BRL), and cloned into pUC19 by electrotransformation of *E. coli* DH5α using standard methods (Sambrook *et al.*, 1989). A colony lift assay (Sambrook *et al.*, 1989) was used to isolate the cloned submolar 5.3 kb fragment using the same probe as described above. Automatic sequencing was performed using an ABI prism 310 genetic analyzer (Perkin Elmer) at the laboratory of Molecular Biology, Wageningen University. Sequence analyses were performed using BLAST (Altschul *et al.*, 1997) from the UWGCG computer programs (release 10.0).

Construction of bacmid cloning vector

The bacmid vector for direct cloning of SeMNPV was constructed by PCR using the Expand long template PCR system (Roche). Custom made primers (Gibco BRL) were designed using DNAstar Primerselect and were based on the sequence of AcMNPV transfer plasmid pVL1393 (Luckow and Summers, 1988), which was the backbone of the transfer vector pMON14272 used to construct the AcMNPV bacmid bMON14272 (Luckow *et al.*, 1993). Primers DZ113 (5'-CCTT*CCTGAGG*TACCTTCTAGAATTCCGGAG-3') and DZ114 (5'-CCTTCC*TCAGG*CCGGGTCCCAGGAAAGGATC-3') were oppositely directed to sequences flanking the *Bgl*II cloning site of pVL1393, and contained additional *Bsu*36I restriction sites (italics) at their 5' end for circularization. DZ114 also contained an internal *San*DI restriction site (underlined) for direct cloning into *San*DI-linearized SeMNPV-US1 DNA. The template for PCR was purified AcMNPV bacmid bMON14272 (Luckow *et al.*, 1993) DNA from the Bac-to-Bac Kit (Gibco BRL). The resulting 8.5 kb PCR product was cloned into the 3.5 kb pCR-XL-TOPO vector (Invitrogen), digested with *Bsu*36I, self-ligated, and cloned into electrocompetent DH10β *E. coli* cells. The obtained bacmid cloning vector was designated BAC-Bsu36I and its identity was verified by restriction analysis.

Direct cloning SeMNPV-US1 as bacmid

SeMNPV-US1 DNA for direct cloning was purified using alkaline treatment of polyhedra and by previously described methods (O'Reilly *et al.*, 1992). Two µg of viral SeMNPV-US1 DNA was linearized at the polyhedrin locus by digestion with 10U of *San*DI (Stratagene) for 16h at 37°C. The restriction enzyme was heat-inactivated for 15 min. at 65°C. One µg bacmid cloning vector BAC-Bsu36I was digested with 10U of *San*DI in a total volume of 35µl for 3 h at 37°C. The 8.5 kb vector was dephosphorylated using 1U HK™ Thermolabile Phosphatase (Epicentre). The enzymes were heat-inactivated for 15 min. at 65°C prior to gel-purification of the linearized cloning vector DNA with Glassmax (Gibco BRL). Ligation was performed for

16h at 15°C with approximately 500ng linearized SeMNPV DNA and 25 ng linearized vector DNA in a total volume of 20 µl using 6U T4 DNA ligase (Promega). Electrocompetent *E. coli* DH10β cells (Gibco BRL) were transformed with 2 µl ligation mix at 1.8 KVolt using a Biorad Gene Pulser. The transformed cells were recovered in SOC medium for 45min at 37°C and spread on agar plates containing kanamycin. A SeMNPV bacmid with the correct restriction profile was selected from 111 putative SeMNPV bacmid clones, and was designated SeBAC10 (Fig. 3.4A).

Deletion SeMNPV non-hr ori by ET-recombination in E. coli

For deletion mutagenesis of the active essential domain of the non-*hr ori* of SeMNPV-US1 bacmid SeBAC10, 68-70 bp long primers were designed with 50 bp 5'-ends flanking the deletion target region on the SeMNPV genome. Forward primer DZ153 was 5' CATTTACTCGAAAACACTGTACACTTCGTCAAAATAAATGACGCAATATTTTTAAGGGCAC CAATAACTG 3', with a viral flanking sequence from nt 83237 to 83286 according to the SeMNPV complete genome sequence (IJkel *et al.*, 1999). Reverse primer DZ154 was 5' ATTTCAAAAATTAGAATCAAAACCCAATTTGCCGGCAACGTTTTAATATTTTCCTGTGCGA CGGTTAC 3', with a viral flanking sequence from nt position 83981 to 83932. The locus to be deleted, which is the essential domain of the SeMNPV non-*hr ori*, is defined by two *Ssp*I sites (Heldens *et al.*, 1997a). These *Ssp*I restriction sites are included in the primers (underlined). The 3'ends of the primers anneal to the chloramphenicol gene of pBeloBAC11 (Shizuya *et al.*, 1992; Wang *et al.*, 1997) from nt position 735 until 1671. PCR on pBeloBAC11 was performed using the Expand long template PCR system (Roche) according to the manufacturer's protocol, giving a product of 1036 bp. The PCR product was purified using the High pure PCR purification kit (Roche), cut with *Dpn*I to eliminate residual pBeloBAC11 template, phenol/chloroform extracted, and ethanol precipitated. Approximately 0.5 µg PCR product was used for transformation of electrocompetent *E. coli* DH10β containing both SeBAC10 and homologous recombination helper plasmid pBAD-αβγ.

DH10β containing SeBAC10 were heat-shock transformed with pBAD-αβγ (Muyrers *et al.*, 1999) and subsequently made electrocompetent according to Muyrers *et al.* (1999). Briefly, 70 ml of LB medium was inoculated with 0.7 ml of an overnight culture. At an OD600 of 0.1-0.15, ET-protein expression from pBAD-αβγ was induced by the addition of 0.7 ml 10% L-arabinose. The cells were harvested at an OD600 of 0.3-0.4 and made electrocompetent by 3 subsequent washes with ice-cold 10% glycerol. The cells were transformed with the purified PCR product in 2mm electroporation cuvets (Eurogentec) using a Biorad Gene Pulser (2.3 kV, 25 µF, 200 Ω). The cells were resuspended in 1 ml LB medium and incubated for 1h at 37°C, and subsequently spread on agar plates containing kanamycin and chloramphenicol (Fig. 3.4A). The altered genotype of the recombinant bacmid, designated SeBAC10Δnonhr, was confirmed by *Pst*I digestion and PCR. The genomic *Pst*I-I fragment of SeBAC10 (7017 bp) was anticipated to be 286 bp bigger in SeBAC10Δnonhr, giving a fragment of 7303 bp (Fig. 3.4B). PCR was performed with forward primer DZ127 and reverse primer DZ128 as

previously described. The PCR product of 1213 bp was cloned into pGEM-Teasy (Promega) and completely sequenced, revealing that recombination had occurred precisely at the anticipated locus via the 50 flanking nucleotides.

Reconstitution SeMNPV polyhedrin gene by pFastBAC1 donor plasmid

To reconstitute the polyhedrin gene in SeMNPV bacmids SeBAC10 and SeBAC10Δnonhr, a donor plasmid pFB1Sepol was constructed. The pFastBac1 vector (Gibco BRL) was digested with *Sna*BI and *Hin*dIII to delete the AcMNPV polyhedrin promoter and the MCS. The SeMNPV polyhedrin gene with its own promoter and the first putative transcription termination signal (Van Strien *et al.*, 1992) was amplified by the Expand long template PCR system (Roche) using forward primer DZ138 5'- CC*CCCGGG*TATATACTAGACGCGATTAC-3' (nt 135475-135494) and reverse primer DZ139 5'-CC*AAGCTT*TGTAATACTTACC-TTTTGTG-3' (nt 757-776), containing *Sma*I and *Hin*dIII restriction sites (italics), respectively. The resulting 930 bp fragment was cloned into a pGEM-Teasy vector (Promega), sequenced and subsequently cloned as a *Sma*I/*Hin*dIII fragment into the pFastBac1 vector to generate pFB1Sepol. The protocol from the Bac-to-Bac manual (Gibco BRL) was followed to transpose the SeMNPV polyhedrin gene from pFB1Sepol into the *att*Tn7 transposon integration site of SeMNPV bacmids SeBAC10 and SeBAC10Δnonhr to generate SeBAC10ph and SeBAC10phΔnonhr, respectively.

Transfection of SeMNPV bacmids

Se301 cells were seeded in a 6-wells tissue culture plate (Nunc) at a confluency of 25% ($5*10^5$ cells). Transfection was performed with approximately 1 μg SeBAC10ph or SeBAC10phΔnonhr DNA using 10 μl Cellfectin (Gibco BRL). As a positive control, 1 μg SeMNPV-US1 DNA was transfected as well. After 5 and 7 days, polyhedra were formed by the cells transfected with SeMNPV-US1 and the bacmids, respectively. Budded virus containing supernatant (defined as P1) and infected cells were harvested 14 days post transfection (90% polyhedra containing cells).

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CHAPTER 4

Cell line-specific accumulation of the baculovirus non-*hr* origin of DNA replication in infected insect cells

Successive viral passage of *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) in the *S. exigua* cell line Se301 leads to the rapid accumulation of the non-*hr* origin of DNA replication (*ori*) as large concatemers. Passage of SeMNPV in two other *S. exigua* cell lines, SeUCR1 and SeIZD2109, did not show the accumulation of such concatemers. When introduced into SeUCR1 and SeIZD2109 cells, the non-*hr ori* concatemers generated in Se301 cells were maintained but did not increase. This suggests that the non-*hr ori* confers a strong selective advantage in Se301 cells, but not or to a lesser extent in the other cell lines. The cell line-specific accumulation of non-*hr ori* concatemers might be due to a higher intrinsic recombination frequency in Se301 cells and may reflect tissue related differences involving some host cell factor(s). Since non-*hr ori* concatemers in Se301 cells were more abundant in intracellular than in extracellular viral DNA preparations, episomal replication and the requirement of a minimal DNA size for packaging into nucleocapsids is hypothesized.

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INTRODUCTION

Spodoptera exigua multicapsid nucleopolyhedrovirus (SeMNPV) belongs to the group II NPVs within the family Baculoviridae. Baculoviruses are large, circular double stranded DNA viruses pathogenic for insects and widely used as bioinsecticides (Inceoglu *et al.*, 2001). Another major application of baculoviruses is their use as vectors for the expression of heterologous proteins in cultured insect and mammalian cells (Kost and Condreay, 1999). More recently, these viruses are exploited as gene-delivery vectors for gene therapy (Loser *et al.*, 2002) and as expression vectors for the surface display of complex proteins (Grabherr *et al.*, 2001).

The development of large-scale insect cell bioreactor systems to produce baculovirus insecticides or heterologous proteins using baculovirus expression vectors is hampered by the passage effect, which causes a significant drop in product yield upon prolonged production (reviewed by Krell, 1996). The passage effect is a result of the accumulation of defective interfering viruses (DIs), which are rapidly generated in cell culture (Pijlman *et al.*, 2001) and which become predominant after prolonged passaging. These DIs interfere with the replication of intact virus, which provides the helper functions in DI replication (Kool *et al.*, 1991; Wickham *et al.*, 1991). DIs are often enriched in cis-acting elements required for viral DNA replication, such as origins of DNA replication (*ori*s), as a result of concatenation (Lee and Krell, 1992; Pijlman *et al.*, 2002). Burand and Summers (1982) were the first to show the accumulation and selective replication advantage of AcMNPV with repeated DNA and suggested that the enriched fragments may act as origins of DNA replication.

Ori activity in baculoviruses is associated with the homologous regions (*hr*s) (Lu *et al.*, 1997), which can also act as transcriptional enhancers (Friesen, 1997). An *ori* not located in the *hr*s and therefore designated non-*hr ori* (Kool *et al.*, 1994), appeared to have a very strong replication advantage in AcMNPV (Lee and Krell, 1992; 1994) and in SeMNPV (Pijlman *et al.*, 2002) infections in cell culture. Non-*hr ori*s are identified in many baculoviruses (Kool *et al.*, 1994; Heldens *et al.*, 1997a; Pearson *et al.*, 1993; Huang and Levin, 1999; Jehle, 2002), and have structural features such as direct/inverted repeats, palindromes, and AT-rich regions in common rather than nucleotide sequences. Besides the accumulation in DIs in cell culture, non-*hr ori*s may also be duplicated *in vivo* (Jehle, 2002), suggesting that they

are utilized during *in vivo* baculovirus DNA replication and contribute to the natural heterogeneity of baculoviruses.

Successive viral passage of SeMNPV in the *S. exigua* cell line Se301 (Hara *et al.*, 1995), led to the rapid intracellular accumulation of the non-*hr ori* as large concatemers. Deletion mutagenesis indicated that the non-*hr ori* was not essential for virus reproduction in cell culture or *in vivo* (Pijlman *et al.*, 2002). In this study, we have investigated whether the generation of these non-*hr ori* concatemers is cell line-specific, by passaging SeMNPV in three different *S. exigua* cell lines.

RESULTS AND DISCUSSION

Serial passage of SeMNPV in Se301, SeUCR1 and SeIZD2109 cells

SeMNPV was serially passaged 25 times in Se301 and SeUCR1 cells and 5 times in SeIZD2109 cells, and viral intracellular DNA was purified. Restriction enzyme analysis of purified viral DNA with *Xba*I showed that *Xba*I-A rapidly disappeared in all cells and that cis-acting elements containing the SeMNPV non-*hr* origin of DNA replication (*ori*) accumulated in Se301 cells as 2.6, 3.0, 4.1, 5.5, and 7.0 kb fragments (Fig. 4.1A, left panel. For comparison taken from Pijlman *et al.*, 2002). These fragments, which occurred in a concatenated form in the viral DNA, did not occur in SeUCR1 and SeIZD2109 cells (Fig 4.1A). The replicative form of the hypermolar (concatenated) non-*hr oris* is as tandem repeats on one physical DNA molecule, as was shown by partial digestion of viral DNA by Pijlman *et al.* (2002).

Hybridizations with a non-*hr ori* probe (not shown) indicated that the fragments generated in Se301 did not appear in SeUCR1 or SeIZD2109, which indicates that they did not pre-exist and thus were *de novo* generated in Se301 insect cells. In contrast to Se301 and SeIZD2109 cells, passaging in SeUCR1 cells resulted in the generation of a stable mutant from passage 5 (P5) to P15, with no hyper- or submolar bands present in the *Xba*I restriction profile. However, the restriction profile changed after P15, indicating that additional deletion mutants and/or DIs were formed. In Se301 and SeIZD2109 cells, a submolar 9 kb band was observed, which appeared to be a truncated form of the hypervariable *Xba*I-A fragment (A*). This 26.5 kb deletion from nucleotides 15301 to 41759 (11.3 to 30.8 m.u.) comprises ORFs 10 to 41, but leaves *hr*1 and *hr*2 intact (Fig. 4.1B). In Se301, the parental *Xba*I-A fragment remains present at a submolar level till P25. In SeUCR1 cells, a major 18 kb genomic deletion was mapped within the hypervariable *Xba*I-A fragment using

additional *Pst*I and *Eco*RI digests (not shown). This deletion from 14.1 to 28.8 m.u. is located in between *hr*1 and *hr*2 (Fig. 4.1B) and comprises at least ORFs 16 to 36 (IJkel *et al.*, 1999). This truncated *Xba*I-A fragment (A**) of 17.5 kb comigrates with the genomic *Xba*I-B fragment.

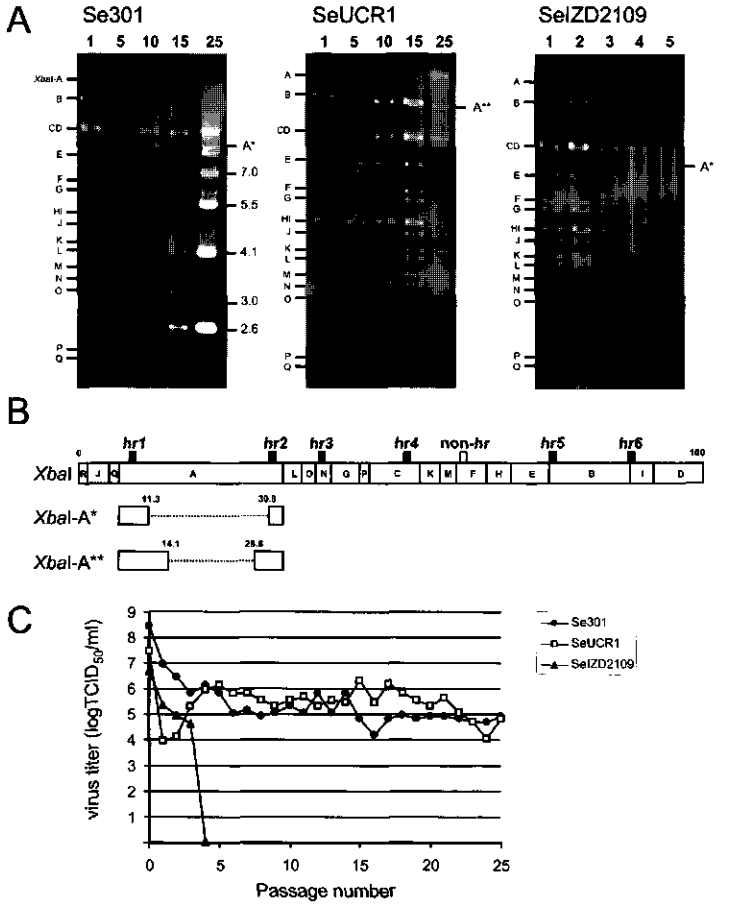

Figure 4.1. Serial passage of wildtype SeMNPV-US1 in *S. exigua* cell lines Se301 (for comparison taken from Pijlman *et al.*, 2002), SeUCR1, and SelZD2109. (A) Restriction profile (*Xba*I) of intracellular viral DNA (3 dpi) from the respective cell lines. Passage numbers are indicated above the lanes. Letters of genomic SeMNPV *Xba*I restriction fragments according to Heldens *et al.* (1996) are indicated on the left. Truncated forms of the *Xba*I-A fragment are indicated with an asterix (A* for the 9 kb band and A** for the 17.5 kb band). (B) Physical map of SeMNPV digested with *Xba*I. The positions of the homologous regions (*hr*s) and the non-*hr ori* are indicated at the top. The truncated *Xba*I-A* and –A** are indicated with the respective deletions in map units. (C) Budded virus titers of serially passaged SeMNPV-US1 in the three different cell lines (titers in Se301 for comparison taken from Pijlman *et al.*, 2002). $TCID_{50}$, 50% tissue culture infective dose.

These major genomic deletions occur in the same genomic region (*Xba*I-A) as the deletions reported in Heldens *et al.* (1996), Dai *et al.* (2000), Muñoz *et al.* (1998). This indicates that in SeMNPV there is a cluster of contiguous ORFs between *hr*1 and *hr*2 that is non-essential for virus replication in cell culture (ORF 15 to 35: Pijlman *et al.*, 2003), although an intact helper virus may still be present. In most cases these deletion mutants were non-infectious to insects upon feeding of polyhedra, indicating that some of the gene products in this cluster are required for virulence *in vivo* (Heldens *et al.*, 1996; Muñoz *et al.*, 1998), making the presence of an intact helper unlikely. Recently, we isolated a bacmid-derived SeMNPV mutant with a deletion of ORFs 15 to 35, which replicated normally in cell culture, but appeared non-infectious *in vivo* (Pijlman *et al.*, 2002). Subsequent studies revealed the essential role of ORF 35 in oral infectivity (Pijlman *et al.*, 2003).

When Se301 cells were infected with SeMNPV, budded virus (BV) titers gradually declined to a low level (Fig. 4.1C. For comparison taken from Pijlman *et al.*, 2002). Virus infectivity in SeUCR1 cells was strongly reduced after the first passage, but was restored in the fourth passage, to reach a stable level in subsequent passages. In SeIZD2109 cells, virus infectivity was rapidly lost within 5 passages, despite attempts to let the infection process develop further. Southern blots with a non-*hr ori* probe (not shown) demonstrated that the non-*hr ori* fragment (*Xba*I-F) also disappears with continued passage, suggesting that SeIZD2109 cells might be less permissive for SeMNPV replication than Se301 and SeUCR1. Duplicate experiments (not shown) indicated that the initial drop in virus titer in SeUCR1 cells and the rapid loss of infectivity in SeIZD2109 cells were reproducible. This suggests a cell-specific response to SeMNPV infection upon passage.

The accumulation of non-*hr ori* concatemers in Se301 cells, but not in SeUCR1 and SeIZD2109 cells, suggests that this is due to differences between cell lines. These differences may act on the level of recombination, which is required to generate the concatemers (or their precursors). Besides host factors, it is possible that virus-encoded recombination factors, such as the genes that promote homologous recombination (Crouch *et al.*, 2002) or very late expression factor 1 (*vlf-1*), are expressed at differential levels in different cell lines and in this way mediate the cell-specific formation of DI molecules. VLF-1 is an integrase involved in very late gene expression (Yang and Miller, 1999) and the excision of unit length monomers from high molecular mass branched concatemers, which are formed during viral DNA replication (Mikhailov and Rohrmann, 2002). Alternatively, there may be a cell line-specific maintenance of such non-*hr ori* concatemers.

Maintenance of Se301-derived non-*hr ori* concatemers in SeUCR1 and SeIZD2109 cells

To test whether non-*hr ori* concatemers from Se301 cells could replicate and be maintained in SeUCR1 and SeIZD2109 cells, the cell lines were inoculated with a virus inoculum from Se301 P19. Subsequently, the virus was serially passaged 5 times in the respective cell lines, and intracellular viral (ICV) DNA was subjected to digestion with *Xba*I and Southern hybridized with a non-*hr ori* probe (Fig 4.2). This showed that the major 2.6, 3.0, 4.1, 5.5, and 7.0 kb bands, indicative of the non-*hr ori* units within the larger concatemers, were still present after 5 successive passages in SeUCR1 and SeIZD2109 cells, albeit a little less abundant in SeIZD2109. This suggests that the replicative advantage of non-*hr ori* concatemers in SeUCR1 and SeIZD2109 cells is much less, if at all, than in Se301, where they predominate in further passages. The minor bands on the blots possibly represent intermediates or subdominant forms of non-*hr ori* concatemers.

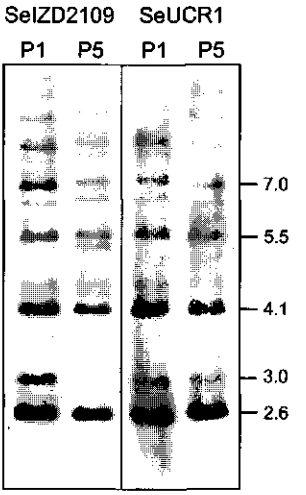

Figure 4.2. Maintenance of concatenated viral DNA containing the SeMNPV non-*hr* origin of DNA replication (non-*hr ori*). SeMNPV-US1 budded virus from Se301 of passage 19 (P19) was used to inoculate SeIZD2109 and SeUCR1 cells. Intracellular viral DNA of subsequent passages 1 and 5 (P1 and P5) was digested with *Xba*I and subjected to Southern blot hybridization with a non-*hr ori* probe. Indicated on the right are the sizes (in kilobases) of the bands, which are indicative for the presence of the non-*hr ori* units within the concatemers.

Differential replication levels of distinct baculovirus genomes in a cell line-specific manner have also been observed in other systems. For example, it was shown that AcMNPV *iap-1* deletion mutants had a strong but unexplainable replication advantage over the wildtype AcMNPV in Tn368 cells, but not in Sf21 cells (McLachlin *et al.*, 2001). Cell-specific responses against virus infection may be related to apoptosis, although this phenomenon was not observed in our experiments. For the cell line-specific generation of non-*hr ori* concatemers observed in our experiments with SeMNPV, an explanation may be found in a higher recombination frequency in Se301, as compared to SeUCR1 and SeIZD2109 cells, which might correlate to tissue-specific

differences in the insect. Unfortunately, the tissue origin of Se301 and SeUCR1 cells is unknown. Se301 cells are adherent spherical cells, derived from the parent cell line Se3FH, which has spindle-shaped and spheroid cells (Hara *et al.*, 1995) and originates from minced neonate larvae of *S. exigua* (Hara *et al.*, 1993). The SeUCR1 cell-line contains both spindle-shaped and epithelial-like cells and was also derived from minced *S. exigua* neonate larvae. The spindle-shaped cells were suggested to originate from muscle tissue, because of rhythmic contraction in early passages (Gelernter and Federici, 1986a). The SeIZD2109 cell-line was established from *S. exigua* haemocytes (Dr. B Möckel and Dr. H. G. Miltenburger, personal information). It would have been of interest to include additional cell lines with different (growth) characteristics, but due to the monospecificity of SeMNPV to its host *S. exigua*, other available lepidopteran cell lines could not be used.

The effect of the multiplicity of infection (MOI) can also affect the specific accumulation of DIs containing the non-*hr ori* in Se301 cells, as it is generally accepted that a high MOI promotes the predominance of DIs (Wickam *et al.*, 1991). In the early passages the highest titers are indeed observed in Se301, as compared to SeUCR1 and SeIZD2109 cells. Still, if a high MOI were the reason, the non-*hr ori* concatemers from Se301 P19 are not expected to be maintained at the same high level during 5 successive passages in the SeUCR1 and SeIZD2109 cell lines.

Relative abundance of non-*hr ori* concatemers in intra- and extracellular viral DNA preparations

Non-*hr ori* concatemers were shown to accumulate in Se301 intracellular viral (ICV) DNA upon serial passaging. Since successive viral infections in cell culture go through BVs, it was investigated whether these concatemers were equally abundant in DNA preparations from BV as from ICV. Viral DNA from ICV and BV of Se301 P10 was digested with *Xba*I, analyzed by agarose gel electrophoresis (Fig 4.3A), and Southern-hybridized with a 650 bp non-*hr ori* probe (Fig 4.3B). The restriction profile indicated that the concatemers made up of multimers of the 2.6 and 3.0 kb fragments were abundantly present in ICV, but in much lower relative amounts in BV.

This suggested that concatenated non-*hr ori* DNA is only partially packaged in BVs. It is not known whether the repeated non-*hr ori* DNA molecules are packaged in BV as concatemers, or whether they are somehow integrated in the viral genome prior to assembly and budding. A study on the fate of plasmid DNA in cotransfection experiments suggested that baculovirus DNA replication is prone to the generation of defective genomes (Wu *et al.*, 1999). The authors showed that randomly integrated plasmids were retained in progeny virions and that high-molecular-weight

concatemers of plasmid DNA were generated in transfected cell, although it remained unclear whether these concatemers itself were efficiently packaged or not.

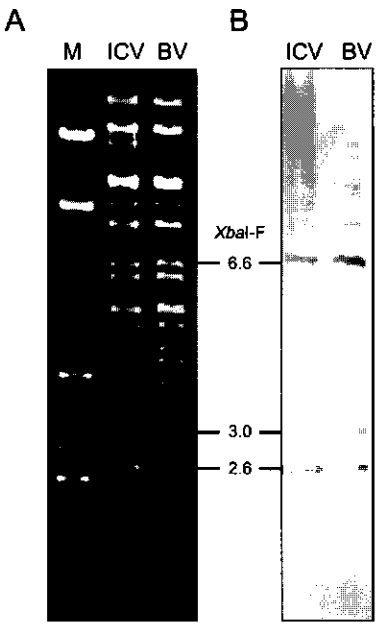

Figure 4.3. Comparative abundance of concatenated DNA containing the SeMNPV non-hr origin of DNA replication (non-*hr ori*). (A) Intracellular viral (ICV) DNA and extracellular budded virus (BV) DNA was purified from Se301 passage 10 (P10) and digested with *Xba*I. The 6.6 kb genomic *Xba*I-F fragment containing the non-*hr ori* is shown, as well as the 2.6 and 3.0 kb fragments, which are indicative for the non-*hr ori* concatemers. (B) Southern hybridization of the *Xba*I digests using a non-*hr ori* probe.

Nevertheless, if the non-*hr ori* concatemers were fully integrated in the viral genome, the proportion of non-*hr ori* sequences is not expected to differ between ICV and BV. Most likely, the non-*hr ori* concatemers coreplicate with the helper virus as episomes of different sizes containing tandem repeats of a defined size (2.6, 3, 4.1, 5.5, or 7 kb: see Fig 4.1A and 4.2). Further, it is assumed that a minimal genome size (approximately 50 kb (Lee and Krell, 1992)) is required for a DNA molecule to become packaged as nucleocapsid and become enveloped as a BV, and that only one circular DNA molecule can be packaged in a BV. Therefore, the difference between BV and ICV may be caused by the presence of a large number of smaller, rapidly replicating non-*hr ori* molecules (including putative minicircles) intracellularly, which are not present in BVs. Consistent with these results, Lee and Krell (1992) also found that the relative amount of the smaller non-*hr ori* DNA molecules in AcMNPV BV of P81 was much lower than in the ICV P81 fraction.

The present results thus indicate that the generation of deletion mutants is a general phenomenon upon serial passage of SeMNPV in *S. exigua* insect cells. However, some host cell factor(s) might be involved in the cell line-specific generation of different baculovirus deletion mutants and DIs that contain sequences with a strong replication advantage, such as non-*hr oris*.

MATERIALS AND METHODS

Virus and cells

The wildtype isolate SeMNPV-US1 (Gelernter and Federici, 1986b) was serially passaged in Se301 (Hara *et al.*, 1995) and two additional *S. exigua* cell-lines, SeUCR1 (Gelernter and Federici, 1986a), and SeIZD2109 (a gift from Dr. B Möckel and Dr. H. G. Miltenburger, Inst. of Zoology, Technische Universität, Darmstadt, Germany). All cells were propagated at 27°C in Grace's supplemented medium (Gibco BRL) containing 10% foetal calf serum (FCS; Gibco BRL) according to established procedures (Summers and Smith, 1987). Haemolymph was collected as previously described (IJkel *et al.*, 2000) and was defined as the passage zero (P0) budded virus (BV) inoculum to initiate serial passage at a multiplicity of infection (MOI) of 10 ($TCID_{50}$ units per cell). Serial undiluted passaging was performed in 25 cm^2 tissue culture flasks (Nunc) by incubation of $2.5*10^6$ cells for 2 h with 1 ml of the virus inoculum of the previous passage. The cells were washed with fresh medium and were further incubated in 4 ml of medium for 72 h.

DNA isolation and Southern hybridization

Budded virus was purified from the cell culture supernatant by centrifugation. BV DNA and total intracellular (ICV) DNA was isolated as described by Summers and Smith (1987). Digested viral DNA was run overnight in ethidium bromide stained 0.6% agarose gels, and Southern blotting was performed with non-radioactive DIG-labelled probes (Roche) of the SeMNPV non-*hr ori* (nt 84027 – 83139, according to IJkel *et al.*, 1999). Hybridization and colorimetric detection with NBT-BCIP (Gibco BRL) were performed according to the manufacturer's recommendations.

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CHAPTER 5

Identification of *pif-2*, a third conserved baculovirus gene required for *per os* infection of insects

Infection of cultured insect cells with *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) results in the generation of mutants with major genomic deletions. Some of the mutants lack the ability to infect *S. exigua* larvae *per os*. The gene(s) responsible for this phenotype in SeMNPV were mapped within a contiguous sequence encoding ORFs 29 to 35. In this paper we show that SeMNPV ORFs 15 to 35 (including genes encoding cathepsin, chitinase, gp37, ptpt-2, egt, pkip-1, and arif-1) are not essential for virus replication in cell culture or by *in vivo* intrahemocoelic injection. By site-specific deletion mutagenesis of a full-length infectious clone of SeMNPV (bacmid) using ET-recombination in *E. coli*, a series of SeMNPV bacmid mutants with increasing deletions in ORFs 15 to 35 was generated. Analyses of these mutants indicated that a deletion of SeMNPV ORF35 (Se35) results in the loss of oral infectivity of polyhedral occlusion bodies. Reinsertion of ORF35 in SeMNPV bacmids lacking Se35 rescued oral infectivity. We propose the name *pif-2* for Se35 and its baculovirus homologues (e.g. AcMNPV ORF22), in analogy to a different gene recently characterized in *S. littoralis* NPV, which was designated *per os* infectivity factor (*pif*). Similar to the *p74* gene, which encodes an essential structural protein of the ODV envelope, *pif* and *pif-2* are part of 30 genes that are conserved among the *Baculoviridae*.

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INTRODUCTION

Spodoptera exigua multicapsid nucleopolyhedrovirus (SeMNPV) infects the single insect species *S. exigua* (Smits & Vlak, 1988) and belongs to the group-II NPVs. Baculoviruses of this group do not contain a GP64 homologue, but a functionally homologous F-protein as structural element of budded viruses (BVs) (IJkel *et al.*, 2000). BVs are required for the systemic spread of infection through the insect whereas occlusion body derived viruses (ODVs) are involved in the horizontal spread of the virus in insect populations (Blissard & Rohrmann, 1990). Occlusion bodies are ingested orally, and the alkaline environment of the midgut causes the release of the ODVs. The ODVs first pass the peritrophic membrane and subsequently fuse to the midgut epithelial cells, thereby causing the initial infection (Funk *et al.*, 1997). Large-scale production of SeMNPV for biological control is carried out *in vivo* using insect larvae, since SeMNPV infection in cell culture leads to the rapid generation and predominance of deletion mutants. These mutants with deletions up to 25 kb often lack the ability to infect *S. exigua* larvae by oral ingestion of occlusion bodies (Heldens *et al.*, 1996). Therefore, the genetic engineering of SeMNPV via recombination in cell culture is complicated (Dai *et al.*, 2000) and precludes the *in vitro* production of biologically active SeMNPV in insect cell bioreactors.

Deletions in the SeMNPV genome predominantly occur within the *Xba*I-A restriction fragment, and these deleted genotypes are present in SeMNPV wildtype isolates where they may act as parasitic genotypes (Muñoz *et al.*, 1998). Heldens *et al.* (1996) described a SeMNPV mutant with a deletion in *Xba*I-A of about 25 kb, spanning ORFs 14 to 41 (IJkel *et al.*, 1999). This mutant replicated efficiently in cell culture but lacked bioactivity *in vivo*. Dai *et al.* (2000) isolated a SeMNPV recombinant with a deletion of 10.6 kb from nucleotides (nt) 18513 to 29106, encompassing ORFs 15 through 28 (Fig. 5.1A). This recombinant (SeXD1) was isolated by alternate cloning between Se301 insect cells and *S. exigua* larvae. A genotypic variant with the same 10.6 kb deletion was shown to exist in the SeMNPV wildtype isolate, and seemed preferentially amplified in cell culture, indicating that deletion mutants may have a replicative advantage in cell culture. The recombinant SeXD1, however, was still infectious *in vivo* by oral ingestion of occlusion bodies, but caused no typical liquefaction of the *S. exigua* larvae, probably due to the deletion of the cathepsin (ORF16) and chitinase (ORF19) genes (Hawtin *et al.*, 1997). More recently, a spontaneous SeMNPV mutant was generated during passaging of

bacmid-derived SeMNPV in Se301 insect cell culture, and this mutant lacked virulence *in vivo* (Pijlman *et al.,* 2002). The mutant contained a major deletion from nt 20162 to 36396, spanning open reading frames (ORFs) 17 to 35, and possibly affecting parts of the promoter region of ORF36. From these preceding studies we deduced that the gene(s) responsible for the observed loss of virulence *in vivo* are located within SeMNPV ORFs 29 to 35 (or perhaps 36).

Untill recently the only baculovirus protein demonstrated to be involved in oral infectivity by ingestion of polyhedra is the ODV-specific P74 (Kuzio *et al.,* 1989), which contains a hydrophobic C-terminus involved in protein localization and transmembrane anchoring. None of the other proteins of the ODV envelope, such as ODV-E18, -EC27, -E35, -E25, -E56, and -E66, were proven to participate in the oral infectivity process (Slack *et al.,* 2001). During the preparation of this paper, however, a conserved baculovirus gene encoded by ORF7 of *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV) was also found to be required for the oral infection of *S. littoralis*. This gene, which is homologous to SeMNPV ORF36 (Se36), was designated *per os* infectivity factor (*pif*) (Kikhno *et al.,* 2002).

The spontaneous SeMNPV bacmid deletion mutant (Fig. 5.1A Pijlman *et al.,* 2002) lacked ORFs 17 to 35 and maybe parts of the promoter of Se36, as only a sequence 18 bp upstream from the translation start of Se36 was left intact. So, it could be that the expression of Se36 was affected explaining the loss of oral infectivity in our mutant. However, more detailed investigation of the Se36 coding sequence showed that there is a late transcription motif (TAAG) present 12 bp upstream (-12) of the ATG. This suggests that Se36 (*pif*) may still be functional in the isolated mutant, as the TAAG motif has been retained. The shortest distance between a functional TAAG motif (at position –6) and the translation start site (ATG) in SeMNPV was reported for the ubiquitin gene (Van Strien *et al.,* 1996).

Using a previously constructed SeMNPV bacmid (Pijlman *et al.,* 2002) and a rapid site-directed mutagenesis protocol in *Escherichia coli*, known as ET-recombination or lambda-red recombination (Muyrers *et al.,* 1999), progressive deletions within the ORF29 to 35 region were made. SeMNPV mutants were tested for their ability to infect *S. exigua* larvae *per os* by oral feeding and by injection into the hemolymph. Analyses of repair mutants resulted in the identification of a novel baculovirus gene that is required for the *per os* infection of *S. exigua* larvae with baculovirus occlusion bodies. This gene was encoded by SeMNPV ORF35 and belongs to a core set of 30 genes shared by all known baculoviruses (Herniou *et al.,* 2003).

RESULTS

Identification of SeMNPV ORFs required for *per os* infectivity of *S. exigua*

From previous studies on SeMNPV performed in our laboratory we concluded that the genomic region from ORF29 to ORF35 contains one or more genes required for oral infectivity (Fig. 5.1A). To identify these ORF(s), progressive site-specific deletions (Fig. 5.1B) were made in the full-length SeMNPV bacmid (SeBAC) using homologous ET-recombination in *E. coli* (Fig. 5.1C). With this method, a sequence of contiguous ORFs from ORF15 to ORF28, 32, 34, or 35 was replaced by a chloramphenicol resistance gene (Cm^R). We also generated a deletion mutant (SeBACΔ15-35*) with the same deletion as a spontaneous mutant found previously (Pijlman *et al.*, 2002; see Fig. 5.1A), which lacked oral infectivity. The identity of the deletion mutant bacmids (as outlined in Fig. 5.1B) was confirmed by their unique *Not*I-*Hin*dIII restriction profile (Fig. 5.1D).

Virus (mutant)	BV injection	OB ingestion
SeMNPV-US1 wt	+	+
SeBACphΔ15-28	+	+
SeBACphΔ15-32	+	+
SeBACphΔ15-34	+	+
SeBACphΔ15-35	+	-
SeBACphΔ15-35*	+	-

Table 5.1. Feeding and injection experiments with *S. exigua* larvae. Infections of Se301 insect cells were carried out with SeMNPV-US1 wildtype and the various deletion mutant SeMNPV bacmids, which had an introduced polyhedrin gene. Twenty-four early 4th instar larvae were injected with budded virus (BV) containing culture medium. Second and 4th instar larvae (24 per treatment) were fed with polyhedral occlusion bodies (OB). A plus (+) indicates mortality, whereas a minus (-) indicates survival and pupation of the *S. exigua* larvae upon treatment.

Since the original SeMNPV polyhedrin gene was disrupted by the insertion of the bacmid vector, this polyhedrin gene was reintroduced by site-specific transposon-mediated integration (Pijlman *et al.*, 2002). Subsequently, Se301 insect cells were transfected with the recombinant SeMNPV bacmids and occlusion bodies were harvested. To check whether all constructed recombinants generated infectious viruses, transfection supernatant containing BVs was injected into the haemocoel of 4th instar *S. exigua* larvae (Table 5.1). All larvae injected with BVs from all recombinants died and produced occlusion bodies, whereas larvae injected with Grace's medium only (negative control) survived. To test the oral infectivity of the

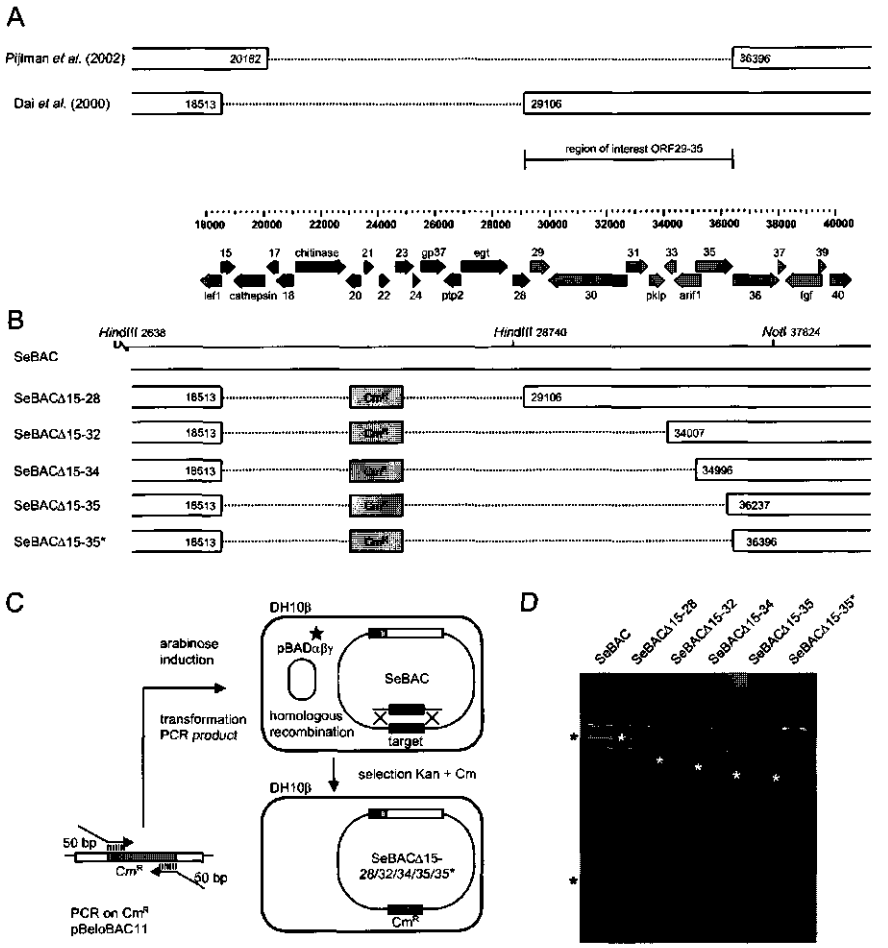

Figure 5.1 A) Genomic organization of the hypervariable region in SeMNPV and schematic representation of SeMNPV deletion mutants. The virus lacking ORFs 17 to 35 is non-infectious *per os* (Pijlman *et al.*, 2002), whereas the virus with retained oral infectivity lacks ORFs 15 to 28 (Dai *et al.*, 2000). The region of interest (ORFs 29 to 35) containing the gene(s) required for oral infectivity, is indicated. B) Schematic overview of SeMNPV deletion mutant bacmids. *Hin*dIII and *Not*I restriction sites are indicated in the full-length bacmid SeBAC. Nucleotide positions according to the complete SeMNPV genome sequence (IJkel *et al.*, 1999) are indicated on either side of the introduced chloramphenicol resistance gene (Cm^R). C) ET-recombination protocol. The PCR product with a CmR gene and viral flanking sequences of 50 bp is electroporated into arabinose-induced *E. coli* DH10β cells, which harbour the homologous recombination helper plasmid pBADαβγ and the full-length SeMNPV bacmid SeBAC. Recombinant bacmids with deletions from ORFs 15 to 28, 32, 34, 35, or 35* are isolated by selection with kanamycin (Kan) and chloramphenicol (Cm). D) *Not*I-*Hin*dIII restriction pattern of the full-length SeMNPV bacmid SeBAC and the SeMNPV deletion mutant bacmids. The 26102 bp *Hin*dIII-*Hin*dIII and 9084 bp *Not*I-*Hin*dIII fragment of SeBAC are indicated with a black asterisk at the left, whereas the *Not*I-*Hin*dIII fragments (24593 bp, 19692 bp, 18703 bp, 17462 bp, 17303 bp) with progressive deletions are indicated with a white asterisk.

Construction and analysis of Se35 repair mutants

To confirm the essential role of Se35 in oral infectivity, the gene was reintroduced (along with the polyhedrin gene) into SeBAC$^{\Delta 15\text{-}35}$ to generate SeBACph$^{\Delta 15\text{-}35}$rep^{35}. After transfection, BV-containing supernatant was injected into 4th instar *S. exigua* larvae and full mortality and formation of OBs in the cadavers was obtained. OBs harvested from infected cells were then fed to 2nd and 4th instar *S. exigua* larvae to check oral infectivity. OBs from the transfection with repair mutant SeBACph$^{\Delta 15\text{-}35}$rep35 resulted in restoration (rescue) of oral infectivity, confirming that a deletion of Se35 leads to a baculovirus phenotype lacking infectivity *in vivo*.

To investigate whether in addition to Se35 also a deletion of the putative promoter region of Se36 in SeBACΔ15-35* could have been responsible for a lack of virulence *in vivo*, Se35 was also reintroduced in SeBACΔ15-35* (by transposon-mediated integration) generating SeBACph$^{\Delta 15\text{-}35*}$rep^{35} (Fig. 5.2), in which the deletion ends at position –18 of the ATG of Se36. Polyhedra from this repair mutant SeBACph$^{\Delta 15\text{-}35*}$rep35 also resulted in restoration of oral infectivity, indicating that the 18 bp sequence (including a TAAG motif) upstream of the ATG start site of Se36 serves as an active promoter in the mutant bacmid SeBACΔ15-35*. Alternatively, Se36 may not be essential for oral infectivity, which would be in contrast to other results (Kikhno *et al.*, 2002).

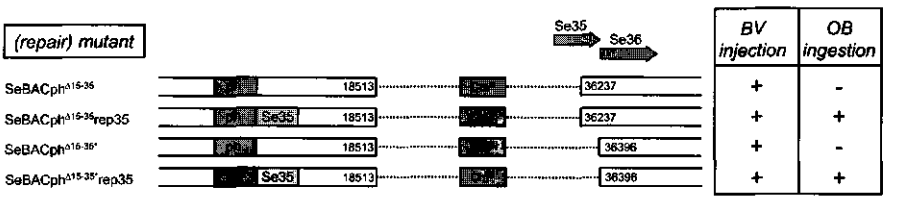

Figure 5.2. Schematic overview SeMNPV bacmid repair mutants and feeding/injection experiments with *S. exigua* larvae. SeMNPV ORF35 (Se35) was reintroduced by transposon-mediated integration into the recombinant bacmids along with the polyhedrin (ph) gene. Culture supernatant from infections of Se301 insect cells were injected in 24 early 4th instar larvae, while polyhedral occlusion bodies were fed to 2nd and 4th instar larvae (24 per treatment). A plus (+) indicates mortality, whereas a minus (-) indicates survival and pupation of the *S. exigua* larvae upon treatment.

Computer-assisted analysis of Se35 and its homologues

The DNA sequence encoding Se35 was first investigated for the presence of putative baculovirus early and/or late promoter motifs, such as TATA(A), the consensus early transcription initiaton motif ATCA(G/T)T(C/T) (Friesen, 1997), and the essential TAAG motif for late genes (Lu & Miller, 1997). No consensus early or

late transcription initiation motifs were found upstream of the first ATG of Se35, but there is a TAAG motif present starting 17 nt before the (third) ATG. This may suggest that Se35 is a late gene, and that it might be translated from the third ATG, although none of the first three ATGs in Se35 is in a favourable Kozak-context. A putative polyadenylation signal (AATAAA) was detected 22 nt after the stop codon.

Amino acid sequences of Se35 and its homologues from all 18 completely sequenced baculovirus genomes and the currently unclassified Hz-1 virus, were aligned with CLUSTALX (Thompson *et al.*, 1997). Alignment of a selection of Se35 homologues from HaSNPV-G4 (a single nucleocapsid NPV), AcMNPV (a group-I MNPV), CpGV (a granulovirus), CuniNPV (a dipteran NPV) and Hz-1V (a more distantly related, lepidopteran virus) is shown in Fig. 5.3. It can be observed that Se35 has a longer ORF than its homologues, and therefore the genuine start of the

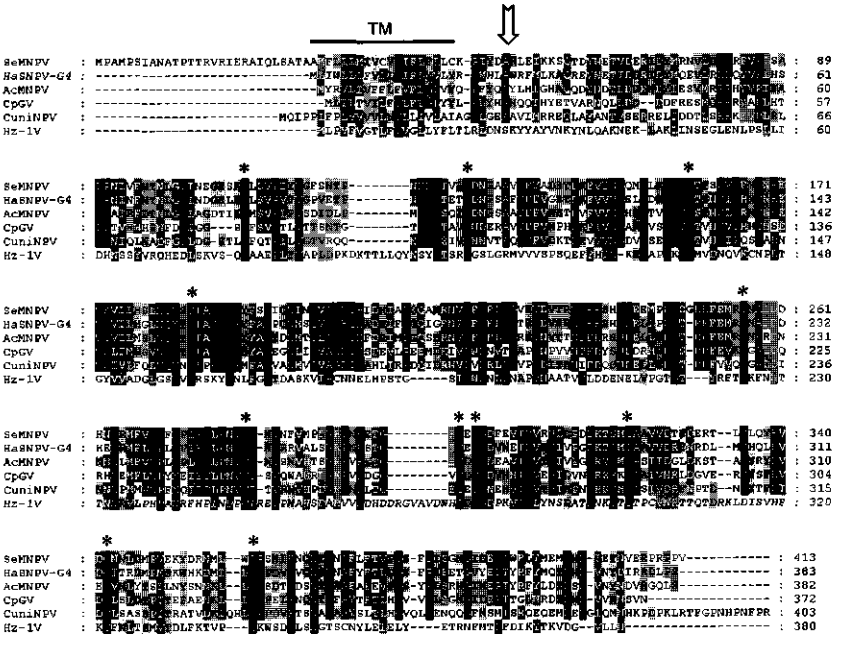

Figure 5.3. Comparison of SeMNPV ORF35 (acc. no. AAF33565) with its homologues HaSNPV-G4 ORF132 (acc. no. AAG53875), AcMNPV ORF22 (acc. no. AAA66652), CpGV ORF48 (acc. no. AAK70708), CuniNPV ORF38 (acc. no. AAK94116), and Hz-1V ORF123 (acc. no. AAN04416). Shading levels indicate amino acid identity/similarity. Conserved cysteïne residues are indicated with an asterisk above the sequence. A hydrophobic, putative transmembrane (TM) domain is indicated with a straight line above the sequences. The predicted signal peptide cleavage sites are the same for SeMNPV (YDA-HL), AcMNPV (YQA-YL), CpGV (YHA-HQ), CuniNPV (GEA-AV), and Hz-1V (DNS-KY) and are indicated with an arrow. The predicted signal peptide cleavage site for HaSNPV-G4 is slightly different (VLY-RP).

protein might be at the third methionine, as suggested before. Hydrophobic, putative transmembrane domains in Se35 were identified with the use of TMpred (Hofmann & Stoffel, 1993). For Se35, a strong hydrophobic domain was predicted at the N-terminus (Fig. 5.3), which is conserved in the other Se35 homologues. By the use of computer prediction program SignalP (Nielsen *et al.,* 1997), it was found that Se35 (and its homologues) has a predicted signal peptide at the N-terminus and a putative cleavage site (indicated with an arrow). The TargetP program (Emanuelsson *et al.,* 2000) predicted that Se35 employes the secretory pathway. In the Se35 alignment (Fig. 5.3) 11 cysteines were found at conserved positions (indicated with an asterisk). These conserved cysteines are also present in all the other homologues (not shown). This indicates that the protein can form multiple disulfide bonds and that it might be heavily folded. No conserved N-glycosylation sites were found in the alignment of Se35.

```
ODV-E66     Se57    MWLYVFMLAVFVIFVILLWQTNIVIVDLESQNQKYYDYF
            Se114   MIAAWFLVVIIVVVIVAIVLYFNNQTNNNINFDNYDDNSN
            Ac46    MSIVLLIVIVVIFLICFLYLSNSNNKNDANKNNAFIDLNP

ODV-E25     Se71    MIGTLVVIVIVLALLYYLWVNNKLNMNSINESSPSLADSS
            Ac94    MWG--IVLLIVLLILFYLYWTNALNFNSLTESSPSLGQSS

PIF         Se36    MHN--IVLIVLLVILIAVTYNNVTLLQYVQQDFIPIVQTF
            Ac119   MHF--AIILLFLLVIIAIVYTYVDLIDVHHEEVRYPITVF

PIF-2       Se35    MFL--LIMIVCVVIFLFLLCKPIYDAHLEIKKSQTDYNET
            Ac22    MYR--VLIVFFLFVFLYIVYQPFYQAYLHIGHAQQDYNDT
```

Figure 5.4. Comparison of N-terminal sequences of ODV-specific proteins and proteins required for oral infectivity. Amino acid sequences of ODV-E66 (AcMNPV acc. no. AAA66676: SeMNPV acc. no. AAF33587 and AAF33643), ODV-E25 (AcMNPV acc. no. AAA66724: SeMNPV acc. no. AAB88623), PIF (AcMNPV acc. no. AAA66749 : SeMNPV acc. no. AAF33566), and the newly identified *per os* infectivity factor PIF-2 (AcMNPV acc. no. AAA66652: SeMNPV acc. no. AAF33565) were aligned with ClustalX. ORF numbers are indicated according to the complete genome sequences (Ayres *et al.,* 1994; IJkel *et al.,* 1999). Se35 is the truncated version of the published sequence (see also Fig. 5.3). Shaded amino acids indicate the strong hydrophobic domain, which is rich in valines (V), leucines (L), and isoleucines (I).

Similar N-terminal hydrophobic domains as in Se35 were also found for AcMNPV ODV-E66 and ODV-E25, which encode ODV-specific structural proteins directly targeted to the nucleus (Hong *et al.,* 1997). Se36 (*pif*) also contains such a hydrophobic domain at the N-terminus. A comparison of homologues of these four proteins from AcMNPV and SeMNPV is shown in Fig. 5.4. All amino acid sequences have a strong hydrophobic domain at the N-terminus that is rich in valines, leucines, and isoleucines.

DISCUSSION

In this paper evidence is presented that SeMNPV ORF35 (Se35) is essential for oral infectivity and that a region of SeMNPV encompassing ORFs 15 to 35 is not required for virus replication in cultured insect cells or *in vivo* after intrahaemocoelic injection. In previous studies involving SeMNPV deletion mutants generated via cell culture or *in vivo* (Heldens *et al.,* 1996; Dai *et al.,* 2000), it could never be excluded that a minor amount of intact helper virus was still present to enable predominant replication of deletion mutants. With the current strategy using bacmids, pure baculovirus mutants are generated from single-copy bacterial artificial chromosomes maintained in *E. coli* (Luckow *et al.,* 1993; Pijlman *et al.,* 2002). The deleted region spanning ORFs 15 to 35 contains genes encoding cathepsin, chitinase, GP37, PTP-2, EGT, PKIP-1 and ARIF-1 (IJkel *et al.,* 1999). Cathepsin (Se16) and chitinase (Se19) are involved in the liquefaction of baculovirus-infected insects, but viruses lacking these genes were not less effective in secondary infections either *in vivo* or in cell culture (Hawtin *et al.,* 1997). GP37 (Se25) may be a chitin binding protein and is homologous to spindolin and entomopoxvirus (EPV) fusolin genes, but it appeared non-essential for baculovirus replication (Cheng *et al.,* 2001). PTP-2 (Se26) is a protein tyrosine phosphatase and not essential for DNA replication (Li & Miller, 1995). EGT (Se27) encodes a non-essential ecdysteroid UDP-glucosyltransferase that is involved in molting of insect larvae (O'Reilly & Miller, 1990). *Pkip-1* (Se32) and *arif-1* (Se34) are located within the genomic region studied in this paper, containing the genes required for oral infectivity (ORFs 29 to 35). *Pkip-1* is a late gene that was found to play an essential role in AcMNPV DNA replication by stimulating the activity of the viral protein kinase-1 (Fan *et al.*, 1998), but is now shown to be non-essential in SeMNPV. A temperature-sensitive mutant, defective in *pkip*, lacks the ability to form plaques and occlusion bodies at non-permissive temperatures (McLachlin *et al.*, 1998), but we found normal OB production with SeMNPV *pkip* deletion mutants. *Arif-1* was identified as an early AcMNPV gene product that is involved in remodeling of the actin cytoskeleton of the infected cell (Dreschers *et al.*, 2001), but is also non-essential and not required for oral infectivity.

Interestingly, a granulovirus from the potato tuber moth called PhopGV, was recently sequenced (Genbank accession no. NC_004062) and appeared to lack exactly the same ORFs as the SeMNPV deletion mutants, namely cathepsin, chitinase, gp37, ptpt-2, egt, pkip, and arif-1. In PhopGV, the gene encoding LEF-1 (ORF66) is adjacent to the Se36 homologue (PhopGV ORF67), as is the case in our SeMNPV 15-35 deletion mutant (Fig. 5.1B). This indicates that this large cluster of

non-essential genes is not conserved throughout the family of Baculoviridae. For the utilization of SeMNPV as expression vector for recombinant proteins, the dispensability of the SeMNPV *Xba*I-A region spanning ORFs 15 to 35 may be advantegous with regard to its biological safety. Furthermore, by deliberate site-specific deletion of ORFs 15 to 34, the intrinsic genetic instability of the SeMNPV genome in cell culture can be eliminated, while the virus is still infectious *per os*. Therefore, the SeBACph15-34 mutant may be an attractive biologically active SeMNPV recombinant, which can be efficiently scaled up both *in vitro* and *in vivo* to be used as bioinsecticide for insect pest control. Further analysis of the infectivity and pathobiology of this mutant should be carried out to evaluate its potential for the use as biopesticide.

With the results presented here we provide genetic and biological evidence that a deletion of SeMNPV ORF35 (Se35) results in the formation of OBs which have lost the ability to infect insects by the oral route. Oral infectivity was rescued by reintroduction of Se35 on a different locus via transposition in the SeMNPV bacmid (Fig. 5.2). These results also support the hypothesis that Se36 (*pif*, Kikhno *et al.*, 2002) was still functional in our mutants, and that Se36 transcription is likely to start from the TAAG at position –12. We did not compare transcription levels of Se36 between the various mutants, since this may only have a quantitative but not a qualitative effecton the identification of Se35 as essential gene for oral infectivity. Se35 belongs to the so-called baculovirus core genes (Herniou *et al.*, 2001, 2003), which are shared by all known baculoviruses. A homologue is also present in the non-assigned *Heliothis zea*-1 virus (Hz-1V) (Cheng *et al.*, 2002). This virus has structural similarities to baculoviruses, but the virions are not occluded. Yet, Hz-1V is infectious *per os*. Se35 was shown to be essential for the *per os* infection of *S. exigua*, but not necessary for systemic infection of *S. exigua* larvae by injection into the heamolymph or for succesful virus replication in cell culture. This indicates that only the Se35 gene product (protein) and thus not the gene itself is needed for the initial steps of midgut infection by ODVs, either by passing the peritrophic membrane, or by fusion with the midgut epithelial cells, or both.

Together with p74 and Se36 (*pif*), Se35 is one of the few baculovirus genes that is conserved among all baculoviruses (Herniou *et al.*, 2003) as well as in the distantly related Hz-1V (Cheng *et al.*, 2002). Moreover, these three conserved proteins are all involved in oral infectivity. Therefore, we speculate on a highly conserved entry mechanism of baculovirus ODVs and Hz-1V virions in insect midgut cells, with p74, Se36 (*pif*) and Se35 as key components. Hz-1V does not produce budded viruses, which is in line with the lack of homologues of gp64 or F-protein groups in the

predicted Hz-1V ORFs. The Hz-1V virions may therefore be structurally quite similar to baculovirus ODV particles containg a single nucleocapsid (similar to GVs and SNPVs), and spread the infection after cell lysis (Burand, 1998).

Analogous to the designation of *pif* to Se36 and its homologues (e.g. AcMNPV ORF119), we propose the name *per os* infectivity factor 2 (*pif-2*) for Se35 and its homologues (e.g. AcMNPV ORF22) in other baculoviruses. Kikhno *et al.* (2002) showed by Western analysis that *pif* is a structural protein of the ODV envelope and they speculated upon a putative interaction between *pif* and *p74*, but now *pif-2* must be considered as well. The overlap of the Se35 (*pif-2*) 3'UTR and the promoter of the neighbouring Se36 (*pif*) may indicate that these two genes in SeMNPV have co-evolved and are closely associated, although their homologues in other baculoviruses (including SpliNPV) are located in separate gene clusters.

Although the strong N-terminal hydrophobic domain in Se35 suggests that the gene encodes a protein associated with membranes, it remains to be elucidated whether *pif-2* is indeed an ODV-specific structural protein. The alignments of Se35 (Fig 5.3) and Se36 with their homologues demonstrated that both proteins have strong hydrophobic domains at their N-terminus (Fig. 5.4). The Se35 and Se36 N-terminal sequences, which are rich in valines, isoleucines, and leucines, are very similar to N-terminal sequences of AcMNPV ODV-E66 (Ac46) and ODV-E25 (Ac94). These latter proteins are conserved among all lepidopteran baculoviruses (Herniou *et al.*, 2003) and were earlier identified as ODV-specific structural proteins. It was proven that their N-terminal hydrophobic domains were sufficient to direct reporter proteins to the nuclear envelope, intranuclear microvesicles and the ODV envelope within baculovirus-infected cells (Hong *et al.*, 1997). It was shown by N-terminal amino acid sequencing that ODV-E66 and ODV-E25 were uncleaved in the ODV envelope. Although Se35 and Se36 have a predicted cleavage site after the hydrophobic domain, it remains unclear whether cleavage occurs. Hong *et al.* (1997) also proposed a model predicting that ODV envelope proteins are incorporated into the endoplasmatic reticulum, and are subsequently transported to the inner and outer nuclear membrane. In agreement with these findings, a Se36 homologue (*pif*; Ac119) may be part of the ODV envelope of SpliNPV (Kikhno *et al.*, 2002). The similar hydrophobic N-terminal amino acid sequences of ODV-E25, ODV-E66, *pif*, and *pif-2* from AcMNPV and SeMNPV (Fig. 5.4), and the involvement of Se35 in oral infectivity, may suggest that Se35 (Ac22) is also a structural protein associated with the ODVs. Further biochemical and immunological studies are required to conclusively demonstrate the location of *pif-2*.

MATERIAL & METHODS

Insect cells, insects, virus

The *Spodoptera exigua* cell line Se301 (Hara *et al.*, 1995) was donated by Dr. T. Kawarabata (Institute of Biological Control, Kyushu University, Japan) and was propagated at 27°C in Grace's supplemented medium (Gibco BRL) containing 10% foetal calf serum (FCS; Gibco BRL). *S. exigua* larvae were reared on artificial diet at 27°C, 70% humidity and a 16:8 h photoperiod. Fourth instar *S. exigua* larvae were infected by contamination of artificial diet with $4x10^5$ SeMNPV-US1 (Gelernter & Federici, 1986b) polyhedra per larva (Smits & Vlak, 1988). Haemolymph was collected as previously described (IJkel *et al.*, 2000). Infectious budded virus titers were determined using the endpoint dilution assay (Vlak, 1979).

Deletion of ORFs by ET-recombination and bacmid DNA preparation

A series of SeMNPV bacmid mutants with progressive deletions of ORF29 through 35 was made by ET-recombination as previously described (Pijlman *et al.*, 2002). To obtain genomic stability of the generated deletion mutant bacmids upon replication in cell culture, ORFs 15 through 28 were also deleted. These ORFs were responsible for genetic instability but were proven to be non-essential for oral infectivity of SeMNPV (Dai *et al.*, 2000). This resulted in bacmids SeBACΔ15-28/32/34/35 (Fig. 5.1B). We also generated a mutant (SeBACΔ15-35*) similar to the spontaneous bacmid SeMNPV mutant lacking most of the upstream (promoter) sequence of Se36, to investigate whether Se36 was still functional or not. Briefly, PCR products with 50bp viral flanking overhangs were generated with the Expand long template PCR system (Roche) and with custom made primers (Invitrogen) listed in Table 5.2. Plasmid pBeloBAC11 (Shizuya *et al.*, 1992) was used as a template for PCR amplification of the chloramphenicol resistance gene. *E. coli* DH10B cells harboring the SeMNPV bacmid SeBAC and the recombination helper plasmid pBADαβγ (Muyrers *et al.*,

Primer	Description	Sequence
DZ 193	ET for Se15	ACGTTCTCTTGATCATGACGCTTTGATTATTGAATGTCGAATGCAGGATA*CCTAGG*TTTAAGGGCACCAATAACTG
DZ 224	ET rev Se28	CCACGCTACTCGACTTTGATATTCGACATAAAACGACGCGGCAATCTTGA*CCTAGG*TTCCTGTGCGACGGTTAC
DZ 225	ET rev Se32	TTGTCTGATTATTATTAATAATAAAATATACGTGTACATATATGAATTTT*CCTAGG*TTCCTGTGCGACGGTTAC
DZ 245	ET rev Se34	GTCGGCGGAGGCATACTGTTCACCGACAAACTACATTGTCTGCAATATTT*CCTAGG*TTCCTGTGCGACGGTTAC
DZ 269	ET rev Se35	GAAACGAGCCGGGCACGGTGAATAGGAAAGCGTTGTCGGTGTTTTGGTTG*CCTAGG*TTCCTGTGCGACGGTTAC
DZ 194	ET rev Se36	CGAGTAGTACTATTAAAACTATATTGTGCATTTTATTAACTTAGAGAGTT*CCTAGG*TTCCTGTGCGACGGTTAC
DZ 241	Se35 for	CAAGCTTGCCAACAACGTTAAACACAC
DZ 242	Se35 rev	CAAGCTTGCGATCAAGATTACGAGTAG

Table 5.2. Oligonucleotides used for site-specific genomic deletions by ET-recombination and PCR amplification of Se35. For the ET-primers, the 50 nt long viral flanking sequence used for homologous recombination (italics) is followed by an *Avr*II restriction site (underlined). The 3'end of the ET-primers is used for PCR amplification of the chloramphenicol resistance gene from pBeloBAC11. The PCR primers for amplification of the coding sequences of Se35 are equipped with *Hin*dIII restriction sites (underlined) for cloning purposes.

1999) were induced with L-arabinose and made electrocompetent with subsequent washes with 10% glycerol (Fig 5.1C). For electrotransformation (200Ω, 25μF, 2.3 kVolt) with a Biorad Genepulser and 2 mm cuvets (Eurogentec), 0.5 μg PCR product was used. Cells were incubated in a shaker at 37°C for 1 h and plated on LB agar plates containing 20 μg/ml chloramphenicol. Bacmid DNA was isolated using an alkali-lysis protocol as earlier described (Pijlman *et al.*, 2002).

Construction of repair bacmids

The coding sequence of Se35 plus the putative promoter region (from position –173 of the ATG) was amplified by PCR using primer pair DZ241/DZ242. *Hin*dIII restriction sites (underlined) were included in the primers for further cloning purposes (Table 5.2). The PCR products were first cloned into a pGEM-Teasy vector (Promega), giving pSe35. The plasmid insert was sequenced (Baseclear, The Netherlands) to confirm that no errors were introduced by PCR. For reintroduction into the SeMNPV bacmid, Se35 was cloned as a *Hin*dIII fragment into pFB1Sepol (Pijlman *et al.*, 2002) using standard methods (Sambrook *et al.*, 1989). pFB1Sepol is a derivative of the pFastBAC1 vector (Invitrogen, Bac-to-Bac) and contains a complete SeMNPV polyhedrin gene. The resulting pFastBAC vector pFB1SepolSe35 was used to construct the repair bacmids using the Bac-to-Bac transposition protocol (Invitrogen).

Transfection, micro-injections and oral infectivity assay

Se301 cells were seeded in 6-wells plates (Nunc), with $5*10^5$ cells per well. Transfection was performed with approximately 0.5 μg bacmid DNA using 10 μl Cellfectin (Invitrogen). BV containing supernatant and polyhedra from infected cells were harvested by centrifugation 14 days post transfection. The micro-injection of BV into 4th instar larvae was performed using a 1.5 ml volume B-D Pen (Becton & Dickinson) and a 28 gauge half-inch NovoFine needle (Novo Nordisk). Ten μl supernatant (or Grace's insect medium for the negative control) was injected into the hemocoel of each larva. The infectivity of recombinant polyhedra was determined with 2nd and 4th instar *S. exigua* larvae by diet contamination, using at least 10^6 occlusion bodies (OBs) per larva. For the injection as well as the feeding experiments, the larvae were kept separately in 24-wells plates (Nunc) and monitored daily untill all larvae had either pupated or died due to SeMNPV infection. At least 24 larvae were tested per treatment.

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CHAPTER 6

Spontaneous excision of BAC vector sequences from bacmid-derived baculovirus expression vectors upon passage in insect cells

Repeated baculovirus infections in cultured insect cells lead to the generation of defective interfering viruses (DIs), which accumulate at the expense of the intact helper virus and compromise heterologous protein expression. In particular, AcMNPV DIs are enriched in an origin of viral DNA replication (*ori*) not associated with the homologous regions (*hr*s). This non-*hr ori* is located within the coding sequence of the non-essential *p94* gene. We investigated the effect of a deletion of the AcMNPV non-*hr ori* on the heterologous protein expression levels upon serial passage in Sf21 insect cells. Using homologous ET-recombination in *E. coli*, deletions within the *p94* gene were made in a bacterial artificial chromosome (BAC) containing the entire AcMNPV genome (bacmid). All bacmids were equipped with an expression cassette containing a green fluorescent protein (GFP) gene and a gene encoding the classical swine fever virus E2 glycoprotein (CSFV-E2). For the parental (intact) bacmid only, a strong accumulation of DIs with reiterated non-*hr ori*s was observed. This was not observed for the mutants, indicating that removal of the non-*hr ori* enhanced the genetic stability of the viral genome upon passaging. However, for all passaged viruses it was found that the entire BAC vector including the expression cassette was spontaneously deleted from the viral genome, leading to a rapid decrease in GFP and CSFV-E2 production. The rationale for the (intrinsic) genetic instability of the BAC vector in insect cells, and the implications with respect to large-scale production of proteins with bacmid-derived baculoviruses are discussed.

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INTRODUCTION

The baculovirus-insect cell expression system is versatile and widely used for the high-level production of heterologous (eukaryotic) proteins (Possee, 1997). The proteins are often properly folded and post-translationally modified to obtain similar biological activities to their authentic counterparts (Vialard *et al.*, 1995). Advances have been made over the last decade to make more convenient and speed up the process of generating baculovirus recombinants, which were initially made via homologous recombination between viral and transfer vector DNA in insect cells (Kitts, 1996). A system based on site-specific transposon-mediated insertion of foreign genes into an infectious baculovirus genome, propagated as a bacterial artificial chromosome (BAC) in *Escherichia coli* (bacmid), has reduced the time taken to obtain pure recombinants from 1-2 months to 1-2 weeks (Luckow *et al.*, 1993). However, for both classical and bacmid recombinants, the rapid generation and accumulation of defective interfering viruses (DIs) upon passage in infected cells is still a major obstacle for the efficient large-scale production of virus and heterologous proteins in cell culture systems (Kool *et al.*, 1991; Wickam *et al.*, 1991). This phenomenon is known as the passage effect and causes a sharp drop in protein production upon serial passage of baculoviruses in cultured insect cells (reviewed by Krell, 1996).

Many reports have shown that genomic deletions and/or insertions of foreign DNA into the viral genome readily occur upon baculovirus infection in cell culture. For example, DIs with deletions of approximately 43% of the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) genome (d43 DIs) are rapidly generated (Pijlman *et al.*, 2001) and subsequently accumulate in cell culture (Kool *et al.*, 1991). Furthermore, DIs with reiterations of small viral sequences become abundant in later stages of passaging (Kool *et al.*, 1993a; Lee and Krell, 1992). These cis-acting sequences were subsequently identified as putative origins of viral DNA replication (*ori*s) by transient replication assays. *Ori* activity in baculoviruses is associated with the homologous repeated regions (*hr*s) (Lu *et al.*, 1997), which are scattered throughout the viral genome and can also act as transcriptional enhancers (Friesen, 1997). The presence of *hr*s is a common feature of baculoviruses, but they are also found in other large circular DNA viruses such as nimaviruses and ascoviruses (Van Hulten *et al.*, 2001; Bigot *et al.*, 2000), implying an important role for these interspersed repetitive sequences in viral DNA replication.

In a detailed study on DI formation following serial passage of AcMNPV in *Spodoptera furgiperda* (Sf21) insect cell culture (Lee and Krell, 1992, 1994), a

specific 2.8 kb AcMNPV sequence predominated in later passages. This fragment contained a viral sequence located on the *Hin*dIII-K restriction fragment of AcMNPV. In an independent study (Kool *et al.*, 1994) it was demonstrated that this AcMNPV *Hin*dIII-K fragment exhibited a strong *ori* activity in transient replication assays. The *Hin*dIII-K sequence was designated non-*hr ori* because it did not contain *hr* sequences. The AcMNPV non-*hr ori* is located within the open reading frame (ORF) encoding the *p94* gene, which is an early gene of unknown function (Friesen and Miller, 1987) and has probably co-evolved with the adjacent apoptosis-inhibiting gene, *p35* (Clem *et al.*, 1994). A related baculovirus, *Bombyx mori* nucleopolyhedrovirus (BmNPV), lacks a *p94*-homologous ORF, but has retained 151 bp of the *p94* gene containing the essential non-*hr ori* regions II and III as identified by Kool *et al.* (1994). This thus suggests that the non-*hr ori* is somehow involved in baculovirus replication. Non-*hr ori*s are identified in many other baculoviruses and share structural similarities rather than sequence homology (Heldens *et al.*, 1997a; Pearson *et al.*, 1993; Huang and Levin, 1999; Luque *et al.*, 2001; Hu *et al.*, 1998; Jehle, 2002). In *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV), the non-*hr ori* was shown to be non-essential for virus replication *in vitro* and *in vivo*. Deletion of the non-*hr ori* even led to enhanced genome stability by preventing DIs from becoming predominant upon passage (Pijlman *et al.*, 2002).

Since it has been clearly shown that the non-*hr* origin of DNA replication of AcMNPV accumulates in DIs upon serial passaging, the question is whether deletion of the non-*hr ori* can prevent the accumulation of DIs and therefore suppress the passage effect. A bacmid-mutagenesis approach was used to make site-specific deletions in the *p94* coding sequence. On analysis of the AcMNPV mutants by serial undiluted passage in Sf21 insect cells, we observed that the viruses became more stable, but that the bacmid insertion containing the foreign genes was specifically lost.

RESULTS

Construction and serial passage of recombinant viruses

To investigate whether a deletion of the AcMNPV non-*hr ori* could prevent the rapid accumulation of DIs and thereby improve the expression of heterologous genes in infected insect cells upon prolonged passaging, three AcMNPV mutants were constructed. Substitutions with a chloramphenicol resistance gene (Cm^R) were made in the *p94* coding sequence by ET-recombination of an AcMNPV bacmid in *E. coli*

(Fig. 6.1A). In AcΔCp94, the essential domains of the non-*hr ori* (according to Kool *et al.*, 1994) located within the C terminus of *p94* were deleted, leaving the N terminus of *p94* intact. As a control for a putative effect of a *p94* disruption, two other mutants were constructed. AcΔNp94 lacked the N terminus of *p94* and thus retained the functional domains of the non-*hr ori*, whereas AcΔp94 lacked the whole *p94* gene including the non-*hr ori*.

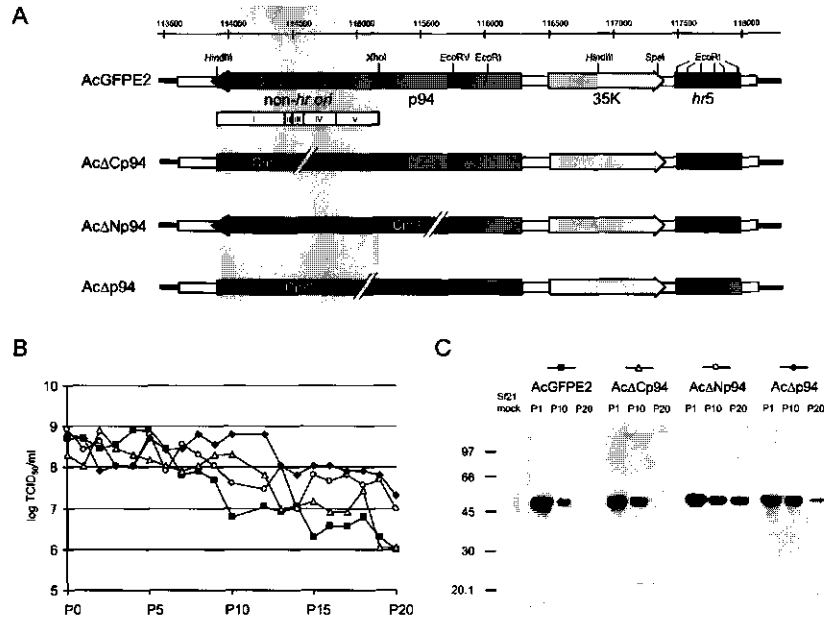

Figure 6.1. A) Schematic overview of (mutant) AcMNPV bacmids. Nucleotide positions according to the complete AcMNPV genome sequence (Ayres *et al.*, 1994) are indicated on top. The genomic organization including endonuclease restriction sites of a region containing *p94* and 35K genes and hr5 is depicted for the intact AcMNPV bacmid AcGFPE2. Three mutants, designated AcΔCp94, AcΔNp94, AcΔp94, were constructed by homologous ET-recombination in *E. coli* in which Cm^R replaced the *p94* C terminus, the *p94* N terminus and the entire *p94* gene, respectively. All viruses were equipped with an expression cassette containing the GFP and CSFV-E2 genes under control of the AcMNPV polyhedrin and *p10* promoters, respectively. B) Infectious budded virus titers upon serial passage in Sf21 cells. $TCID_{50}$/ml values were based on GFP expression. AcGFPE2 (■), AcΔCp94 (Δ), AcΔNp94 (O), AcΔp94 (◆). C) Immunodetection intracellular CSFV-E2 levels upon passage in Sf21 cells. Molecular mass markers (kDA) are indicated on the left.

The parental and the three mutant AcMNPV bacmids were equipped with an expression cassette containing GFP under the control of the polyhedrin promoter to visualize infection, and a gene encoding the classical swine fever virus E2 glycoprotein (CSFV-E2) under the control of the *p10* promoter to measure protein

production. Recombinant bacmid DNA was transfected into insect cells, budded virus (BV) titers were determined, and serial undiluted passage was carried out with an initial MOI of 100. Virus titers (monitored by GFP expression) were determined during serial passage (Fig. 6.1B).

As expected, the GFP-based titers for AcGFPE2 dropped rapidly from almost 10^9 at P0, to less than 10^7 at P10 and to almost 10^6 $TCID_{50}$/ml at P20. Similarly, GFP expression for the AcΔNp94 mutant (containing the non-*hr ori*) also dropped to lower levels upon passaging, although less rapidly than for AcGFPE2. In sharp contrast to what was expected, namely that a non-*hr ori* deletion would prevent the formation of DIs and therefore result in enhanced protein expression, a similar rapid decrease in GFP expression was observed for the non-*hr ori* deletion mutants AcΔCp94 and AcΔp94. Coinciding with this overall drop in GFP-based virus titers, a major decrease in the production of CSFV-E2 following virus passage was observed by Western blot analysis (Fig. 6.1C) for AcGFPE2, AcΔCp94 and AcΔp94 and to a lesser extent for AcΔNp94.

In summary, this experiment showed that the presence or absence of the non-*hr ori* was not correlated with the level of recombinant protein expression upon serial passage.

Analysis of genomic alterations upon serial passage

To investigate the putative formation of DIs and to find the molecular basis for the unexpected rapid decrease in protein production upon serial passage of the parental virus and the three mutants, intracellular viral (ICV) DNA of passage 1 (P1), P10 and P20 was subjected to digestion with *Hin*dIII (Fig. 6.2). For all viruses, two bands of 10.9 and 9.2 kb were submolar in P20 with respect to the other fragments. The genetic content of these bands was investigated in more detail (see Fig. 6.4). Typically, novel fragments of 3.2, 1.8 and 1.6 kb appeared in P20 of AcGFPE2 (Fig. 6.2A), but not in the DNA preparations of the other (mutant) viruses (Fig. 6.2B-D). These fragments accumulated relative to the genomic *Hin*dIII fragments of the parental virus, suggesting that these sequences were part of DI molecules. According to the hypothesis, we investigated whether these novel fragments contained the non-*hr ori*. Therefore, the viral DNA was digested and transferred to a membrane and Southern hybridized with a DIG-labeled non-*hr ori* probe (Fig. 6.2). The original non-*hr ori*-containing *Hin*dIII-K fragment in AcGFPE2 of 2971 bp hybridized to the non-*hr ori* probe (Fig. 6.2A). Moreover, the results showed that the novel 3.2, 1.8 and 1.6 kb bands in AcGFPE2 hybridized strongly with the non-*hr ori*

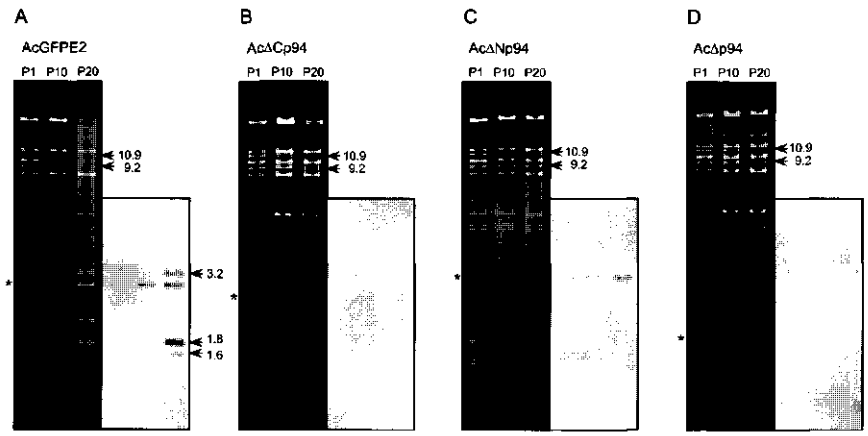

Figure 6.2. Restriction analysis of intracellular viral DNA upon serial passage in Sf21 cells and Southern detection with a non-*hr ori* probe. Viral DNA isolated from Sf21 cells infected with AcGFPE2, AcΔCp94, AcΔNp94, and AcΔp94 at P1, P10, and P20 was subjected to digestion with *Hin*dIII. Restriction fragments containing the non-*hr ori* and/or a Cm^R insertion are indicated with an asterisk. The two submolar fragments of 10.9 and 9.2 kb are indicated with an arrow on the right. Novel, hypermolar fragments of 3.2, 1.8 and 1.6 kb hybridizing to the non-*hr ori* probe are indicated with arrows on the right of the AcGFPE2 Southern blot.

probe (Fig. 6.2A), indicating that the accumulated sequences indeed contained the non-*hr ori.* As expected, the non-*hr ori* probe did not hybridize to AcΔCp94 (Fig. 6.2B) and AcΔp94 (Fig. 6.2D), but did hybridize to the fragment with Cm^R insertion in AcΔNp94 (Fig. 6.2C). Thus, deletion of the non-*hr ori* (in AcΔCp94 and AcΔp94) or a non-*hr ori* flanking sequence (in AcΔNp94) prevented the generation and accumulation of DIs, but apparently did not prevent the decrease in recombinant protein production.

Sequence and replicative form of hypermolar non-*hr ori* fragments

Subsequently it was investigated whether the novel 3.2, 1.8 and 1.6 kb *Hin*dIII fragments hybridizing to the non-*hr ori* originated from ordinary deletions in the 2971 bp *Hin*dIII-K fragment, or, alternatively, were derived from non-*hr ori* mini-circles or concatenated non-*hr ori* repeats within DI molecules, as previously found with SeMNPV (Pijlman *et al.,* 2002). Viral DNA from AcGFPE2 P20 was digested with *Bam*HI, *Hin*dIII, and *Xho*I and Southern hybridized with the same non-*hr ori* probe as described above (Fig. 6.3A). The 3.2 fragment appeared in the *Bam*HI, *Hin*dIII, and *Xho*I digests, whereas the 1.8 and 1.6 kb fragments appeared in the *Hin*dIII and *Xho*I digest only (Fig. 6.3A). This indicated that the novel non-*hr ori* containing fragments

must appear in a circular form or as tandem repeats in larger concatemers. The *Hin*dIII fragments of 3.2, 1.8 and 1.6 kb were subsequently cloned and sequenced (Fig. 6.3B). As expected from the Southern blots, all fragments contained the five essential domains (I, II, III, IV and V) of the non-*hr ori* sequence between the *Hin*dIII and *Xho*I sites. The sequence results also demonstrated the presence of a junction site between sequences from either side of the non-*hr ori* (Fig. 6.3B, asterisks). The sequence overlaps at the junction sites may indicate the imprints of the recombination steps involved in the formation of these circular or concatenated molecules.

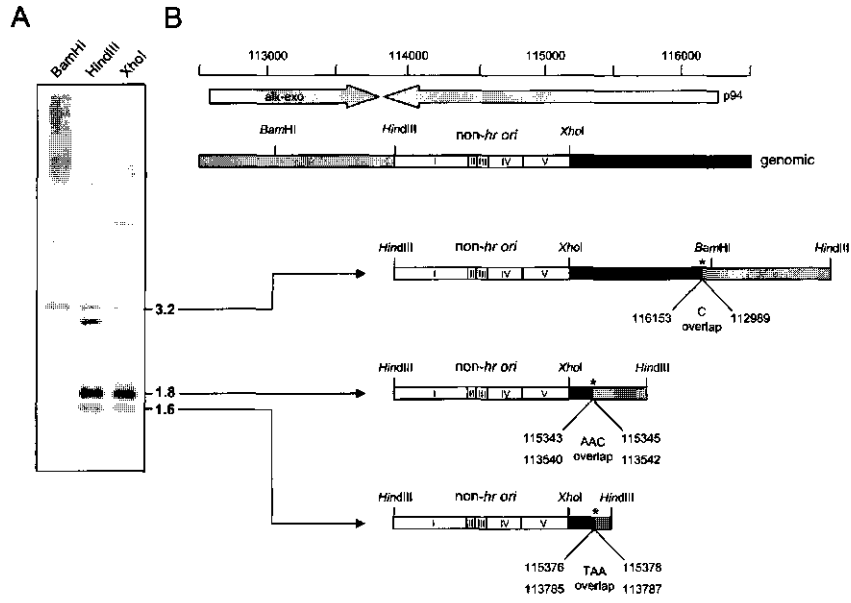

Figure 6.3. Genetic organization of novel hypermolar non-*hr ori* fragments. A) Intracellular viral DNA of AcGFPE2 P20 was digested with *Bam*HI, *Hin*dIII and *Xho*I and Southern detected with a DIG-labeled non-*hr ori* probe. B) Schematic representation of the (*Hin*dIII-linearized) sequences of the hypermolar non-*hr ori* fragments of 3.2, 1.8 and 1.6 kb, compared with the genomic organization of the parental virus. Nucleotide positions according to the complete AcMNPV genome sequence (Ayres *et al.*, 1994) are indicated on top, as well as endonuclease restriction sites and genes encoding alk-exo and *p94*. The five essential domains of the non-*hr ori* (after Kool *et al.*, 1994) are represented by a white bar below the *p94* gene. The sequence left of the *Hin*dIII site is depicted as a grey bar, whereas the sequence right of the *Xho*I site is depicted as a black bar. Corresponding sequences in the hypermolar fragments have the same colours. Nucleotide positions and sequence overlaps are indicated at the junction sites (asterisk).

Specific deletion of BAC vector sequences upon serial passage

To find an explanation for the overall decrease in protein production, the genetic content of the 10.9 and 9.2 kb *Hin*dIII fragments, which became submolar upon serial passage of all viruses (Fig. 6.2A-D), was determined. The fragments were mapped on computer-predicted digests of *in silico* reassembled bacmid sequences. Both 10.9 and 9.2 kb *Hin*dIII fragments were located on the BAC vector insertion, which contains a kanamycin resistance gene, a LacZ-mini-attTn7 cassette, and a bacterial mini-F replicon (Luckow *et al.*, 1993). More specifically, the 10.9 *Hin*dIII fragment was located entirely on the mini-F replicon and the 9.2 kb *Hin*dIII fragment on the kanamycin resistance gene (Fig. 6.4B). To map the deletions of the BAC vector sequences in more detail, a *Pst*I digest was performed and a DIG-labeled probe specific for the BAC vector (Fig. 6.4B) was used specifically to distinguish the BAC-derived fragments from co-migrating genomic fragments (Fig. 6.4A). The results showed that all *Pst*I fragments in the BAC vector from the kanamycin resistance gene (KanR) to the end of the mini-F replicon were deleted in AcGFPE2, AcΔCp94 and AcΔp94 upon passage and were submolar in AcΔNp94 (Fig. 6.4A). In AcΔp94, only the *Pst*I fragment of 1240 nt, which is localized in the kanamycin resistance gene, was not fully deleted. This indicated that the moment of deletion and the actual size of the BAC vector deletions may differ between separate experiments and that there is probably not a well-defined deletion mechanism.

To investigate whether in addition to a deletion of the BAC vector parts, the expression cassette containing GFP and CSFV-E2 genes was also deleted, restriction digests with *Xho*I and *Kpn*I were carried out (Fig. 6.4C). Again, the results showed that all *Xho*I and *Kpn*I fragments derived from the BAC vector as well as from the GFP and CSFV-E2 genes (sizes of fragments on the left in Fig. 6.4C correspond to sizes on the physical map in Fig. 6.4B) were deleted upon passage in AcGFPE2, AcΔCp94 and AcΔp94 and were submolar in AcΔNp94 (Fig. 6.4C). In contrast to the deletions in the BAC vector and the expression cassette, the genomic *Xho*I-J, -K, -L and *Kpn*I-E fragments of viral origin (named after digests of the complete AcMNPV genome sequence; Ayres *et al.*, 1994) remained at nearly equimolar levels in the three mutants. In AcGFPE2, however, all fragments became submolar as a consequence of predominating non-*hr ori* concatemers with 3.2, 1.8, and 1.6 kb units (arrows, Fig. 6.4C). The results clearly indicated that, irrespective of the presence or absence of the non-*hr ori*, sequences from the BAC vector and the expression cassette were systematically deleted and that sequences of viral origin were retained upon passage in insect cells.

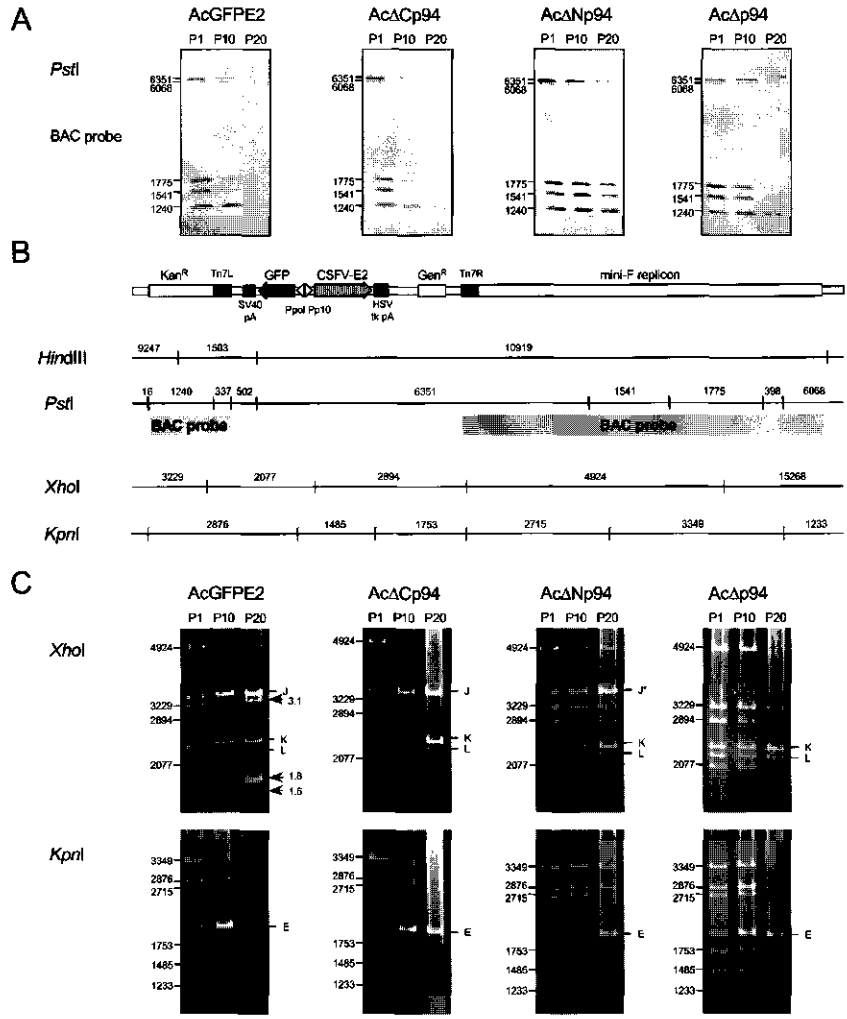

Figure 6.4. Restriction mapping of specific deletions in the BAC vector. A) Southern detection of a *Pst*I digest with a probe specific for the BAC vector. Sizes of BAC vector-specific restriction fragments are indicated on the left and correspond to the sizes in B. B) Schematic representation of the organization of the bacterial expression cassette containing GFP and CSFV-E2 genes under the control of AcMNPV polyhedrin and *p10* promoters, respectively. Sizes of *Hin*dIII, *Pst*I, *Xho*I and *Kpn*I restriction fragments (nt) are indicated. C) Restriction analysis of intracellular viral DNA with *Xho*I and *Kpn*I. Genomic *Xho*I and *Kpn*I fragments are indicated at the right with letters according to the complete genome sequence of AcMNPV (Ayres *et al.*, 1994). The increased size of the *Xho*I-J fragment due to a Cm^R insertion is indicated with an asterisk (J*). In AcΔp94 the *Xho*I-J fragment is not present, because of a disruption of the *Xho*I site (see also Fig. 1). Sizes of BAC vector-specific restriction fragments are indicated on the left and correspond to the sizes in B.

DISCUSSION

In this paper we investigated whether a deletion of the non-*hr ori* from the AcMNPV genome could prevent the accumulation of DIs. Three mutants were constructed with deletions in the *p94* coding sequence, of which the C terminus comprises the non-*hr ori*. Similar to SeMNPV (Pijlman *et al.*, 2002), the results showed that the AcMNPV non-*hr ori* is dispensable for *in vitro* replication and confirmed that *p94* is not essential for AcMNPV replication in cultured insect cells (Friesen & Nissen, 1990). Also, a *p94* gene is not present in the closely related BmNPV, which is a baculovirus with a very high sequence homology to AcMNPV (Kamita *et al.*, 1993). However, BmNPV has retained (essential) parts of the non-*hr ori*, implying that the non-*hr ori* may be important for virus replication (Kool *et al.*, 1994).

Passaging of the parental AcMNPV bacmid (AcGFPE2) resulted in the predominance of DI molecules containing reiterated non-*hr ori*s (Figs 2 and 3). Sequence overlaps on the junction sites of the concatenated non-*hr ori* molecules suggested that they were generated by homologous recombination during viral DNA replication (Pijlman *et al.*, 2002). The generation of non-*hr ori* DIs was also expected to occur for AcΔNp94, because this mutant only lacks the *p94* N-terminal part but still contains the five non-*hr ori* subdomains, which are required for optimal *ori* activity (Kool *et al.*, 1994; Fig. 6.1A). However, this was not the case. It may be that auxiliary sequences located in the adjacent N-terminal part of *p94* are necessary either for the excision of non-*hr ori*s from the genome, or are responsible for a higher *ori* activity, thereby giving the DIs a stronger replicative advantage. The latter hypothesis is supported by data from transient replication assays by Kool *et al.* (1994), who showed that plasmids containing the entire sequence from *Hin*dIII to *Eco*RV (see Fig. 6.1A) had a slightly greater replication ability than the smaller *Hin*dIII-*Xho*I region which is retained in the AcΔNp94 mutant. Thus, the AcMNPV non-*hr ori* is not essential for virus replication *in vitro*, and deletion of the non-*hr ori* and/or flanking auxiliary sequences prevent the accumulation of DIs enriched in non-*hr ori*s upon serial passage in insect cells.

In spite of the fact that the formation of non-*hr ori* DIs did not occur in the three recombinants, BV titers (based on GFP expression) and CSFV-E2 production decreased rapidly upon passaging of all viruses (Fig. 6.1B,C). We demonstrated that the non-essential BAC vector including the expression cassette was spontaneously deleted from the viral genome upon passage in insect cells (Fig. 6.4). This cassette

comprises a bacterial mini-F replicon (or BAC vector), two antibiotic resistance genes and two foreign genes (CSFV-E2 and GFP) under the control of baculovirus *p10* and polyhedrin promoters. Spontaneous baculovirus mutants carrying this deletion quickly became predominant upon passage, which explained the drop in foreign protein production levels.

Instability of mini-F plasmids, which are also known as bacterial artificial chromosomes (BACs), in eukaryotic cells has been reported to occur in several other cases. An infectious clone of the pseudorabies virus (PRV) was maintained as a stable BAC in *E. coli*, but reconstitution of the virus led to the spontaneous deletion of the BAC vector insertion upon transfection of mammalian cells (Smith and Enquist, 1999). Approximately 5-6 kb of flanking viral sequence was deleted along with the BAC vector sequence. In contrast, when the BAC vector was inserted at a different locus, the virus was stable, suggesting that the location of BAC vector insertion might also be important (Smith and Enquist, 2000). Wagner *et al.* (1999) showed that during construction of a mouse cytomegalovirus (MCMV) BAC, overlength genomes were not stable in mammalian cells. To overcome this problem, they designed duplicated viral sequences flanking the BAC vector insertion, allowing spontaneous excision by homologous recombination. Adler *et al.* (2001) further showed that excision of BAC vector sequences (by Cre-lox recombination) from cloned MHV-68 genomes was critical for reconstitution of wildtype properties. Similarly, insertion of the BAC vector in CMVs requires a deletion of non-essential genes, because CMVs only tolerate 5 kb of additional sequence in their genomes (Brune *et al.*, 2000).

Most likely, these properties of herpesvirus BACs are the result of physical limitations of the virus capsid, which can only package a genome of a defined (maximum) size. Maximum packaging capacity is also observed for other DNA viruses. An overlength of only 5% leads to unstable adenovirus and Epstein-Barr genomes (Bett *et al.*, 1993; Bloss and Sugden, 1994). For baculoviruses, a maximum packaging capacity may also exist, although the rod-shaped baculovirus nucleocapsids are presumed to be more flexible with respect to DNA content as they contain genomes of up to almost 180 kb (XcGV; Hayakawa *et al.*, 1999) and allow inserts of up to 25 kb (Roosien *et al.*, 1986). Still, the occurrence of spontaneous (major) deletions in baculoviruses is a general phenomenon. In SeMNPV, deletions up to 25 kb of non-essential sequences are routinely observed upon infection of cultured insect cells (Heldens *et al.*, 1996; Dai *et al.*, 2000; Pijlman *et al.*, 2002) and are located in the largest region between two adjacent *hrs* (SeMNPV *hr1* and *hr2*; IJkel *et al.*, 1999). In AcMNPV, deletions are frequently found in the EGT/DA26 locus (Kumar and Miller, 1987), which is located in the middle of AcMNPV inter-*hr* region

*hr*1-2 (Ayres *et al.*, 1994). Since *hr*s are believed to be involved as *ori* in viral DNA replication, we hypothesize that the occurrence of genomic deletions is more likely in regions with a low *ori* density. This may explain why deletions in the BAC vector sequence are likely to occur. Yet the BAC vector itself may also display a certain intrinsic genetic instability. Alternatively, the heterologous gene may confer a certain level of toxicity to the infected cells, thereby creating an added selection pressure against intact bacmids. However, toxicity in insect cells has never been observed with CSFV-E2, which is a commercialized baculovirus expression product used as the major constituent of a marker vaccine against classical swine fever (Van Rijn *et al.*, 1999). In addition, bacmids equipped with an expression cassette not containing CSFV-E2 also showed specific loss of BAC vector sequences (G.P. Pijlman, unpublished results).

In this paper we have shown that reconstitution in insect cells of infectious baculovirus from a bacmid is accompanied by genetic instability of BAC vector sequences. Recently we have obtained similar results with SeMNPV bacmid-derived viruses in cultured insect cells (G.P. Pijlman, unpublished results). Once the instability is removed by spontaneous deletion of the (non-essential) BAC vector during viral DNA replication, a more stable virus is generated, which predominates subsequent passages. The present observations may constitute a major concern for the utilization of bacmid-derived baculoviruses for the large-scale production of heterologous proteins, especially in insect-cell bioreactors involving many virus passages. Although the generation of mutant baculoviruses by the classical method is more time-consuming than the generation of a recombinant bacmid, it may yield a virus with greater stability. An improvement for the bacmid strategy would be to introduce the heterologous gene(s) at a more stable locus remote from the BAC vector insertion. Alternatively, a bacmid could be developed in which the BAC vector is deliberately excised (using Cre-lox recombination) upon replication in insect cells, while leaving the introduced heterologous gene(s) intact.

MATERIALS AND METHODS

Cells and virus

Spodoptera frugiperda (Sf-AE-21) cells (Vaughn *et al.*, 1977) were maintained at 27°C in Grace's supplemented insect medium (Gibco-BRL) with 10% foetal calf serum (FCS; Gibco-BRL). Routine cell culture maintenance was performed according to established procedures (Summers & Smith, 1987; King & Possee, 1992). Isolation and transfection of bacmid DNA to

Sf21 cells was done according to the Bac-to-Bac baculovirus expression system manual (Gibco-BRL). The bacmid-derived budden virus (BV) inoculum was defined as passage 1 (P1) and was used to initiate undiluted serial passaging in Sf21 cells at an initial MOI of 100 $TCID_{50}$ units/cell. Serial undiluted passaging was carried out as previously described (Pijlman *et al.*, 2001). Infectious BV titers were determined using the endpoint dilution assay (Vlak, 1979).

Deletion mutagenesis by ET-recombination in E. coli

For deletion mutagenesis of the *p94* coding sequence of the AcMNPV bacmid, 74-76 bp primers were designed with 50 bp 5'-ends flanking the deletion target region on the AcMNPV genome (Table 6.1). The 3'-ends of the primers annealed to the chloramphenicol gene of pBeloBAC11 (Shizuya *et al.*, 1992) from nt position 735 to 1671. PCR on pBeloBAC11 was performed using the Expand long-template PCR system (Roche) according to the manufacturer's protocol, giving a product of 1048 bp. The PCR product was purified using the High pure PCR purification kit (Roche), digested with *Dpn*I to eliminate residual pBeloBAC11 template, phenol/chloroform extracted and ethanol precipitated. For ET-recombination, 70 ml of LB medium was inoculated with 0.7 ml of an overnight culture of *E. coli* DH10β containing the AcMNPV bacmid and homologous recombination helper plasmid pBAD-αβγ (Muyrers *et al.*, 1999). At an OD_{600} of 0.1-0.15, ET-protein expression from pBAD-αβγ was induced by the addition of 0.7 ml 10% L-arabinose. The cells were harvested at an OD_{600} of 0.3-0.4 and made electrocompetent by 3 subsequent washes with ice-cold 10% glycerol. The cells were transformed with approximately 0.5 μg purified PCR product in 2 mm electroporation cuvettes (Eurogentec) using a Biorad Gene Pulser (2.3 kV, 25 μF, 200 Ω). The cells were resuspended in 1 ml LB medium and incubated for 1h at 37°C and subsequently spread on agar plates containing kanamycin and chloramphenicol. Colonies were picked and screened by restriction analysis and PCR.

prime	*location*	*description*	*sequence*	*recombinant*
DZ 209	113868 -113917	**ET for p94**	*TTTTATTTACGACAAATATTGACTCGTTGTTCAGAAAGT* *TTAATAAGCTTCCTTAGGTTTAAGGGCACCAATAACTG*	AcΔCp94, AcΔp94
DZ 210	115178 - 115227	**ET rev Cp94**	*TGTTTGAAACAATTCAGGGCAGTTTGACCAAGTTTAAAA* *GCCGACTCGAGCCTAAGGTTCCTGTGCGACGGTTAC*	AcΔCp94
DZ 211	115973 - 116022	ET rev p94	*AGAATATAAACTTGCTCGACGGCAAGTTGAAACTTTTGT* *ACATGGTGACCCCTAAGGTTCCTGTGCGACGGTTAC*	AcΔNp94, AcΔp94
DZ 223	115134 - 115183	ET for Np94	*ATTGAATTTTTAATCTCTTTATTTTGGCTCCATAAAAGA* *GGAAACTCGAGCCTTAGGTTTAAGGGCACCAATAACTG*	AcΔNp94

Table 6.1. Oligonucleotides used for site-specific genomic deletions by ET-recombination. The 50 nt long viral flanking sequence used for homologous recombination (italics) is followed by a *Bsu*36I restriction site (underlined). The 3'end of the ET-primers is used for PCR amplification of the chloramphenicol resistance gene from pBeloBAC11. Locations of primers are according to the complete AcMNPV genome sequence (Ayres *et al.*, 1994).

Donor plasmid construction for GFP and CSFV-E2 expression

To introduce the GFP and CSFV-E2 genes in the (mutant) AcMNPV bacmids, a donor plasmid pFBD-GFPE2 was constructed. First, the red-shifted GFP gene (Davis and Vierstra, 1998) was cloned as an *Eco*RI/*Hin*dIII fragment into the pFastBac-DUAL vector (Invitrogen) under the polyhedrin promoter to generate pFDB-GFP. To create a translational start codon for CSFV-E2, pSeMO7 (Dai *et al.*, 2000) was digested with *Bam*HI and *Xba*I and a linker was inserted. This linker was made with oligonucleotides DZ195 (5'-GATC*ATCGAT*-TATGGATCCT-3') and DZ196 (5'-CTAGAGGATCCAT*AATCGAT*-3'), and is *Bam*HI compatible, contains a *Cla*I site (italics) followed by a ATG-*Bam*HI fusion (underlined) and an *Xba*I site. The CSFV-E2 gene was then cloned as a *Bam*HI fragment from pAcE2 (Van Oers *et al.*, 2001) behind the ATG of the inserted linker. The CSFV-E2 gene was subcloned as a *Cla*I fragment into pBluescript, and then cloned downstream of the *p10* promoter as a *Xho*I-*Xba*I fragment into pFBD-GFP digested with *Xho*I and *Nhe*I, generating pFBD-GFPE2. Before transposition, *E. coli* DH10β containing the recombinant AcMNPV bacmids were transformed with transposition helper plasmid pMON7124 (Luckow *et al.*, 1993). The protocol from the Bac-to-Bac manual (Gibco-BRL) was followed to transpose the GFP and CSFV-E2 genes from pFBD-GFPE2 into the attTn7 transposon integration site of the AcMNPV bacmids.

Viral DNA isolation, Southern hybridization, molecular cloning and sequencing

Intracellular viral DNA was isolated as previously described (Summers and Smith, 1987). Digested viral DNA was run overnight in ethidium bromide-stained 0.6% agarose gels, and Southern blotting was performed by standard capillary upward blotting (Sambrook *et al.*, 1989) using Hybond-N (Amersham Pharmacia) filters. As a DNA size marker, λ DNA digested with *EcoRI*/*Hin*dIII/*Bam*HI was used. Random-primed DNA probes for Southern hybridization were made using the DIG non-radioactive nucleic acid labelling and detection system (Roche). For the non-*hr ori* probe, a PCR product (1036 bp) of the AcMNPV non-*hr ori* was made with primers DZ123 5'- TGCGGCCAGGTTTTGTAGAATG-3' (nt 114056 - 114077: Ayres *et al.*, 1994) and DZ124 5'- GCATGGAACGCGTTTGTCAC-3' (nt 115072 - 115091), purified using the High pure PCR purification kit (Roche), and DIG-labeled overnight. For the BAC vector probe, BAC-Bsu36I (Pijlman *et al.*, 2002) was DIG-labeled overnight. Hybridization and colorimetric detection with NBT-BCIP (Roche) were performed according to the manufacturer's recommendations. Hypermolar viral *Hin*dIII bands were cut from the gel, purified with the Matrix gel extraction system (Marlingen), and cloned into pBluescript by electrotransformation of *E. coli* DH5α using standard methods (Sambrook *et al.*, 1989). Automatic sequencing was performed at Baseclear, The Netherlands. Sequence analyses were performed using BLAST (Altschul *et al.*, 1997). *In silico* reassembling of bacmid sequences and computational predictions of restriction digests were done using the Lasergene DNAStar package.

SDS-PAGE and immunodetection

Protein samples were analyzed in 12% SDS-PAGE gels as described in Sambrook *et al.* (1989). Protein masses were determined using the Low Molecular Weight protein marker (Amersham Pharmacia Biotech). Semi-dry blotting was performed onto an Immobilon™-P membrane (Millipore) using a Tris-Glycine buffer (25 mM Tris base, 192 mM glycine, 10% (v/v) methanol, pH 8.3). Immobilon-P membranes were blocked in 2% low-fat milk powder (Campina, The Netherlands) in TBS (0.2 M NaCl, 50 mM Tris-HCl, pH 7.4). The marker was visualized on the membrane by Ponceau-red staining (Sambrook *et al.,* 1989). Immunodetection of CSFV-E2 was performed by incubation with a monoclonal antibody (mAb A18, Intervet International B.V.) diluted 1:10000 in TBS with 1% low-fat milk powder for 1 h at room temperature. Subsequently, anti-mouse antibody conjugated with horseradish peroxidase (Amersham) was used at a concentration of 1:10000 and detection was performed with an Enhanced Chemiluminescent-light (ECL) Detection Kit (Amersham).

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CHAPTER 7

Validation of baculovirus expression vectors with enhanced stability in continuous cascaded insect-cell bioreactors

Continuous protein production with baculovirus expression vectors in insect-cell bioreactors is characterized by a dramatic drop in heterologous protein production within a few weeks. This is mainly due to the spontaneous deletion of the heterologous gene(s) from the baculovirus genome and/or to the rapid accumulation of defective interfering baculoviruses (DIs). Cell culture experiments with bacmid-derived baculoviruses showed that spontaneous deletions in the foreign bacterial artificial chromosome (BAC) sequences of the bacmid-derived viruses readily occurred, causing the deletion mutants to predominate at the expense of the parental virus. The spontaneous deletions correlated with a low density of baculovirus homologous (repeat) regions (*hr*s), which contain multiple repeats and which are located dispersed throughout the baculovirus genome. These *hr*s are believed to act as origins of viral DNA replication (*ori*s) and as enhancers of transcription. To test the hypothesis that deletions are more likely to occur in regions with a low *ori* density, the properties of bacmid-derived baculoviruses with an additional *hr* in the unstable BAC sequences were compared to the standard bacmid-derived baculovirus in a continuous cascaded insect-cell bioreactor configuration. All viruses were equipped with an expression cassette containing a green fluorescent protein (GFP) gene and a gene encoding the classical swine fever virus E2 glycoprotein (CSFV-E2). The insertion of an extra *hr* in the BAC vector led to improved genetic stability of adjacent sequences, resulting in prolonged protein expression. The maintenance of the BAC sequences appeared to be dependent on the orientation of the inserted *hr*. The advantages of the utilization of *hr*s to improve the stability of baculovirus expression vectors for the large-scale protein production in insect-cell bioreactors are discussed.

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INTRODUCTION

The baculovirus insect-cell expression system is highly versatile and widely used for the expression of recombinant proteins in a eukaryotic environment (Kitts, 1996; Kost and Condreay, 1999). Pure stocks of recombinant baculoviruses can nowadays be obtained within two weeks (Luckow *et al.*, 1993), specific production levels are high, and large-scale insect-cell culture in bioreactors is well established (Agathos, 1996; Ikonomou *et al.*, 2003). Moreover, as a result of proper folding and post-translational modification, the produced proteins often show biological activities similar to their authentic counterparts (Vialard *et al.*, 1995), which is of great importance for their utilization as diagnostics, (subunit) vaccines and pharmaceuticals.

However, continuous production of baculovirus-expressed proteins in insect-cell bioreactors is limited to a few weeks. The dramatic decrease in production has been ascribed to the interference of defective virus mutants with the intact baculoviruses (Kool *et al.*, 1991). The phenomenon is known as the passage effect (reviewed by Krell, 1996) and is apparent in continuous and large-scale (batch) productions involving many virus passages. The mutant viruses often lack the heterologous gene of interest (Van Lier *et al.*, 1992) or other genes involved in major late gene expression (Van Lier *et al.*, 1994), which in the latter case makes them dependent on an intact helper virus for replication. Defective interfering baculoviruses (DIs) accumulate due to their replication advantage, because the replication of smaller genomes with a higher density in origins of DNA replication (*ori*s) is favoured.

Continuous baculovirus production in cascaded insect-cell bioreactors is analogous to serial virus passage in insect cell monolayers, with the difference that in the latter case the virus passages are discrete (De Gooijer *et al.*, 1992). In serial passage experiments in monolayers, DIs are rapidly generated (Pijlman *et al.*, 2001), coinciding with a sharp drop in heterologous protein production.

Baculoviruses contain two types of *ori*s, the first type being the well-characterized homologous regions (*hr*s) (Pearson *et al.*, 1992), which are repeated sequences scattered throughout the genome. Beside their *ori* activity, *hr*s are also active as transcriptional enhancers (Theilmann and Stewart, 1992). The second type of *ori* is not related to *hr*s and therefore designated non-*hr ori* (Kool *et al.*, 1994). In particular, these non-*hr oris* appeared to be preferentially amplified in DIs during serial passage experiments with AcMNPV (Lee and Krell, 1992; Pijlman *et al.*, 2003b) and SeMNPV (Pijlman *et al.*, 2002). Subsequent deletion of baculovirus non-

*hr ori*s resulted in recombinants with enhanced stability of virus and protein production upon serial passage in monolayers (Pijlman *et al.*, 2002, 2003b).

Although the generation of baculovirus DIs could be prevented to a large extent by elimination of the non-*hr ori*, non-essential sequences comprising the BAC insert and heterologous gene expression cassette were spontaneously deleted and led to a rapid decrease in heterologous protein production (Pijlman *et al.*, 2003b). Similarly, passage of baculovirus SeMNPV in cell culture is prone to major deletions in the hypervariable *Xba*I-A restriction fragment containing a contiguous series of non-essential genes (SeMNPV ORFs 15 to 36; Pijlman *et al.*, 2003a).The occurrence of deletions in this particular genomic region is a common phenomenon of SeMNPV infection in cell culture, but these deletions do not compromise BV or protein production (Heldens *et al.*, 1996; Dai *et al.*, 2000; Pijlman *et al.*, 2003a).

The common feature of spontaneous deletions in this SeMNPV *Xba*I-A fragment and the BAC vector is that the deleted sequences are not required for virus replication in cell culture, and are located in regions with a large distance between *hr*s (Pijlman *et al.*, 2003b). The deletions in SeMNPV *Xba*I-A are located in the largest inter-*hr* region (*hr*1-*hr*2 encompassing 30.2 kb) present in the wildtype SeMNPV genome (IJkel *et al.*, 1999). Since *hr*s are thought to act as *ori* in viral DNA replication (Pearson *et al.*, 1992), incomplete replication may lead to deletions of sequences in between two adjacent *ori*s. Therefore the hypothesis was put forward that genomic deletions are more likely to occur in regions with a low *ori* density. This may explain why deletions in the SeMNPV *Xba*I-A fragment and in the BAC vector sequence readily occur upon infection of insect cells (Pijlman *et al.*, 2003b).

In this report, we evaluated the application of improved SeMNPV expression vectors (with a deleted non-*hr ori*) in a continuous cascaded insect-cell bioreactor configuration and investigated the effect of additional *hr*s on the genetic stability of the flanking sequences.

RESULTS

Construction of recombinant baculovirus expression vectors

To test the role of *hr*s in the genetic stability of their flanking sequences, an additional copy of *hr*1 was inserted in the (genetically unstable) BAC vector sequence of the SeMNPV bacmid, with almost equal distance between *hr*6 and the authentic *hr*1 of the original SeMNPV bacmid spanning a region of about 35.3 kb

(Fig. 7.1A, left). This insertion generates two smaller inter-*hr* fragments of 18.2 and 21.4 kb, respectively (Fig. 7.1A, right).

In the first experiment, the extra *hr*1 was inserted in the opposite orientation relative to the parental *hr*1, thereby reducing the possibility of a spontaneous deletion of the intermediate region through homologous recombination between the added and the parental *hr*1 sequences. This bacmid (SeBAC$^{\Delta nonhr}$+hr^{inv}) was compared to the original SeMNPV bacmid (SeBAC$^{\Delta nonhr}$). For both bacmids the non-*hr ori* was

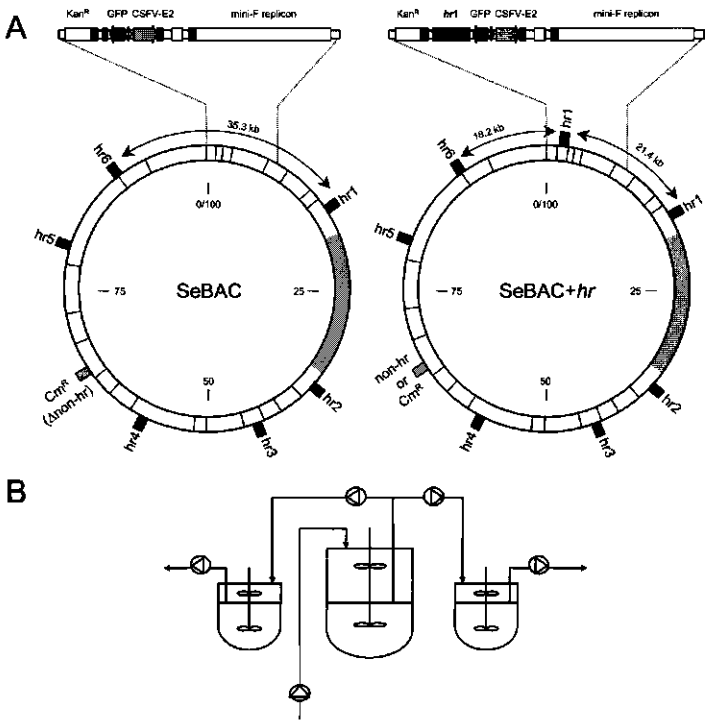


Figure 7.1. A) Schematic representation of SeMNPV bacmids. The circle represents the viral dsDNA genome with *Xba*I restriction fragments. The 6 homologous regions (*hr*s) and the non-*hr ori* are indicated with bars on the outside of the circular genome. The BAC vector insertion containing a kanamycin resistance gene (Kan^R), a bacterial mini-F replicon and the gene expression cassette containing GFP and CSFV-E2 genes are enlarged at the top. The distance (in kb) between *hr*6 and *hr*1 is indicated with a double arrow (left). On the right, the additional copy of *hr*1 is indicated, without orientation, and distances between *hr*6-and the extra *hr*1 and the authentic *hr*1 are indicated with double arrows. The commonly deleted region between *hr*1 and *hr*2 in the *Xba*I-A fragment (Dai *et al.*, 2000) is indicated in grey. The SeMNPV bacmids either have retained the non-*hr ori* or this sequence has been replaced by a chloramphenicol resistance gene (Cm^R). B) Schematic representation of the cascaded continuous insect-cell bioreactor system. The system consists of two head-space aerated parallel infection reactors (0.75 dm^3 each) connected to a cell-growth reactor (1.5 dm^3) and has a mean residence time of 72h. Pumps and stirrers are indicated.

deleted to prevent the formation of DIs. In the second experiment, the role of the orientation of the extra *hr-ori* was further investigated. Therefore, a bacmid was constructed with a copy of *hr*1 inserted in the same orientation as the parental *hr*1 (SeBAC$^{\Delta nonhr}$+hr). SeBAC$^{\Delta nonhr}$+hr was compared to SeBAC+hr^{inv}, which contained the *hr*1 in the opposite orientation (similar to the first experiment), but the latter virus retained the non-*hr ori* as an additional control to rule out unexpected effects of a non-*hr ori* deletion.

The four different bacmid-derived viruses were tested in parallel in two subsequent runs in a continuous cascaded bioreactor configuration with Se301 insect cells (Fig. 7.1B). The cells were cultured in a 1.5 liter continuous stirred-tank reactor (CSTR). The effluent was led to two separate reactors, each containing half the volume of the cell-growth reactor, where the cells were infected with the respective viruses (Van Lier *et al.,* 1992). Both recombinant viruses were equipped with an expression cassette containing a green fluorescent protein (GFP) gene to follow the infection levels by intracellular fluorescence and to measure virus titers, and a gene encoding the secreted form of classical swine fever virus E2 surface glycoprotein (CSFV-E2) as a model for (glyco)protein production (Hulst *et al.,* 1993).

EXPERIMENT 1. EFFECT OF *HR-ORI* INSERTION

Exp 1. Cell concentration and viability

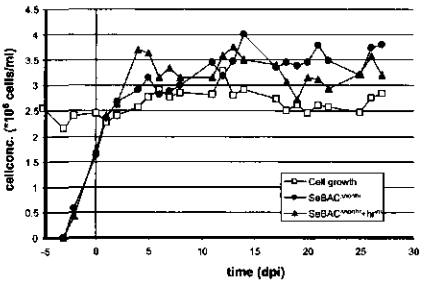
In this experiment, the effect of the insertion of an *hr-ori* on the stability of the adjacent BAC sequences with a protein expression cassette was investigated in continous cascaded bioreactor system (Van Lier *et al.*, 1994). The first reactor was infected with SeBAC$^{\Delta nonhr}$ (Fig. 7.1A and 7.1B, left), while the second reactor run in parallel was infected with SeBAC$^{\Delta nonhr}$+hr^{inv}, which contains a *hr*1 insertion (Fig. 7.1A and 7.1B, right) in the opposite orientation relative to the authentic *hr*1 still present in the virus.

The cell-growth reactor was operated continuously for more than a month and reached a steady-state level of approximately $2.5*10^6$ cells/ml (Fig. 7.2A). The infection reactors were coupled to the infection reactor at –3 dpi and were operated continuously. After three days the infection reactors (~$1.6*10^6$ cells/ml) were infected with an MOI of 0.01 (0 dpi; Fig. 7.2A). Until 5 dpi the cell concentration in the SeBAC$^{\Delta nonhr}$+hr^{inv} containing reactor was higher than in the reactor containing SeBAC$^{\Delta nonhr}$, but dropped after 5 dpi due to virus infection. After 5 dpi, the cell

concentrations in both infection reactors remained slightly higher than the cell-growth reactor.

The viability in the cell-growth reactor was 90% or higher during the entire experiment (Fig. 7.2B). The cell viability in both infection reactors dropped directly after virus inoculation to approximately 70-75% (SeBAC$^{\Delta nonhr}$) and 60-65% (SeBAC$^{\Delta nonhr}$+hr^{inv}). The sharp drop in viability for SeBAC$^{\Delta nonhr}$ occurred earlier (at 2 dpi) than for SeBAC$^{\Delta nonhr}$+hr^{inv} (at 7 dpi), indicating that the infection with SeBAC$^{\Delta nonhr}$ developed faster, which is in accordance with the lower cell densities until 5 dpi.

A



B

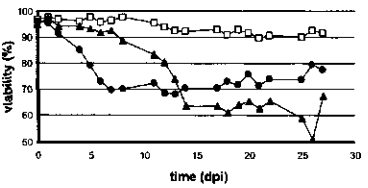


Figure 7.2. A) Viable cell concentrations of the three reactors during the first experiment. The point of virus inoculation is indicated with a vertical line (0 dpi). B) Cell viabilities (in % living cells) in the three reactors as determined by trypan blue exclusion.

Exp 1. Budded virus and heterologous protein production

Budded virus (BV) titers in the supernatant were determined by endpoint dilution and were scored in two ways, based on GFP expression of infected cells, and based on cytopathologic effect (CPE) and cell lysis due to baculovirus infection (Fig. 7.3A). The GFP-based titers for SeBAC$^{\Delta nonhr}$ increased after infection till 10^4 $TCID_{50}$/ml at 2dpi, and became zero at 8 dpi (Fig. 7.3A, left panel). When the cells were scored for infection only, higher BV titers were determined for the same supernatants, suggesting that infectious viruses had been generated that did not express GFP. The SeBAC$^{\Delta nonhr}$+hr^{inv} titers based on GFP expression and those based on cytopathology were similar until 18 dpi (Fig. 7.3A, right panel). After that, GFP-based titers of SeBAC$^{\Delta nonhr}$+hr^{inv} decreased with time, whereas the infectious BV titers stayed at a similar level above 10^5 $TCID_{50}$/ml, which was higher than with SeBAC$^{\Delta nonhr}$.

Secreted CSFV-E2 was measured by ELISA in samples from the supernatant (Fig. 7.3B). The E2 production with SeBAC$^{\Delta nonhr}$ increased to a maximum at 5 dpi, reaching a level of only 1.3 mg/l. After that, the production rapidly declined to become zero at 14 dpi (Fig. 7.3B, left panel). In contrast, the E2 production levels with SeBAC$^{\Delta nonhr}$+hr^{inv} at 5 dpi were much higher than SeBAC$^{\Delta nonhr}$ (Fig. 7.3B, right panel). The production even further increased to a maximum of almost 30 mg/l at 12 dpi. After that, the production eventually decreased to low levels at the end of the experiment (27 dpi).

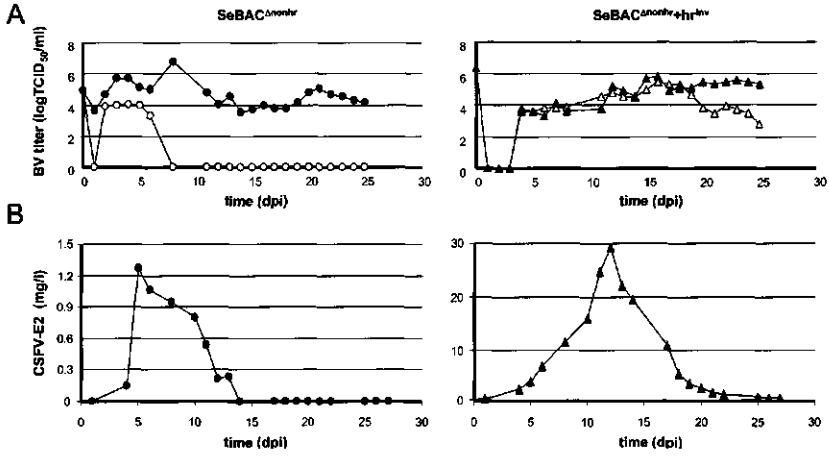


Figure 7.3. A) Budded virus (BV) titers determined by end point dilution (logTCID$_{50}$/ml) in the two infection reactors, scored on GFP-expression (open symbols), or on infection only (solid symbols). B) CSFV-E2 production levels in the two infection reactors determined by ELISA (in mg/l).

Exp 1. Viral DNA analysis

Intracellular viral DNA was purified from both infection reactors, digested with *Xba*I, *Xho*I and *Pst*I and the fragments separated in 0.6% agarose gels (not shown). The restriction patterns at 3 dpi indicated that SeBAC$^{\Delta nonhr}$ and SeBAC$^{\Delta nonhr}$+hr^{inv} contained major deletions in the hypervariable *Xba*I-A fragment of 15 and 10 kb, respectively. Deletions in this genomic region are common upon replication of SeMNPV in vitro (Heldens *et al.*, 1996; Dai *et al.*, 2000). For both viruses also fragments corresponding to the BAC vector insertion became submolar relative to genomic restriction fragments of the parental virus. This indicated that BAC vector sequences were specifically removed from the bacmid-derived viral genome upon passage.

To investigate in more detail whether the BAC vector sequences were fully or only partially deleted, a *Pst*I digest of viral DNA was hybridized with a probe specific for BAC vector sequences (Fig. 7.4). For SeBAC$^{\Delta nonhr}$ (Fig. 7.4, left), the 6293 bp fragment was only present until 12 dpi at submolar levels, whereas the fragments of 1775, 1541 (both from the mini-F replicon) and 1240 bp were already gone at 3 dpi. The largest band on the blot corresponds to the 10396 and/or 10782 bp viral fragment(s) on either side of the BAC vector (Fig. 7.4, center), which were retained. For SeBAC$^{\Delta nonhr}$+hr^{inv} (Fig. 7.4, right), the 1240 bp fragment containing the kanamycin resistance gene, was retained until the end of the experiment (27 dpi). The 6293 bp fragment was present until 12 dpi, whereas the fragments of 1775 and 1541 (mini-F replicon) became submolar with increasing dpi. Similar to SeBAC$^{\Delta nonhr}$, the largest band corresponds to the 10396 and/or 10782 bp viral fragment(s) on either side of the BAC vector (Fig. 7.4, center). Similar blots hybridized with GFP and CSFV-E2 specific probes (not shown) indicated that for SeBAC$^{\Delta nonhr}$ the GFP gene could only be detected until 5dpi, and the CSFV-E2 gene until 8 dpi. In contrast, the SeBAC$^{\Delta nonhr}$+hr^{inv} blots showed that both GFP and CSFV-E2 genes were still present at 22 dpi, although less abundant than at the start. This explains the eventual decrease in GFP-based titers and CSFV-E2 production after 13 dpi with this recombinant.

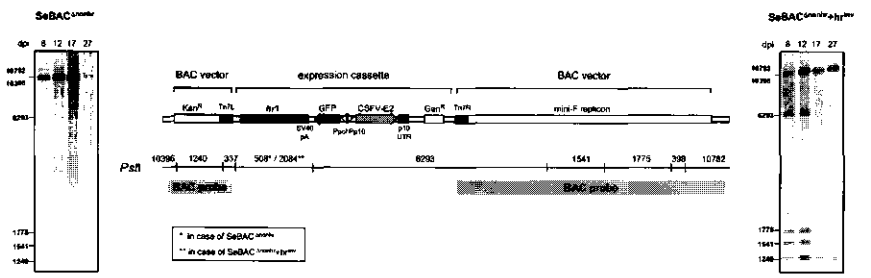


Figure 7.4. Analyis of BAC vector deletions generated upon infection with the bacmid-derived viruses. Intracellular viral DNA was purified at several days post infection (dpi) and digested with *Pst*I, separated in agarose gels and transferred to a nylon membrane. The DNA on the blots was detected with a DIG-labelled probe specific for BAC vector sequences. Sizes of BAC vector *Pst*I fragments are indicated on the left of the blots (left and right, resp.) and correspond to the *Pst*I fragment sizes in the schematical representation of the BAC vector region in the SeMNPV bacmids (center). The BAC vector sequence contains a kanamycin resistance gene (Kan^R), Tn7L and Tn7R transposition sites, and a bacterial mini-F replicon. The internal expression cassette contains a gentamycin resistance gene (Gen^R), and GFP and CSFV-E2 genes under control of SeMNPV polyhedrin and p10 promoters, respectively. An additional copy of *hr*1 is present in SeBAC$^{\Delta nonhr}$+hr^{inv} only. The relative sizes of the *Pst*I fragments and the length of the BAC vector DIG-probe are indicated at the bottom.

Thus, the entire BAC vector including the GFP and CSFV-E2 genes was deleted within 8 days upon infection with SeBAC$^{\Delta nonhr}$, whereas for SeBAC$^{\Delta nonhr}$+hr^{inv} GFP and E2 were still detected at 22 dpi although clearly less abundant. This suggested that increased genetic stability was obtained after insertion of an extra *hr-ori* in the BAC vector of the SeMNPV bacmid.

EXPERIMENT 2. EFFECT ORIENTATION OF *HR-ORI* INSERTION

Exp 2. Cell concentration and viability

In the second experiment, the effect of the orientation of the inserted *hr-ori* on the stability of the non-essential BAC vector was studied. One reactor was infected with SeBAC$^{\Delta nonhr}$+hr, which contains a *hr*1 insertion in the same orientation relative to the original *hr*1. The parallel reactor was infected with SeBAC+hr^{inv}, which contains a *hr*1 insertion in the opposite (inv) orientation relative to the original *hr*1 still present in the virus. In the first experiment it was shown that such a *hr*1 insertion enhanced the genetic stability of adjacent sequences. In contrast to the first experiment, it was decided to leave the non-*hr ori* intact in SeBAC+hr^{inv}, as an additional control for unexpected effects of a non-*hr ori* deletion. The bacmid-derived viruses from the second experiment can then be compared with the first experiment, where the infection of SeBAC$^{\Delta nonhr}$ +hr^{inv} was studied.

The cell-growth reactor was inoculated with Se301 cells at a concentration of $3*10^5$ cells/ml (Fig. 7.5A). After the cascaded bioreactor system was operated continuously for 7 days, the cell-growth reactor reached a stable viable-cell concentration of approximately $0.7*10^6$ cells/ml (0 dpi). Both infection reactors had a cell concentration between $1.3*10^6$ and $1.5*10^6$ cells/ml and were infected with an MOI of 0.01 (Fig. 7.5A). After 8 dpi the cell concentrations in all three reactors were similar throughout the experiment, indicating that cell growth was (partially) arrested in the infection reactors. Between 5 and 20 dpi, the cell concentration in the cell-growth reactor increased to reach a maximum of $3*10^6$ cells/ml (Fig. 7.5A), but this did not compromise the comparison between the two (parallel) infection reactors.

The viability in the cell-growth reactor was higher than 90% during the entire experiment (Fig. 7.5B). Directly after virus inoculation the cell viabilities in both infection reactors sharply dropped to approximately 55% at 10 dpi (Fig. 7.5B). After 20 dpi the viability of the SeBAC$^{\Delta nonhr}$+hr infected cells was higher (70%) than the SeBAC+hr^{inv} infected cells (60-65%), suggesting that the infection level with SeBAC+hr^{inv} remained higher.

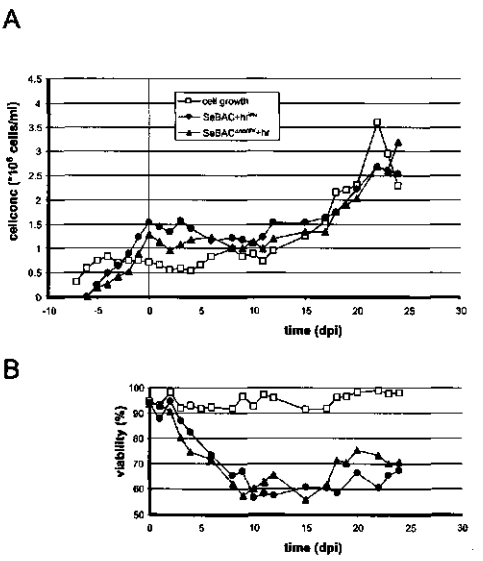


Figure 7.5. A) Viable cell concentrations of the three reactors during the second experiment. The point of virus inoculation is indicated with a vertical line (0 dpi). B) Cell viabilities (in % living cells) in the three reactors as determined by trypan blue exclusion.

Exp 2. Budded virus and heterologous protein production

The SeBAC+hr^{inv} BV titers based on GFP expression and those based on virus infection only were similar until 8 dpi (Fig. 7.6A, left panel). After that, GFP-based titers decreased and were lower than the BV titers based on cytopathology. Both titers were similar at 18 dpi. The GFP-based titers for SeBAC$^{\Delta nonhr}$+hr increased after

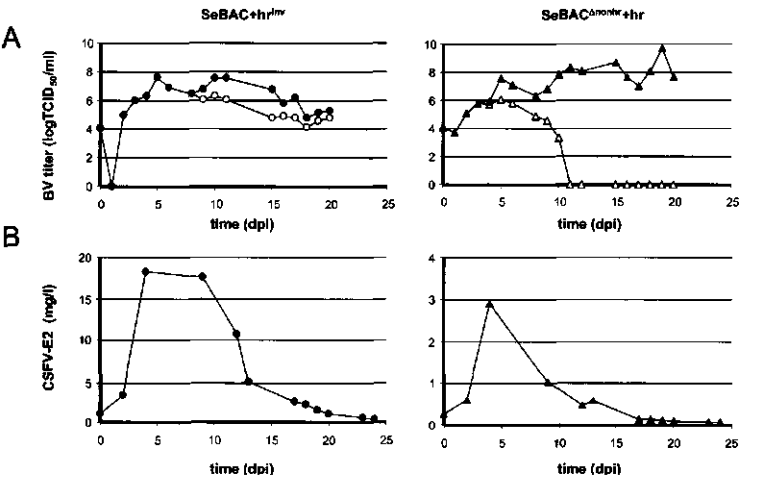


Figure 7.6. A) Budded virus titers determined by end point dilution (logTCID$_{50}$/ml) in the two infection reactors, scored on GFP-expression (open symbols), or on infection only (solid symbols). B) CSFV-E2 production levels in the two infection reactors determined by ELISA (in mg/l).

infection till 10^6 $TCID_{50}$/ml at 3 dpi, and after that decreased rapidly to become zero at 11 dpi (Fig. 7.6A, right panel). When the cells were scored for virus infection only, high BV titers of 10^8 $TCID_{50}$/ml were determined, suggesting that (mutant) viruses had been generated that did not express GFP. Noticeably, these titers increased significantly at the same moment the GFP expression was rapidly declining. This may be the result of a gain in replication speed due to spontaneous deletions leading to smaller genomes.

The E2 production with SeBAC+hr^{inv} was the highest from 4 till 9 dpi, reaching a level of nearly 18 mg/l (Fig. 7.6B, left panel). After that, the production decreased to lower levels at the end of the experiment (24 dpi). The maximum E2 production level with SeBAC$^{\Delta nonhr}$+hr at 4 dpi was much lower (3 mg/l) than SeBAC+hr^{inv}, and rapidly declined to low levels (Fig. 7.6B, right panel).

Exp 2. Viral DNA analysis

Intracellular viral DNA was purified from both infection reactors, digested with *Xba*I, *Xho*I and *Pst*I and separated in 0.6% agarose gels (not shown). Similar to the first experiment, at 3 dpi both SeBAC+hr^{inv} and SeBAC$^{\Delta nonhr}$+hr contained major deletions in the hypervariable *Xba*I-A fragment of 18 and 10 kb, respectively.

To examine putative deletions of the BAC vector sequences, *Pst*I digest of SeBAC+hr^{inv} and SeBAC$^{\Delta nonhr}$+hr were hybridized with a BAC vector probe as in Fig. 7.4 (Fig. 7.7). For SeBAC+hr^{inv}, the 6293, 1775, 1541 (all from the mini-F replicon) and 1240 bp fragments were retained until 19 dpi, although less abundant after 6 dpi (Fig. 7.7, left). The second largest band on the blot corresponds to the 10396 and/or 10782 bp viral fragment(s) on either side of the BAC vector (Fig. 7.7, left). A novel fragment of around 14 kb appeared at 10 dpi (Fig. 7.7, left panel, arrowhead) and became more abundant at 19 dpi. This fragment was the result of a deletion of the BAC vector, thereby joining the remnants of *Pst*I fragments of 6293 and 10782 bp. For SeBAC$^{\Delta nonhr}$+hr, the 6293, 1775, 1541 (both from the mini-F replicon) and 1240 bp fragments were only present until 10 dpi (Fig. 7.7, right). A novel fragment of around 20 kb appeared (Fig. 7.7, right, arrowhead), which could only be the result of a deletion in the BAC vector, thereby joining the remnants of *Pst*I fragments of 10396 and 10782 bp. Blots hybridized with GFP and CSFV-E2 specific probes showed that both GFP and CSFV-E2 genes could be detected until 22 dpi for SeBAC+hr^{inv}, although slightly less abundant than at 6 dpi (not shown). The SeBAC$^{\Delta nonhr}$+hr blot showed that the GFP gene was only present until 12 dpi, and the CSFV-E2 gene until 22 dpi, although much less abundant than at 6 dpi (not shown). In conclusion,

these results showed that the increase in genetic stability was dependent on the orientation of the extra *hr-ori*. Moreover, the presence of the non-*hr ori* in SeBAC+hr^{inv} did not lead to the generation of DIs and had no influence on the stabilizing effect of the extra *hr-ori*.

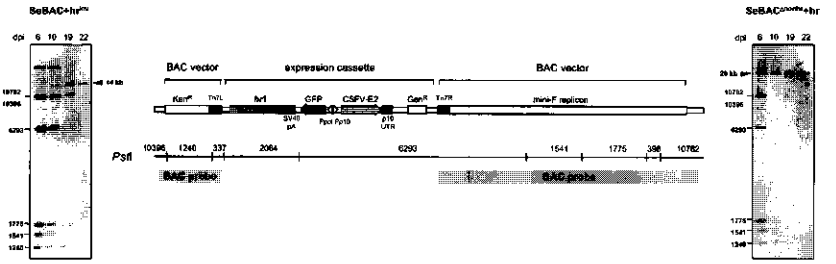


Figure 7.7. Analyis of BAC vector deletions generated upon infection with the bacmid-derived viruses. DNA on Southern blots were detected with a DIG-labelled probe specific for BAC vector sequences. Sizes of BAC vector *Pst*I fragments are indicated on the left of the blots (left and right, resp.) and correspond to the *Pst*I fragment sizes in the schematical representation of the BAC vector region in the SeMNPV bacmids (center). Both bacmids contain an additional copy of *hr*1, but in a different orientation (+hr vs. +hr^{inv}). The relative sizes of the *Pst*I fragments and the length of the BAC vector DIG-probe are indicated at the bottom.

DISCUSSION

Previously it was shown that the genetic stability of SeMNPV and AcMNPV in cell culture could be improved by the deletion of the non-*hr ori*. Such a modification prevents the accumulation of DIs (Pijlman *et al.*, 2002; Pijlman *et al.*, 2003b). However, the heterologous genes present in the BAC vector insertion of the AcMNPV bacmid appeared to be spontaneously deleted upon passage in insect cells. It was hypothesized that this spontaneous deletion is correlated with the low *ori* density of the deleted region (Pijlman *et al.*, 2003b).

In this paper we validated this hypothesis by studying the effect of baculovirus homologous regions (*hr*s) on the genetic stability of adjacent sequences. This was done in continuous insect-cell bioreactors with the bacmid-derived SeMNPV expression vectors, which did not accumulate DIs in cell culture experiments (Pijlman *et al.*, 2002). The insertion of an extra copy of *hr*1 in the BAC vector of SeMNPV bacmids in an opposite orientation relative to the native SeMNPV *hr*1 delayed the deletion of adjacent sequences (Figs 7.3 and 7.4). The stabilized sequences included the heterologous GFP and CSFV-E genes, and therefore the expression of these

recombinant proteins in a continuous insect-cell bioreactor configuration was improved.

The increase in genetic stability of adjacent heterologous gene upon insertion of the additional *hr* is most likely due to the activity of the *hr* as origin of DNA replication (*ori*) (Kool *et al.*, 1994). During baculovirus infection, DNA replication also is likely to be initiated from the extra *hr*, and therefore the flanking sequences are less likely to be deleted. The importance of *hr* density and the spatial distribution along the baculovirus genome in the generation of deletions may also be exemplified by the occurrence of spontaneous deletions of non-essential sequences in the largest inter-*hr* fragment of SeMNPV (*Xba*I-A, see also Fig. 7.1). These deletions readily occur upon infection in insect cells (Heldens *et al.*, 1996; Dai *et al.*, 2000; Pijlman *et al.*, 2002). Other indications for a role of *hr*s in the occurrence of genomic deletions are found for the nimavirus WSSV, which also contains a large circular dsDNA genome and with *hr*s equally distributed along the genome. The Thailand isolate of WSSV (Van Hulten *et al.*, 2001) contains a major deletion of 12 kb, which is located in the largest inter-*hr* region of the Chinese WSSV isolate (Yang *et al.*, 2001).

Apart form a smaller chance of deletion of adjacent sequences, the insertion of an extra *hr-ori* in the BAC vector may also have another advantage. A supposed deletion of the BAC vector does not only result in a smaller genome (thereby gaining a replicative advantage), but it simultaneously results in a loss of the extra *hr-ori* (thereby loosing a replicative advantage). This would mean that a deletion of the BAC vector containing an extra *hr-ori* will not gain the same replicative advantage as in case that this *hr-ori* was not inserted. Therefore, next to the lower chance of deletion, the smaller gain in replicative advantage may explain why the BAC vector is less rapidly deleted from SeBAC$^{\Delta nonhr}$+hr^{inv} (Fig. 7.4, right) and SeBAC+hr^{inv} (Fig. 7.7, left) than from SeBAC$^{\Delta nonhr}$ (Fig. 7.4, left).

Apparently, the *hr* activity is orientation dependent, although it remains unclear why the obtained genetic stability is dependent on the orientation of the extra *hr*, since a rapid deletion of the BAC vector was observed upon infection with SeBAC$^{\Delta nonhr}$+hr (Fig. 7.7, right), which contains the duplicated *hr*1 in the same genomic orientation as the native *hr*1. It is possible that homologous recombination between the two *hr*1 sequences leads to the deletion of the inter-*hr* sequence. However, an entire deletion between the inserted *hr*1 and the native *hr*1 would result in a deletion of ORF1629, which is an essential baculovirus nucleocapsid gene, which is essential for virus infectivity (Pham *et al.*, 1993). Consequently, such a deletion would not yield a viable virus but could result in a defective interfering virus.

Although rearrangements and/or deletions occurred in the BAC vector region, the GFP and CSFV-E2 coding sequences were retained in the viral genome for an extended timeframe as compared to the bacmids lacking an extra *hr*. This suggests that the presence of the extra *hr* in close vicinity to the heterologous genes has a positive effect on its maintenance and ultimate expression levels in insect-cell bioreactors. The expression is possibly further improved by the supposed transcriptional enhancer function of *hr*s (Theilmann and Stewart, 1992). Unfortunately, quantification of the production per infected cell due to the enhancer function of the extra *hr* was not possible in these experiments, since the marker genes (GFP and CSFV-E2) were spontaneously deleted. Putative negative effects of the presence of a non-*hr ori* in one of the experiments (SeBAC+hr^{inv}) were not observed, indicating that for SeMNPV infections in bioreactors the accumulation of DIs enriched in non-*hr ori*s may not constitute a major problem. This is in contrast to what was expected from *in vitro* cell culture experiments (Pijlman *et al.,* 2002) and might be related to lower overall multiplicities of infection (MOI) or other conditions (such as kinetics of virus infection) in a bioreactor environment.

The genetic instability of BAC vector sequences may prove to constitute a major constraint for the utilization of bacmid-derived baculoviruses for the large-scale production of heterologous proteins in insect-cell bioreactors (Pijlman *et al.*, 2003b). The utilization of *hr*s to enhance either transcription levels for the foreign gene and/or the genetic stability of flanking sequences may not only lead to stabilized bacmid-derived baculoviruses, but also to the further robustness of the baculovirus-insect cell expression system in general.

MATERIALS AND METHODS

Insect cells, media, and culture maintenance

Experiments were carried out with *Spodoptera exigua* Se301 cells (Hara *et al.*, 1995). The cells were maintained at 27°C in Grace's medium, supplemented with 10% foetal bovine serum (FBS heat-inactivated, Life Technologies). Medium was made by dissolving Grace's powder medium (Gibco), lactalbumin hydrolysate (DIFCO), yeastolate (Becton-Dickinson &Company) and Pluronic® F68 (0,1% w/v) in Millipore water. Gentamycin, penicillin and streptomycin (50 mg/l per antibiotic) were added prior to filtration (0.2 μm).

Se301 cells were adapted to this medium and propagated at 27 °C as monolayers in 25F (25 cm^2, 4 ml medium) tissue culture flasks or in suspension in 250 ml polycarbonate Erlenmeyer flasks (Nalgene, 30 ml medium) on an orbital

shaker at 100 rpm. T-flask cultures were passaged every week by resuspending 100-200 µl of a 100% confluent monolayer in a new T-flask. Erlenmeyer cultures were subcultured by diluting cells in the exponential growth phase (approx. $1,5*10^6$ cells/ml) to a minimal inoculation density of 3.10^5 cells/ml.

Cell concentrations were determined with a Neubauer haemocytometer or a Casy1-TTC cell counter (Schärfe System, Reutlingen, Germany). Viabilities were determined with the trypan blue exclusion method. Scaling-up of the insect cells was done in 300 ml (50 ml medium) glass Erlenmeyers. In total 8 Erlenmeyers provided the cell inoculum for the cell growth reactor resulting in an initial concentration of $4*10^5$ cells/ml in the reactor.

Cascaded bioreactor system

The continuous bioreactor system consisted of one 3 dm^3 (working volume: 1.5 dm^3) flat-bottomed fermentor (Applikon) to culture the insect cells and two 1 dm^3 (working volume: 0.75 dm^3) round-bottomed fermentors (Applikon) connected to the cell-growth reactor for production of virus and recombinant protein. In all reactors temperature, dissolved oxygen (DO) concentration and pH of the medium were continuously measured using a AD 1030 Bio Controller (Applikon) and data were logged every minute. The Bio Controller regulated the temperature at 27°C via a Thermo Circulator (ADI 1018, Applikon) connected to a closed water circuit through the reactor. DO was controlled at 30% through head-space aeration with a mixture of nitrogen and oxygen using mass-flow controllers connected to the Bio Controller. The gas mixture was passed through 0.2 µm filters into the headspace of the reactors to prevent contamination.

Medium was pumped into the 1.5 dm^3 cell-growth reactor at a rate of 0.5 dm^3/day, establishing a mean residence time of 72 h. The medium vessel was stored in a refrigerator. Via a single chemostat tube in the cell-growth reactor cell suspension was pumped into both 0.75 dm^3 infection reactors at a rate of 0.25 $dm^3.day^{-1}$ leading also to a residence time of 72 h. Superfluous cell suspension was continuously pumped out of the infection reactors at the same rate via chemostat tubes. Low overall pump rates and equal distribution of the cell suspension between the infection reactors were attained by pumping at discrete time-intervals. The individual flows towards the infection reactors were checked regularly. The reactors were equipped with a turbine impeller in the headspace and a marine impeller in the medium. Stirring occurred at a speed of 230 rpm (infection reactors) and 430 rpm (cell growth reactor). To monitor the continuous process, samples of about 20 ml from all reactors were taken on a daily basis.

Construction pFastBAC donor plasmids

To introduce the GFP and CSFV-E2 genes in the (mutant) SeMNPV bacmids, a donor plasmid pFB1Se-GFPE2 was constructed. First, the AcMNPV polyhedrin promoter was removed from pFastBAC1 by digestion with *Bst*1107I and *Stu*I, generating pFB1ΔAcPpol. The SeMNPV polyhedrin promoter was amplified by PCR using primers DZ168 (5'-GGGGTACC-

TATATACTAGACGCGATAAC-3') and DZ169 (5'-TGCCATGGTTATATTTATTTT-3'), introducing *Kpn*I and *Nco*I sites (underlined). The promoter was subsequently cloned as *Not*I fragment into the *Not*I site of pFB1ΔAcPpol, generating pFB1PSepol. The red-shifted GFP gene (Davis and Vierstra, 1998) was cloned as an *Xba*I/*Hin*dIII fragment into pFB1PSepol downstream of the polyhedrin promoter, generating pFB1SeGFP. To create a translational start codon for CSFV-E2, pSeMO7 (Dai *et al.*, 2000) was digested with *Bam*HI and *Xba*I and a linker was inserted. This linker was made with oligonucleotides DZ195 (5'-GATC*ATCGAT*TATGGATCC*T*-3') and DZ196 (5'-CTAGAGGATCCAT*AATCGAT*-3'), is *Bam*HI compatible and contains a *Cla*I site (italics) followed by a ATG-*Bam*HI fusion (underlined) and an *Xba*I site. The SeMNPV p10 promoter and 3'UTR were PCR amplified using primers DZ191 (5'-CGTCGACCCGCGACCTGCCACGATAC-3') and DZ192 (5'-CGTCGACTCAATTTACGACGACAAACCAAC-3'), introducing *Sal*I sites (underlined). This PCR product was cloned into pGEM-Teasy and sequenced. The CSFV-E2 gene was then cloned as a *Bam*HI fragment from pAcE2 (Van Oers *et al.*, 2001) downstream of the ATG of the inserted linker. The p10-driven CSFV-E2 gene with p10 3'UTR was finally cloned as *Sal*I fragment into pFB1SeGFP, generating pFB1SeGFPE2.

SeMNPV *hr*1 (nt 10235-12098 of the SeMNPV genome; IJkel *et al.*, 1999) was amplified by PCR using forward primer 5'-CGCCCCTTTCTCAAGAATACAGTG-3' and reverse primer 5'- CGCATCGCCGGCTTCGAGTGTGAC-3', and was subsequently cloned into pGEM-T (Promega). The 1.9 kb product was subcloned with *Sac*II and *Sac*I into pBluescript (Stratagene). *Hr*1 was finally cloned as a compatible 1.5 kb *Spe*I fragment into an *Avr*II linearized pFB1SeGFPE2.

Bacmid construction and virus preparation

Before transposition, *E. coli* DH10β containing the recombinant SeMNPV bacmids (Pijlman *et al.*, 2002) were transformed with transposition helper plasmid pMON7124 (Luckow *et al.*, 1993). The protocol from the Bac-to-Bac manual (Gibco BRL) was followed to transpose the GFP and CSFV-E2 genes from pFB1SeGFPE2 into the attTn7 transposon integration site of the SeMNPV bacmids. Isolation and transfection of bacmid DNA to Se301 cells was done according to the Bac-to-Bac baculovirus expression system manual (Gibco BRL). The bacmid-derived BV inoculum was used to scale-up the virus preparation in 75F T-flasks with Se301 cells. Infectious budded virus titers were determined using the endpoint dilution assay (Vlak, 1979).

Viral DNA isolation, Southern hybridization and CSFV-E2 quantification

Intracellular viral DNA was isolated as previously described (Summers and Smith, 1987). Digested viral DNA was run overnight in ethidium bromide stained 0.6% agarose gels, and Southern blotting was performed by standard capillary upward blotting (Sambrook *et al.*, 1989) using Hybond-N (Amersham Pharmacia) filters. As a DNA size marker, λ-DNA digested with *Eco*RI/*Hin*dIII/*Bam*HI was used. Random-primed DNA probes for Southern hybridization

were made using the DIG non-radioactive nucleic acid labelling and detection system (Roche). For the BAC vector probe, BAC-*Bsu*36I (Pijlman *et al.,* 2002) was DIG-labeled overnight. Hybridization and colorimetric detection with NBT-BCIP (Roche) were performed according to the manufacturer's recommendations. *In silico* reassembly of bacmid sequences and computational predictions of restriction digests were done using the Lasergene DNAStar package. CSFV-E2 quantification was carried out with the Ceditest CSFV (CEDI Diagnostics, Lelystad, The Netherlands) as previously described (Van Oers *et al.*, 2001).

ACKNOWLEDGEMENTS

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CHAPTER 8

General discussion

1. DEFECTIVE INTERFERING BACULOVIRUSES AND DELETION MUTANTS

Origin of defective interfering baculoviruses

The occurrence of defective interfering viruses (DIs) has been a recognized phenomenon for many years (von Magnus, 1954), and is an intrinsic feature of successive virus passage in cell culture. Although DIs are believed to exist in virtually all known virus families, their generation mechanism(s) and biological importance in virus infectivity remain enigmatic up to the present day. DIs are also generated during baculovirus infection in insect cells (Kool *et al.*, 1991; Lee and Krell, 1992) and are responsible for the interference phenomenon known as the passage-effect (Krell, 1996). Because of the major limitations baculovirus DIs pose on the production of recombinant proteins in insect cells, in this thesis a fundamental study was undertaken to investigate the origin, nature and generation mechanism(s) of DIs upon virus passage.

Two baculoviruses were subject of investigation, the prototypic and best studied baculovirus AcMNPV with its broad host range and productive *in vitro* replication, and the monospecific SeMNPV, which displays a hign pathogenicity to its host but is slightly unstable in cell culture. The origin of AcMNPV DIs was investigated using a sensitive PCR-based assay, and it was shown that DIs were readily detectable in plaque purified baculovirus stocks, and could persist during *in vivo* multiplication in insect larvae (Chapter 2). The same DIs were, however, not found in the authentic AcMNPV isolate (Vail *et al.*, 1973), suggesting either that AcMNPV DI formation might be an artifact of insect cell culture, or that DIs were present *in vivo* in undetectable amounts. It must be noted, however, that the abundance of DIs in intracellular DNA preparations is higher than in BV (and presumably polyhedra), as a minimal genome size may be required for baculovirus packaging (Lee and Krell, 1994; Chapter 4).

By the use of infectious baculovirus clones maintained as bacterial artificial chromosomes in *E. coli* (bacmids), homogenous DNA preparations consisting of intact full-length genomes could be obtained and were used to show that AcMNPV

DIs were generated in a single passage in cell culture. Moreover, the use of bacmids showed that the genesis of SeMNPV and AcMNPV DIs with reiterated non-*hr ori*s repeatedly occurred in infected insect cells (Chapter 3 and 6). Although SeMNPV DIs may occur *in vivo* as part of natural genetic variation (Muñoz *et al.*, 1998), their prevalence *in vitro* indicates that cell culture systems in general promote the generation, preferential replication and maintenance of baculovirus DIs.

Generation mechanism of deletions and baculovirus DIs

Prior to the design of strategies to prevent or diminish the passage effect, a more detailed investigation of the DI generation mechanism was carried out. By analysis of the junctions sequences in DI molecules, homologous recombination was found to be one of the mechanisms involved in DI formation. This was not surprising since baculovirus DNA replication promotes high frequency homologous recombination (Martin and Weber, 1997). However, homologous recombination was not always involved as evidenced by the lack of imprints at some junction sequences. Homologous recombination could have occurred via short sequence overlaps (>3 nt) that were present at remote locations on the viral genome (as shown in Chapter 2, 3 and 6), resulting in excision of the intermediate sequence (deletion) or a sequence containing the *ori*. It is unknown whether the short sequence overlaps itself are sufficient for homologous recombination or that flanking sequences are involved in (stabilizing) the recombination mechanism. Excised *ori*s are subsequently assumed to undergo rolling circle type of DNA replication (Kornberg and Baker, 1992), which results in the formation of large concatenated molecules that interfere with intact virus replication (Fig. 8.1). In Chapter 3, this concatenation was shown by the presence of multiples of non-*hr ori* units in partial digests of intracellular viral DNA.

Thus, it appears that the generation of DI molecules could involve hetero- and homologous recombination or possibly even sequential recombination steps.

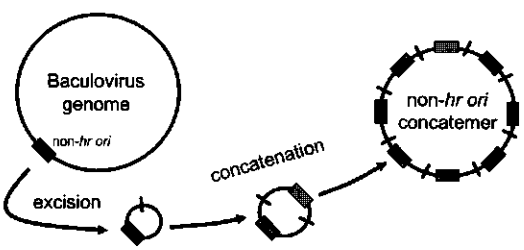

Figure 8.1. Proposed model of generation mechanism of DIs with enriched non-*hr ori*s. The non-*hr ori* is excised from the viral genome by heterologous or homologous recom-bination. Subsequent concatenation of the circular molecule takes place during baculovirus DNA replication, resulting in the formation of large concatenated molecules with reiterated non-*hr ori*s.

Other examples of homologous recombination leading to genetic rearrangements involve host transposable elements, which can integrate into the baculovirus genome. Two or more copies of such a transposon can lead to inversion (Arends *et al.*, 2002) or deletion of the intermediate region, with the possibility of creating DI molecules. Baculovirus sequences shown to be associated with transposon integration include the FP25K en EGT/DA26 loci (Kumar and Miller, 1987).

Biological role of baculovirus deletion mutants

During the cloning of SeMNPV as a BAC in *E. coli* (Chapter 3) it was observed that many genotypic variants, some with large deletions, were present in a natural SeMNPV isolate. This finding suggested that baculovirus deletion mutants exist in natural isolates and may have an important biological role in the infection process of baculoviruses. For instance, deletion mutants and DIs may be modulators of virulence and as such play a key role in the establishment of persistent baculovirus infections in insects. Also, a mixed population of genotypes (including DIs) in a baculovirus isolate may provide genetic flexibility to obtain a higher level of viral fitness. Paradoxically, due to their smaller genome and consequent faster replication speed, they may also be able to enhance the pathogenicity of the virus to the host organism.

The predominance of deletion mutants of SeMNPV in cell culture lacking genes for liquefaction (*chiA* and *v-cath*) and oral infectivity (*pif* and *pif-2*) suggest that these genotypes indeed have a replicative advantage and consequently suppress or outcompete the intact virus (Chapter 5). A possible scenario is that the faster-replicating deletion mutants are responsible for the rapid spread of infection through the insect. The intact virus may then only be required in low abundance to serve as a helper for e.g. liquefaction (*chiA* and *v-cath*) and oral infectivity (*pif* and *pif-2*) to degrade the insect and to produce infectious polyhedra, respectively. The relative high proportion of genotypic variants in SeMNPV infections *in vivo* may consequently offer an explanation for the high pathogenicity of SeMNPV to its host *S. exigua*.

Novel tools to study genetic diversity in natural isolates

Cloning of baculovirus genomes as BACs is a relatively novel and very useful strategy to identify the genetic diversity in natural occurring baculovirus isolates. Moreover, defective genotypes, which can only replicate with the aid of a (intact) helper virus and thus cannot be purified by plaque assay and/or *in vivo* cloning methods, can now be easily isolated and their genetic and biological properties

evaluated. In some cases such defective baculovirus genomes are described as parasitic genotypes (Muñoz *et al.*, 1998), and it is of great fundamental interest to understand the molecular basis of this phenomenon and investigate their role in baculovirus pathogenicity, biodiversity and evolution.

Out of the 111 SeMNPV bacmids generated by direct cloning, only 3 contained the entire genome (Chapter 3); the remainder contained smaller-sized SeMNPV genotypes. This relatively low number of complete genomes in the bacmid library may reflect the high degree of genetic variation in natural isolates. However, it must also be taken into account that smaller genomes (containing deletions) may be easier transformed into *E. coli*, giving an overrepresentation of smaller genomes in the bacmid library. Therefore, the resulting library of BAC clones might not give directly a quantitative, but rather a qualitative reflection of the heterogeneity of natural baculovirus isolates. Also, genotypes lacking the unique restriction site used for cloning are not represented in the library. Nevertheless, further investigation of the genetic and biological properties of the genotypic variants in the bacmid library will give valuable information about the role of deleted (both defective and non-defective) genomes in natural baculovirus isolates.

For instance, a genotypic variant (SeBAC72) with a small (few kb) deletion in the hypervariable *Xba*I-A restriction fragment, could be readily reconstituted as virus upon transfection with bacmid DNA in three cell lines Se301, SeUCR and SeIZD. Surprisingly, the full-length SeMNPV bacmid (SeBAC10, Chapter 3), could only be reconsituted in Se301 and SeIZD but not in SeUCR, despite extensive attempts. This could indicate that the deleted part contains a sequence or encodes a protein that prohibits efficient virus replication in SeUCR. Alternatively, the (small) deletion is only readily generated in Se301 and SeIZD cells, but not in SeUCR cells due to a putative lower recombination frequency in this particular cell line. This is supported by experiments indicating that the passage effect more readily occurs in certain cell lines than in others (Chapter 4). After mapping the deletion in SeBAC72, site-directed mutations in the full-length bacmid could be used to more specifically identify the genes or sequences involved, and explain this interesting observation. Potentially, it could shed a light on the rationale for the occurrence of spontaneous deletions upon SeMNPV infection in cultured insect cells, and determine the role of deleterious mutants in *in vivo* infections.

2. KEYS TO IMPROVE BACULOVIRUS EXPRESSION VECTORS

Non-hr oris and defective interference

Baculovirus DIs with reiterated non-*hr oris* were shown to interfere with parental virus replication in serial passage experiments with AcMNPV (Lee and Krell, 1992; 1994; Chapter 6) and SeMNPV (Chapter 3), illustrating a pivotal role for non-*hr oris* in the defective interference phenomenon. The SeMNPV and AcMNPV non-*hr oris* were previously shown to have a high *ori* activity in transient replication assays (Heldens *et al.*, 1997a; Kool *et al.*, 1994). Although these findings may suggest that non-*hr oris* are key components in baculovirus DNA replication, these sequences appeared not to be essential for SeMNPV and AcMNPV DNA replication (Chapter 3 and 6), and thus it leaves the question why baculoviruses have such sequences. The finding that deletion of the non-*hr ori* from AcMNPV or SeMNPV resulted in significantly stabilized genomes, is an important improvement to construct baculovirus expression vectors, as they do not generate DIs in *in vitro* production systems including insect-cell bioreactors.

Hrs and spontaneous genomic deletions

Hrs act as transcriptional enhancers (Friesen, 1997) and as *oris* in viral DNA replication (Pearson *et al.*, 1992; Kool *et al.*, 1993a,b; Kool *et al.*, 1995). As baculovirus DNA replication is thought to be initiated from multiple *oris* at the same time, the distribution of *hrs* may be correlated with the likelyhood that deletions occur in the viral genome. In Chapter 6 and 7 a hypothesis was put forward that deletions are more likely to occur in regions with a low *hr* density. This view is supported by the occurrence of spontaneous deletions of BAC vector sequences (Chapter 6). These relatively large but non-essential sequences had a low density of *hrs*, thereby potentially increasing the change for deletions to occur. Insertion of an *hr* was shown to enhance the stability of adjacent sequences in the viral genome (Chapter 7) upon virus passage, and may boost the expression of recombinant proteins due to its enhancer function.

Bicistronic baculovirus vectors with selective pressure on gene of interest

The previous two stategies to improve baculovirus expression vectors focussed on the prevention of DI formation and stabilizing the viral genome. In this section another but entirely different way to resolve the problems associated with the passage effect is proposed, namely by providing baculovirus vectors with a bicistronic expression cassette, which maintains a selective pressure on the gene of

interest. Irrespective of the generation of DIs, protein production will maintain upon prolonged passage. A preliminary study was performed to investigate the feasibility and future potential of such a system.

The physical difference between DIs and BVs is their smaller size and genetic content, which in DIs can be as little as a few kb viral DNA (Lee and Krell, 1992; Chapter 3). It is likely that the composition of structural proteins of the DI BVs is very similar if not identical to BVs containing the intact viral DNA. Therefore, it is thought that direct coupling of the heterologous protein expression to the expression of an essential (structural) baculovirus encoded gene, has the potential to solve the problems of decreased heterologous protein production as a consequence of defective interference. This essential viral gene must be required both for the production of intact BVs as well as the production of DI BVs. As long as infection is maintained upon BV passage, the essential protein is produced. Consequently, the heterologous protein is produced as well.

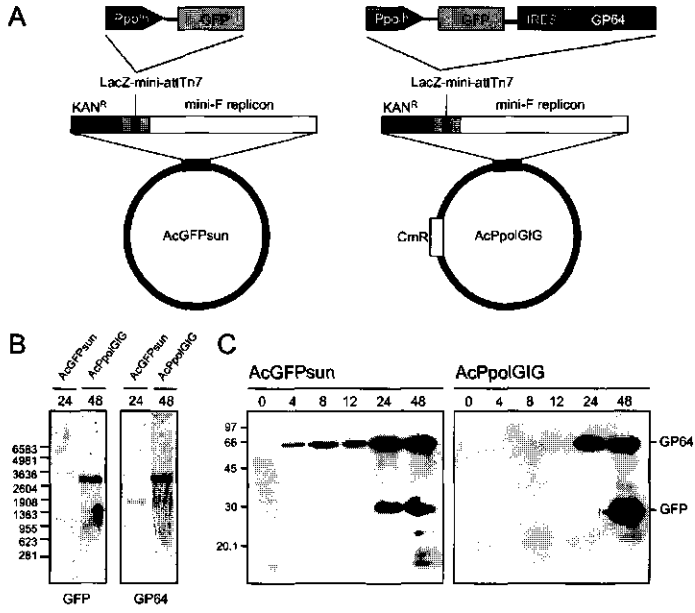

Figure 8.2. A) Schematic overview of the bicistronic and control baculovirus expression vectors. The authentic gp64 gene in the bicistronic bacmid (right) is replaced by a choramphenicol resistance gene (Cm^R). The control bacmid (left) contains a GFP gene under control of the polyhedrin promoter and has retained its authentic gp64 gene. B) Northern detection of bicistronic transcripts. Polyadenylated mRNAs from AcGFPsun and AcPpolGIG were purified and detected with 32P-radiolabeled GFP (left) and GP64 (right) DNA probes at 24 and 48 h p.i., respectively. C) Western blot time-course analysis of GP64 and GFP expression. Intracellular GP64 and GFP proteins were detected on the same blot with monoclonal antibodies.

To couple the expression of the heterologous gene to an essential baculovirus gene, bicistronic constructs were made using an internal ribosome entry site (IRES) from an insect picorna-like virus, *Rhopalosiphum padi* virus (RhPV) (Woolaway *et al.*, 2001), thereby establishing a positive selection pressure on a single mRNA encoding two genes. This IRES was proven to be active in lepidopteran (Sf21) cells (Domier and McCoppin, 2003). The essential baculovirus envelope fusion protein GP64 was chosen as a target for positive selection, as this glycoprotein is present in both DI BV as well as in BV containing intact genomes, and is required for the cellular entry of the virion (*Monsma et al., 1996*).

An AcMNPV bacmid with a deleted GP64 gene (Lung *et al.*, 2002), which does not produce BVs and therefore cannot spread the infection by itself was used as the parent genotype. With this construct, a positive selection pressure was established on the transcription of the full-length (3.3 kb) mRNA encoding both the heterologous GFP and the native GP64, of which the latter gene was translated by internal ribosome entry (Fig. 8.2A). Northern blotting with GFP and GP64 probes on mRNA from Sf21 cells was carried out (Fig. 8.2B). A single 3.3 kb full-length GP64 transcript was detected for the bicistronic construct, indicating that GP64 was translated from the full-length mRNA by internal ribosome entry. Western blot time-course analysis (Fig. 8.2C) showed that the GP64 expression in AcPpolGIG was slightly delayed as compared to AcGFPsun, but this did not compromise virus spread.

Overall, the results indicate that the RhPV 5'IRES is able to drive the expression of foreign genes in Sf21 insect cells. The results also show that sufficient amounts of GP64 are produced by internal ribosome entry for establishement of a successful spread of baculovirus infection.

Conclusion

The generation of DIs can be prevented to a large extent by the deletion of the non-*hr ori* from the viral genome, which can lead to a major improvement for the large-scale baculovirus-mediated production of proteins in insect cells. Furthermore, the insertion of *hrs* may contribute to generate baculovirus expression vectors with enhanced stability. Although the preliminary results with bicistronic constructs are promising, this strategy remains to be validated in serial passage experiments and/or insect-cell bioreactors with respect to maintenance of protein expression upon passage.

3. FURTHER IMPROVEMENTS FOR THE BACULOVIRUS-INSECT CELL EXPRESSION SYSTEM

The major problem of large-scale application of the baculovirus-insect cell expression system is the loss of production due to the passage effect. Therefore diminishing or preventing this phenomenon through engineered expression vectors would be a major improvement. The passage effect can also be minimized by using low MOIs, which may be practical in small-scale batch operations, but difficult to avoid in continuous or fed-batch bioreactor systems. The development of transgenic cell lines and the improvements in bioreactor and medium design are also of great importance and are therefore shortly discussed in this section. Finally, a proposal for a supreme baculovirus expression vector is presented, which is (partially) based on the strategies to improve baculovirus vectors described in this thesis.

Transgenic insect cell lines and novel media

Baculovirus expression vectors are widely considered to be excellent tools for the production of recombinant (glyco)proteins in cultured insect cells, although there are still some fundamental differences between the glycoprotein processing pathways of insects and higher eukaryotic organisms (Marchal *et al.*, 2001). In addition to the occurrence of the passage effect, the inability of insect cells to produce authentic mammalian glycans, and thus biologically active glycoproteins, might be another significant limitation of the baculovirus expression system.

Current research is therefore focussed on "humanizing" the insect glycoprotein processing pathways by transgenic engineering of commonly used insect cell lines (Jarvis, 2003). These cell lines are equipped with mammalian key enzymes from metabolic pathways that enable the glycoproteins to be properly modified (Hollister *et al.*, 2002). Also, stable insect cell cultures constitutively expressing recombinant protein(s) can be developed (reviewed by Jarvis and Guarino, 1995). This is mainly interesting for very specific glycoproteins for therapeutic use, of which the quality is compromised by the viral infection process, more specifically as a result of proteolytic degradation and limited glycosylation. This system, however, is less flexible and more complicated to operate than baculovirus-mediated expression, but is usefull in applications where high yields are less important.

For high yields of properly glycosylated recombinant proteins, fetal bovine serum (FBS) has been standardly used for many years as a supplement in insect cell media. However, with respect to safety concerns (e.g BSE), regulatory issues and problems with purification of the recombinant protein, FBS has become an

undesirable medium component. With the development of novel (defined) media and/or medium supplements, the use of FBS is not required anymore. Because of the relatively low insect cell densities (as compared to yeast or bacteria) and high medium costs, there is still a need for medium improvement to obtain recombinant protein for a lower cost. Marteijn *et al.* (2003) demonstrated that with the use of genetic algorithms an increase in viable (HzAM1) cell density of 550% ($19.5*10^6$ cells/ml) could be obtained, as compared to a fermentation in an optimized commercial medium. It remains to be seen, however, whether these extremely high cell densities still allow efficient baculovirus infection, and result in a higher yield of biologically active recombinant protein per unit medium.

Improved insect-cell bioreactors

Wickam *et al.* (1991) already showed that baculovirus defective interfering particles are responsible for variations in recombinant protein production as a function of multiplicity of infection. In the bioreactor experiments with SeMNPV expression vectors (Chapter 7) DI formation was not observed, very likely because the MOI was never higher than 1. These findings imply that optimal recombinant protein production in a continuous bioreactor can be obtained by maintenance of a low MOI (<1) during the entire production run (Liebman *et al.*, 1999). This may be achieved by the use of adequate virus-cell combinations, or otherwise by the development of MOI-controlled bioreactor systems. Such a system could for example be based on the inducible expression of a baculovirus gene required for the formation of BV, such as the baculovirus BV glycoprotein GP64. Provided that the amount of infectious BV can be real-time monitored, the expression of GP64 may be regulated by an inducible transactivation system, such as the Drosophila metallothionin promoter (Hegedus *et al.*, 1998) or tetracycline-regulated gene expression (Gossen and Bujard, 1992; Wu *et al.*, 2000).

An alternative way to develop a MOI-controlled bioreactor could involve the use of neutralizing antibodies to modulate the virus infection pressure, or a system that inactivates a certain percentage of the infectious BV in the culture medium. For instance, the virus inactivation in a continuous recirculation medium flow (by for example UV light) could establish the desired (lower) MOI in the bioreactor. These strategies, however, may require a detailed investigation of parameters of the production system, the most important being the number and the time-course of BV release. Initial attempts to gain more insight in the kinetics of baculovirus infections by quantitative PCR were conducted by Rosinksi and Reid (2002).

Advanced Baculovirus Expression Vectors: a proposal

Combined with current developments in establishing improved transgenic cell lines, medium optimalization, and process conditions, the design of enhanced baculovirus expression vectors is essential to establish successful large-scale production systems. Several aspects to improve existing baculovirus expression vectors are described and summarized in Fig. 8.3.

Efficient construction and versatility

The construction of recombinant baculoviruses for recombinant protein expression should be simple, fast and efficient. The bacmid system is a rapid and efficient method to generate recombinant baculoviruses by using site-specific transposition to insert foreign genes into a baculovirus genome propagated as a bacterial artificial chromosome (BAC) in *E. coli* (Luckow *et al.*, 1993). Recombinant bacmid DNA isolated from *E. coli* is genetically homogeneous, thereby eliminating the time-consuming steps of plaque purification, which reduces the time to generate recombinant viruses from several weeks to 7-10 days. Bacmids also have the advantage that they can be repeatedly reconstituted to produce new virus stocks (Hahn *et al.*, 2003), and that site-specific mutagenesis protocols in *E. coli* can be used to modify the viral genome. A problem with the current bacmid technology, however, is the impurity of recombinant bacmid DNA after transposition. Restreaking of positive clones is always necessary to remove a background of so-called "empty bacmids", which have a non-occupied tranposition locus. A solution would be to construct a non-infectious empty bacmid by deletion of an essential gene (e.g. ORF1629, ie1, GP64, p6.9 or p35). Along with the heterologous gene(s), the essential gene can then be restored via transposition using modified pFastBAC vectors (Fig. 8.3). Thus, as backbones for baculovirus expression, bacmids are the vectors of choice, because of their highly versatile nature and ease of construction.

Stability upon passage in insect cells

Strategies explored in this thesis to prevent the passage effect were the deletion of non-*hr ori*s (Chapter 3 and 6), the insertion of *hr*s to enhance the genome stability (Chapter 7), and the use of bicistronic expression cassette to select for the gene to be expressed (Chapter 8). The problem of DI formation can be overcome to a large extent by the deletion of sequences with a replicative advantage (e.g. non-*hr ori*s) from the viral genome (Fig. 8.3). To prevent the formation of DIs as a result of host

transposon integration, the non-essential EGT/DA26 and/or 25K genes may be deleted (Fig. 8.3).

To use baculovirus expression vectors for protein expression only, genes dispensable for *in vitro* replication can be deleted as well (e.g. *p74*, *pif* and *pif-2*: Fig. 8.3). This will prevent spontaneous deletion of these non-essential genes. In addition, it will also further improve the biological safety of the vector, although most baculovirus expression vectors are already biologically contained due to a lack of the polyhedrin gene.

Another major stability problem is the spontaneous deletion of BAC vector sequences and the heterologous gene(s) from the viral genome upon passage (this thesis Chapter 6 and 7). A solution could be the insertion of *hrs*, to decrease the likelyhood that adjacent sequences are deleted (Chapter 7). The heterologous gene(s) can also be inserted in between essential genes, or their expression can be coupled to the expression of an essential gene using an internal ribosome entry mechanism. However, a more definite solution is the deliberate removal of BAC vector sequences upon reconstitution in insect cells by Cre-Lox recombination (Fig. 8.3A) as soon as the BAC replicates in cell culture, allowing the generation of viruses with wildtype genome properties (Smith and Enquist, 2000; Adler *et al.*, 2000). *In vitro* "Gateway" recombination (Invitrogen) could also be used to remove BAC vector sequences prior to transfection (Fig. 8.3B). These or comparable strategies could now be utilized for bacmids to generate recombinant baculoviruses with wildtype properties, lacking any bacterial sequence, antibiotic resistance genes, or marker genes.

Proteolysis, glycosylation and yield

Product quality as well as product quantity are affected by proteases, which are encoded by the baculovirus itself (Slack *et al.*, 1995) or are produced by the host cell after infection (Ikonomou *et al.*, 2003). The addition of protease inhibitors in the culture medium or during protein purification is therefore common practice. Two baculovirus genes involved in the liquefaction of insects, *cathepsin* (*v-cath*) and *chitinase* (*chiA*), have proteolytic and chitinolytic activity in cell lysates after baculovirus infection. An enhanced baculovirus vector lacking *v-cath* and *chiA* did not show these enzymatic activities. This vector system gave greater protein stability and was subsequently commercialised as BacVector-3000 (Monsma and Scott, 1997), indicating that *v-cath* and *chiA* should be removed from the viral genome for better protein expression (Fig. 8.3).

Glycosylation is often required to yield biologically active proteins. Transgenic cell lines can nowadays be equipped with novel (mammalian) glycosylation pathways (Jarvis, 2003), which can also be engineered in the baculovirus vector itself (Tomiya *et al.*, 2003). Although initially the strong very late promoters such as polyhedrin and p10 were used to drive protein expression, for proper post-translational modifications (including glycosylation) to occur, promoters from early or late genes (e.g. *ie1, gp64, p6.9*) may be preferred. In the early stages of infection the cell is not yet in a metabolic (shut-down) state and the cellular glycosylation pathways are still functional.

With respect to quantity instead of quality, the yield of protein from the very late promoters (p10 and polyhedrin) can be enhanced by the removal of the competing native baculovirus polyhedrin or p10 promoters/genes (Van Oers *et al.*, 1992). Another way to enhance the yield is the insertion of *hr*s, which serve as transcriptional enhancers (Friesen, 1997) and may also stabilize the flanking sequences (Chapter 7).

4. CONCLUDING REMARKS

With the ongoing developments in eukaryotic protein expression, especially the improvements in stable (mammalian) cell lines and novel virus-based systems, the baculovirus-insect cell expression system has maintained its strong position as efficient tool for recombinant glycoprotein production, functional protein studies, as well as for commercial production of (subunit) vaccins, diagnostics and pharmaceuticals. This is mainly because of its current status of proven technology and well-established experimental procedures, but also due to recent improvements in the ease of recombinant construction and the development of better cell lines and culture media.

In this thesis, an intrinsic but major problem of prolonged baculovirus infections, the passage effect, was studied in detail. The determination of the molecular mechanims responsible for the generation and subsequent accumulation of defective interfering baculoviruses has led to a better understanding of the passage effect but also gave more insights into baculovirus DNA replication in general. Finally, this research has led to several improvements in the stable and large-scale production of recombinant proteins in baculovirus-infected insect cells.

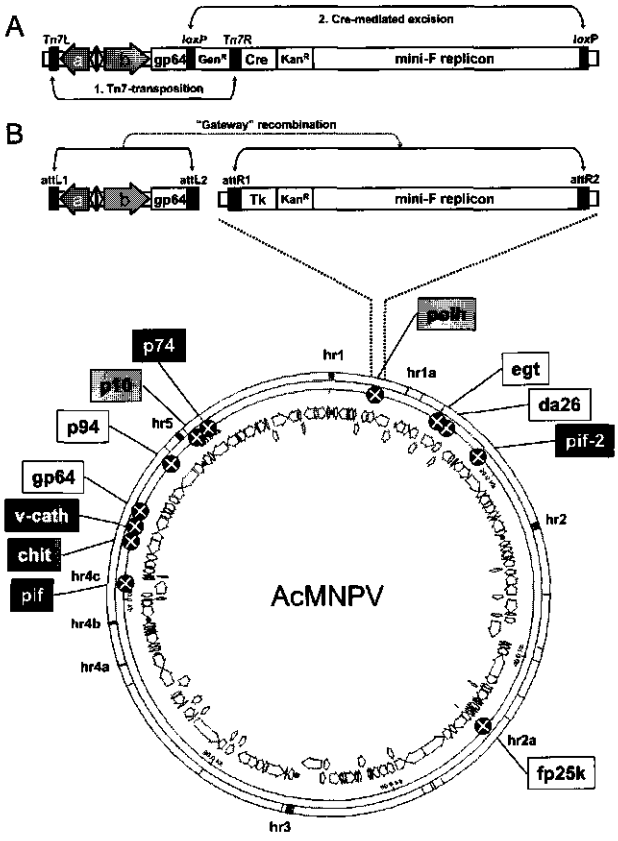

Figure 8.3. Baculovirus expression vector optimized for genetic stability, ease of construction, site-directed mutagenesis, protein quality and yield, and biological safety. To improve the genetic stability in insect cells, the non-hr ori located in the p94 gene is deleted, as well as the transposon integration sites located at FP25K and EGT/DA26 loci. P10 and polyhedrin genes are deleted to eliminate competition with the expression of recombinant genes. P74, pif and pif2 genes are deleted to enhance the biological safety of the expression vector. V-cath and chiA genes are deleted to enhance the stability of the expressed proteins. GP64 is deleted from its original location to serve as a positive selection marker after transposition. Two strategies to construct the expression cassette while removing antibiotic resistance genes and the unstable mini-F replicon are shown at the top. A) The BAC vector contains an attTn7 tranposon integration site, a mini-F replicon, a kanamycin resistance gene (KanR) and a Cre recombinase under control of an early baculovirus promoter. The genes of interest (a and b), a gentamycin resistance gene (GenR), and GP64 (for positive selection in insect cells) are tranposed to the bacmid by Tn7 transposition. Upon transfection of insect cells, the sequence between the loxP sites is removed by the expressed Cre-recombinase, resuslting in a pure virus stock. B) The Bac vector contains a mini-F replicon, a kanamycin resistance gene (KanR), and a HSV thymidine kinase gene under control of an early baculovirus promoter (for negative selection in insect cells). After in vitro "Gateway" recombination, the sequence between attR1 and attR2 is replaced by the sequence between attL1 and attL2. Negative selection against the HSV Tk gene results in a pure virus stock upon transfection of insect cells.

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SUMMARY

Baculoviruses have great potentials as highly specific bioinsecticides, as surface display vectors, and as future gene delivery vehicles for gene therapy purposes. Nevertheless, their main application to date is their use as gene expression vector for the production of eukaryotic (glyco)proteins in cultured insect cells (Chapter 1). Although many improvements to the baculovirus-insect cell expression system have been made over the last decade(s), the major limitation for large-scale virus and/or recombinant protein production in insect-cell bioreactors is still the occurrence of the so-called passage effect, which causes a sharp decrease in production with increased virus passage number. This phenomenon is the result of the generation and subsequent accumulation of defective interfering (DI) baculoviruses, which specifically interfere with the parental (intact) virus and compete for replication factors. As a consequence, low passage virus seed stocks must be repeatedly generated and kept to ensure product quality and quantity. At the same time, large-scale productions require a tight control on limiting virus passage numbers, in order to keep the level of DI accumulation to a minimum. DIs lack considerable parts of the viral genome and are often enriched in origins of DNA replication (*ori*s), which are either associated with the baculovirus homologous regions (*hr*s), or with sequences of the non-*hr* type. This gives DIs a competitive advantage upon replication over time.

At the onset of the research described in this thesis, it was unclear whether the generation of baculovirus DIs was solely an artifact of insect cell culture systems, or that they were also present in natural virus isolates. In Chapter 2, the origin of AcMNPV DIs was investigated by a (nested) PCR approach. The results showed that DIs with major genomic deletions were present in low-passage AcMNPV virus stocks and polyhedra, but not in an authentic AcMNPV isolate obtained prior to passage in cell culture. Supporting evidence that DIs were readily generated upon infection in cell culture was obtained upon reconstitution and subsequent serial passage of a (genetically homogeneous) bacterial artificial chromosome (BAC) containing the entire AcMNPV genome (bacmid). Imprints of homologous and heterologous recombination steps involved in the generation of DIs were found at deletion junction sites in the DI genomes. The overall data suggested that the generation of DIs is an intrinsic property of baculovirus infections in insect cell culture, and that DIs can persist *in vivo*.

In order to study the mechanisms involved in the generation of baculovirus DIs in more detail, a model system of SeMNPV infections in cultured *S. exigua* (Se301) cells was established, which is more rapid and reproducible than AcMNPV in Sf21 cells (Chapter 3). Serial passage of SeMNPV in cultured *S. exigua* (Se301) cells resulted in a dramatic drop in budded virus and polyhedron production within 10 passages. This drop concurred with the rapid accumulation of DIs containing reiterated SeMNPV non-*hr ori*s and which replicated as concatemers. A genetically homogeneous bacterial clone of SeMNPV (bacmid) was generated to demonstrate that similar DIs with concatenated non-*hr ori*s were readily generated upon serial passage of the bacmid-derived virus. Using a novel site-directed mutagenesis protocol involving homologous recET-mediated recombination in *E. coli*, an enhanced SeMNPV bacmid lacking the non-*hr ori* was constructed, which showed greater stability and improved polyhedron production upon serial passage. These results revealed a pivotal role for non-*hr ori*s in the generation and maintenance of baculovirus DIs, as a result of their strong replicative advantage in cell culture. Moreover, a stable protein expression vector based on SeMNPV was obtained, which could be used for large-scale heterologous protein production and SeMNPV functional genomics.

Subsequently, it was investigated whether the accumulation of non-*hr ori*-enriched DIs was dependent on the cell-line. Therefore, SeMNPV was passaged in two other *S. exigua* cell lines, SeUCR1 and SeIZD2109 (Chapter 4), but no accumulation of concatenated non-*hr ori*s was observed. However, the non-*hr ori* concatemers generated in Se301 could be maintained for several passages in SeUCR and SeIZD2109. An explanation for the observed difference in the generation of DIs could be the greater replicative advantage of the non-*hr ori* in Se301 cells or the involvement of some host factor. It was also found that non-*hr ori* concatemers were more abundant in intracellular than in extracellular viral DNA preparations, suggesting that a minimal DNA size is required for packaging into nucleocapsids or for budding.

SeMNPV infections *in vitro* consistently resulted in the generation of (major) deletion mutants, which often lacked the ability to infect *S. exigua* larvae *per os*. Based on earlier studies involving SeMNPV deletion mutants a contiguous sequence encoding ORFs 29 to 35 was predicted to contain one or more gene(s) required for *per os* infectivity. The gene responsible for this phenotype in SeMNPV was mapped via the generation of progressive deletions from ORF 29 to 35 of a SeMNPV bacmid using homologous recombination in *E. coli* (Chapter 5). First, it was shown that SeMNPV ORFs 15-35 (including *cathepsin, chitinase, gp37, ptpt-2, egt, pkip-1,* and

arif-1 genes) were not essential for *in vitro* replication or *in vivo* replication after intrahaemocoelic injection. Subsequent feeding of *S. exigua* with occlusion bodies of the mutants indicated that a deletion of SeMNPV ORF35 (Se35) caused loss of oral infectivity and that reinsertion of Se35 in SeMNPV bacmids lacking Se35 rescued oral infectivity. Se35 was named *per os* infectivity factor-2 (*pif*-2), analogous to a different gene from *S. littoralis* NPV, which was designated *per os* infectivity factor (*pif*) and is a homologue of Se36. *Pif-2, pif* and *p74* belong to a relatively small group of 30 genes that are conserved among members of the *Baculoviridae* family.

Repeated infections with AcMNPV or SeMNPV (Chapter 3) in cultured insect cells led to the generation of DIs containing reiterations of a non-*hr ori*. The location of this non-*hr ori* in AcMNPV is within the coding sequence of the non-essential *p94* gene. In Chapter 6 the effect of a deletion of the AcMNPV non-*hr ori* on recombinant protein expression levels upon serial passage in Sf21 insect cells was studied. Deletions within the *p94* gene were made in an AcMNPV bacmid using homologous recombination in *E. coli*, and the bacmids were equipped with a green fluorescent protein (GFP) gene and a gene encoding the *Classical swine fever virus* E2 glycoprotein (CSFV-E2) to serve as markers for expression. For the parental (intact) bacmid but not for the mutants, a strong accumulation of DIs with reiterated non-*hr oris* was observed. This indicated that deletion of the non-*hr ori* enhanced the genetic stability of the AcMNPV genome upon passaging, confirming previous findings with SeMNPV (Chapter 3). Nevertheless, for all viruses a rapid decrease in GFP and CSFV-E2 production upon passaging was observed as a result of a spontaneous deletion of the entire BAC vector insert including the expression cassette for GFP and CSFV-E2. These results suggested that the BAC vector sequence harbours an (intrinsic) genetic instability in infected insect cells. This should be of major concern for the utilization of bacmid-derived baculoviruses for large-scale production of proteins.

The major genomic deletions in SeMNPV (ORF 15 to 35), subject of investigation in Chapter 5, and those in the BAC vector of AcMNPV bacmids (Chapter 6) have in common that the deleted sequences are located in regions with a large distance between neighbouring *hrs*. In Chapter 7, the hypothesis is tested that genomic deletions are more likely to occur in regions with a low *hr* density, since *hrs* are thought to act as *ori* in viral DNA replication. Therefore, the properties of the standard bacmid-derived SeMNPV expression vector (generated in Chapter 3) were compared to those of bacmid-derived viruses with an additional *hr* engineered into the BAC vector in bioreactor-driven expression experiments (Chapter 7). All bacmid-derived viruses contained a GFP marker gene for detection of infection and a CSFV-

E2 gene as an example for secreted glycoprotein production. The level and maintenance of heterologous protein production with SeMNPV *hr-ori* insertional mutants was compared with that of the control viruses in a so-called "continuous cascaded" insect-cell bioreactor configuration. The insertion of an extra *hr* in the BAC vector led to improved genetic stability of adjacent sequences (including GFP and CSFV-E2 genes), resulting in enhanced and extended protein expression. However, this result was only obtained when the *hr* was placed in opposite orientation to the authentic *hr* present in the genome, suggesting that the orientation of the inserted *hr* is critical for maintenance of the BAC vector.

In Chapter 8 the overall results on the origin and generation mechanism(s) of baculovirus DIs and the implications for large-scale protein production in insect cells are discussed. More specifically, the elucidation of the role of non-*hr ori*s in the generation of DIs and the effect of *hr*s on genetic instability of the viral genome, provide keys to engineer baculovirus expression vectors with improved stability. Also, a novel approach using bicistronic baculovirus vectors to maintain protein expression upon virus passage is described and experimentally validated. Current developments to improve the baculovirus-insect cell expression system are described, and a proposal for the design of a superior baculovirus expression vector is presented. This vector is based on an infectious BAC clone (bacmid) that can readily be manipulated in *E. coli*. Bacmids are excellent tools to study genetic diversity in natural baculovirus isolates and baculovirus gene function, and can be used as well-defined backbones for the design of enhanced expression vectors or bioinsectides. This research has led to improvements for the large-scale production of heterologous proteins with stabilized baculovirus expression vectors in insect cells.

SAMENVATTING

Baculovirussen kunnen op diverse wijzen worden toegepast, b.v. als hoogspecifieke bio-insecticiden of als zogenaamde "surface display" vectoren, en in de toekomst wellicht in de gentherapie. Op dit moment worden baculovirussen echter met name gebruikt als eukaryote (gen)expressievector voor de productie van (glyco)proteïnen in insectencellen (Hoofdstuk 1). Hoewel het baculovirus-insectencel expressiesysteem gedurende de laatste tientallen jaren op velerlei wijzen verbeterd en verfijnd is, vormt het z.g. "passage-effect" nog steeds een groot probleem waar het gaat om grootschalige productie van virus en/of recombinant eiwit in insectencel-bioreactoren. Dit passage effect, dat een sterke productieafname veroorzaakt, treedt op bij een toenemend aantal viruspassages en is het gevolg van het ontstaan en de daaropvolgende vermenigvuldiging van defecte interfererende (DI) baculovirussen. Deze DI deeltjes interfereren met het oorspronkelijke, intacte virus ondermeer door concurrentie voor replicatiefactoren. Om de productkwaliteit en –kwantiteit te kunnen verzekeren moeten er herhaaldelijk virusvoorraden ("stocks") van lage passage worden aangemaakt en bewaard. Verder dient bij producties op grote schaal het aantal viruspassages gering te blijven, om de vorming van DI's tot een minimum te beperken. DI's missen aanzienlijke delen van het virale genoom en zijn vaak verrijkt met startpunten van DNA-replicatie (zogenaamde "*ori*'s"), die ofwel verbonden zijn met de homologe regio's ("*hr*'s") van baculovirussen, ofwel met nucleotidenvolgorden van het niet-*hr* ("non-*hr*") *ori* type. Dit geeft DI's een competitievoordeel dat bij elke passage nadrukkelijker wordt.

Aan het begin van het in dit proefschrift beschreven onderzoek was het niet duidelijk of het ontstaan van baculovirus DI's slechts een artefact van de (*in vitro*) insectencelkweekmethoden is, of dat deze ook aanwezig zijn in natuurlijke virusisolaten. In Hoofdstuk 2 is daarom allereerst de herkomst van DI's bij het qua toepassingsmogelijkheden thans belangrijkste baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) onderzocht. Dit geschiedde met behulp van een zogenaamde dubbele ("nested") PCR-methode. De resultaten tonen aan dat DI's met grote deleties aanwezig zijn in viruspreparaten en polyeders na een beperkt aantal passages, maar niet in het authentieke isolaat vóórafgaand aan passage in celcultuur. Dit toont aan dat AcMNPV DI's kennelijk onder laboratoriumcondities in celkweken ontstaan en niet van nature voorkomende deletiemutanten zijn. Ondersteunend bewijs hiervoor werd verkregen door middel van seriële passage van

een genetisch homogene virale kloon, d.w.z. een "bacterial artificial chromosome (BAC)", dat het volledige AcMNPV-genoom bevat (bacmide). Daarbij werden aanwijzingen gevonden voor het optreden van zowel homologe als heterologe recombinatiestappen uiteindelijk resulterend in DI's met zeer grote deleties. De resultaten illustreren dat het ontstaan van DI's een intrinsieke eigenschap is van baculovirusinfecties in insectencelkweken en dat DI zich kunnen handhaven *in vivo* (in het hele insect).

Om het ontstaan van baculovirale DI's nauwkeuriger te bestuderen, werd in Hoofdstuk 3 overgegaan op een ander modelvirus, te weten *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV), dat in *S. exigua* (Se301) cellen sneller en reproduceerbaarder DI's produceert dan AcMNPV in Sf21. Seriële passage van SeMNPV in Se301 cellen resulteert binnen 10 passages reeds in een sterke afname van extracellulair virus en van polyhedrine productie. Deze afname valt samen met de sterke vermenigvuldiging van DI's die meerdere kopieën van de SeMNPV non-*hr ori* bevatten en die zich vermenigvuldigen als concatemeren. Ook voor SeMNPV werd een genetisch homogene, bacteriële kloon (bacmide) van SeMNPV gemaakt om aan te tonen dat soortgelijke DI's met meerdere non-*hr ori*s gemakkelijk en *de novo* kunnen worden gegenereerd. Gebruikmakend van een nieuwe mutagenesetechniek gebaseerd op homologe, recET-afhankelijke recombinatie in *E. coli*, werd vervolgens een SeMNPV-bacmide zonder non-*hr ori* geconstrueerd, die gedurende seriële passage stabieler bleek te zijn dan wildtype virus en als gevolg een duurzamere polyhedrineproductie gaf. Hieruit kon geconcludeerd worden dat de non-*hr ori*'s een cruciale rol spelen bij het ontstaan van baculovirus DI's, kennelijk als gevolg van de bijdrage van deze *ori* bij het sterke replicatievoordeel in celcultuur. Tevens werd een stabiele SeMNPV-expressievector verkregen, die gebruikt kan worden voor grootschalige heterologe eiwitproductie en voor functionele studies aan het SeMNPV-genoom.

Vervolgens is onderzocht of de ophoping van DI's (in feite ook uit te drukken als "non-*hr ori*-concatemeren") afhankelijk is van de gebruikte cellijn. Daartoe werd SeMNPV 'gepasseerd' in 2 andere *S. exigua*-cellijnen, SeUCR en SeIZD2109 (Hoofdstuk 4). Echter, in deze lijnen werd geen ophoping van non-*hr ori* concatemeren waargenomen. De in Se301-cellen gevormde non-*hr ori* concatemeren konden zich echter wel gedurende enkele passages in SeUCR en SeIZD2109 cellen handhaven. Het cellijn-specieke verschil m.b.t. de vorming van DI's zou verklaard kunnen worden door ofwel een replicatievoordeel van de non-*hr ori* in alleen de Se301 cellen, of door de aan- of afwezigheid van bepaalde gastheerfactoren. Omdat bij dit onderzoek tevens werd aangetoond dat non-*hr ori*

concatemeren meer voorkomen in intra- dan in extracellulair gezuiverd viraal DNA, lijkt het er op, dat voor assemblage (als extracellulair virus) het in te pakken DNA een minimale grootte moet hebben.

Een ander effect van het optreden van DI's bij SeMNPV in celcultures is dat vaak het vermogen om *S. exigua*-rupsen via de reguliere, orale route te infecteren sterk vermindert. Dit kan hinderlijk zijn wanneer dit virus grootschalig gekweekt wordt t.b.v. biologische bestrijdingstoepassingen. Gebaseerd op eerder onderzoek met SeMNPV-deletiemutanten kon worden voorspeld dat één of meer genen (ook wel "open reading frames" (ORFs) genoemd), die naast elkaar op het SeMNPV genoom liggen (ORFs 29 tot 35), nodig zijn voor orale infectuositeit. De genen in SeMNPV verantwoordelijk voor dit fenotype werden in kaart gebracht door het aanbrengen van stapsgewijze deleties van de ORFs 29 tot 35, gebruikmakend van een SeMNPV-bacmide en homologe recombinatie in *E. coli* (Hoofdstuk 5). Aldus werd allereerst aangetoond dat ORFs 15 tot 35 (hieronder bevinden zich de genen *cathepsine, chitinase, gp37, ptp-2, egt, pkip-1* en *arif-1*) niet essentieel zijn voor *in vitro*-vermenigvuldiging of *in vivo*-replicatie na injectie in de hemolymfe. Vervolgens toonden voedingsproeven met de mutante polyeders aan dat deletie van het SeMNPV-ORF35 (Se35) leidt tot verlies van orale infectuositeit. Derhalve wordt Se35 aangeduid als "*per os* infectivity factor-2" (*pif*-2), naar analogie van een ander gen van *S. littoralis* NPV, dat "*per os* infectivity factor" (*pif*) wordt genoemd en een homoloog is van Se36. *Pif-2, pif* en *p74* behoren tot een relatief kleine groep van (30) genen, die geconserveerd is binnen de familie *Baculoviridae*.

In Hoofdstuk 3 is beschreven dat opeenvolgende infecties met AcMNPV of SeMNPV in insectencellen leidt tot het ontstaan van DI's met repetities van de non-*hr ori*. In AcMNPV overlapt deze non-*hr ori* met het coderende deel van het (niet-essentiële) *p94* gen. In Hoofdstuk 6 is het effect van een deletie van de AcMNPV non-*hr ori* op de expressieniveaus van recombinante eiwitten bestudeerd gedurende seriële passage in Sf21-cellen. Daartoe werden deleties in het *p94*-gen aangebracht d.m.v. homologe recombinatie in *E. coli*, en de resulterende mutante bacmiden uitgerust met 2 merkergenen voor het volgen van heterologe expressie, te weten het groen-fluorescerende eiwit-gen (GFP) en een gen, coderend voor het E2-glycoproteïne van het klassieke varkenspestvirus (CSFV-E2). Alleen bij het oorspronkelijke (intacte) bacmide werd een sterke vermenigvuldiging van DI's met repeterende non-*hr oris* waargenomen, maar niet voor de mutanten. Dit toont opnieuw aan dat een deletie van de non-*hr ori* bij *in vitro*-vermenigvuldiging de genetische stabiliteit van een baculovirus genoom vergroot, hetgeen de eerdere resultaten met SeMNPV bevestigt (Hoofdstuk 3). Echter, voor alle mutante virussen

werd gedurende passages een sterke afname in GFP en CSFV-E2 productie waargenomen. Dit werd veroorzaakt door een spontane deletie van de gehele BAC-vector-insertie, inclusief de expressiecassette, die de GFP en CSFV-E2 genen bevat. Dit geeft aan dat de winst m.b.t. genetische stabiliteit, verkregen door deletie van de non-*hr ori*, in dit geval teniet wordt gedaan door de toename van genetische instabiliteit van de ingebrachte BAC-vector-nucleotidevolgorde. Met dit effect dient nadrukkelijk rekening gehouden te worden bij het gebruik van bacmiden als uitgangsmateriaal voor eiwitproductie op grote schaal.

Een overeenkomst tussen de grote deleties in het SeMNPV-genoom (ORF 15 tot 35, zie Hoofdstuk 5) en die van de BAC-sequentie in AcMNPV-bacmiden (Hoofdstuk 6), is dat de verdwenen nucleotidenvolgorden gelegen zijn in gebieden met grote afstand tot naburige *hr*'s. Een mogelijke verklaring hiervoor zou kunnen zijn dat er deleties in het genoom ontstaan in gebieden met een lage "*hr* dichtheid". In Hoofdstuk 7 is deze hypothese getest door de standaard SeMNPV-expressievector (zie Hoofdstuk 3) in bioreactor experimenten te vergelijken met (van bacmide–afgeleide) virussen met een extra *hr* in de BAC-vector-insertie (Hoofdstuk 7). Om het infectieproces te kunnen volgen werden alle virussen uitgerust met een GFP-merkergen, en tevens met een CSFV-E2- gen als voorbeeld voor productie van een uit te scheiden glycoproteïne. De insertie van een extra *hr* in de BACvector leidde inderdaad tot een toegenomen genetische stabiliteit van de naburige nucleotidenvolgorden (inclusief GFP en CSFV-E2 genen), resulterend in een verhoogd eiwitexpressieniveau. Dit resultaat werd echter alleen verkregen wanneer de *hr* in de tegengestelde oriëntatie ten opzichte van de authentieke, in het genoom aanwezige, *hr* werd geplaatst.

Tenslotte worden de in dit proefschrift beschreven resultaten in hoofdstuk 8 besproken met betrekking tot herkomst en ontstaansmechanisme van baculovirale DI's en hun implicaties voor grootschalige eiwitproductie in insectencellen. Het aantonen van de rol die non-*hr ori*'s spelen bij het ontstaan van DI's en het effect van *hr*'s op de genetische stabiliteit van het virale genoom bieden uitstekende aanknopingspunten voor het ontwerpen van baculovirale expressievectoren met een sterk verbeterde stabiliteit. Tevens is in dit hoofdstuk een nieuwe strategie beschreven (en gedeeltelijk experimenteel gevalideerd), gebaseerd op het gebruik van bicistronische baculovirusvectoren t.b.v. duurzame eiwitexpressie in bioreactoren. Tevens worden in Hoofdstuk 8 een aantal andere, recent beschreven verbeteringen van het baculovirus-insectencel expressiesysteem besproken, en wordt een ontwerp voor een hoogwaardige baculovirale expressievector naar voren gebracht.

NAWOORD

Een proefschrift kost je vier jaar van je leven. Bij de start lijkt het project overzichtelijk en goed behapbaar, maar naarmate de tijd verstrijkt en je lekker aan het pipetteren slaat, komt opeens het moment dat je lastig gevallen wordt door het continu opdoemen van nieuwe ideeën. Gelukkig vallen er veel af door logisch afstrepen (te prematuur, te duur, te weinig handen of gewoon dom), en de rest blijft over om je mee te vermaken. Na de eerste publicatie (en bijbehorende taart) komen opeens de studenten aangestormd, die altijd de projecten willen doen die je voor jezelf gepland had. Hoe dan ook, het werk wordt gedaan en de vraagstelling beantwoord. Na het nodige focusseren en afmaken van essentiële proeven blijft er aan het einde van de rit toch een bergje onafgemaakt experimenteel werk over, wat je moet laten liggen omdat je moet schrijven. Maar, er is altijd licht (schuimend bier) aan het einde van de tunnel, en dan is daar opeens het boekje, fris gedrukt en scherp gesneden.

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CURRICULUM VITAE

Gorben Peter Pijlman werd geboren op 19 november 1975 in Terwispel. In 1994 behaalde hij het VWO diploma aan de Rijksscholengemeenschap in Heerenveen. In datzelfde jaar werd aangevangen met de studie Bioprocestechnologie aan Wageningen Universiteit, waar hij in januari 1999 het ingenieursdiploma behaalde. Tijdens de doctoraalfase heeft hij afstudeeronderzoek verricht bij achtereenvolgens het Laboratorium voor Virologie aan Wageningen Universiteit (prof. dr J.M. Vlak), de groep Dierlijke celkweek van de sectie Proceskunde van Wageningen Universiteit (dr. D.E. Martens) en bij de Division of Entomology van het CSIRO Canberra, Australië (dr. David J. Dall). Van april 1999 tot april 2003 was hij werkzaam als onderzoeker in opleiding bij het Laboratorium voor Virologie in samenwerking met de sectie Proceskunde van Wageningen Universiteit, onder begeleiding van prof. dr. J.M. Vlak, prof. dr. R.W. Goldbach en dr. D.E. Martens. Van het daar uitgevoerde onderzoek, dat gefinancierd werd door de Stichting Technische Wetenschappen (STW) van de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO), staan de resultaten beschreven in dit proefschrift. Sinds april 2003 is hij werkzaam als Post-Doc bij het Sir Albert Sakzewski Virus Research Centre, Brisbane, Australië.

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