Molecular genetics of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome

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VOORWOORD

Vreemd genoeg wordt een voorwoord voor een proefschrift meestal achteraf geschreven. Het is dan een soort terugblik op vier jaar of soms langer onderzoek. Dat is in dit geval ook zo. De experimenten zijn gedaan en het manuscript ligt dukklaar naast me. Langzaam laat ik vier jaar promotie onderzoek bij de vakgroep Virologie van de Landbouwuniversiteit door mijn gedachten gaan. Allerlei leuke ervaringen en mensen schieten mij te binnen. Zonder iemand te willen vergeten zou ik hier graag een aantal van die mensen willen noemen.

Allereerst denk ik dan aan mijn begeleiders, de beide hoogleraren van de vakgroep, Just Vlak en Rob Goldbach en co-promotor Douwe Zuidema. Zij hebben mij al die tijd de ruimte geboden om het project naar eigen inzicht richting en inhoud te geven en keken als het ware over mijn schouder mee. Daarnaast stimuleerden zij mij de resultaten van het onderzoek voor een breed internationaal publiek in artikelen en voordrachten te presenteren

Dan waren er mijn kamergenoten. Bep van Strien en Rene Broer, ofwel het Se-promotie team. Dit proefschrift is mede dankzij jullie discussies over proefopzetten en "regenachtige zaterdagmidddag" experimenten geworden tot wat het nu is. Aan onze wekelijkse Royal Baculovirus Beer Society meetings, om de scherpe kantjes van de week weg te spoelen, zal ik nog lang met veel plezier terugdenken.

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Alle studenten die gewerkt hebben aan onderdelen van het proefschrift in het kader van hun afstuderen. Jan Koenderink, Henri Kester, Eric Coenen, Suzanne Luppens, Femke Bijlsma, Michel Kooijman, Corinne Kruiswijk en Adriënne Oomen. Een speciaal woord van dank aan Yi Liu (Jenny) die onder andere gezorgd heeft voor het sequencen van *Xba*I-C en met wie ik twee jaar perfect heb mogen samenwerken.

Tenslotte, mijn familie en vrienden die steeds maar weer begrip hebben opgebracht voor het feit dat ik weer eens een proefje moest doen, en dus niet gezellig was. Met jullie had ik misschien dit voorwoord wel moeten beginnen, want zonder jullie morele ondersteuning, geduld en vertrouwen had ik dit niet af kunnen maken.

Stellingen

behorende bij het proefschrift:

"Molecular genetics of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome"

1. Het feit dat uit het baculovirus isolaat SeMNPV-US zo snel een deletiemutant gezuiverd kan worden, duidt erop dat selectiedruk op handhaving van het volledige genoom wegvalt bij passage van het virus in cellijnen.
   Dit proefschrift.

2. De grootte van het genoom van het SeMNPV-isolaat dat Hara *et al.* bestudeerden en de afwezigheid van 11.5 en 13.8 kb fragmenten in het *Pst*I digest van dit virus doen vermoeden dat ook hier sprake is van een deletiemutant van SeMNPV.
   Hara *et al.*, (1995). Acta Virologica 39, 215-222.
   Dit proefschrift.

3. Er is onvoldoende bewijs geleverd voor de conclusie dat AcMNPV-p143 aspecifiek aan *hr*s bindt.
   Laufs *et al.*, (1997). Journal of Virology 228, 98-106.

4. De conclusie dat de genetische organisatie van SpliNPV zeer afwijkend is van die van andere baculovirussen is voorbarig.
   Faktor *et al.*, (1997). Archives of Virology 142, 1-15.
   Van Strien, (1997). Proefschrift Landbouwuniversiteit Wageningen.

5. Het enkelstrengs DNA bindend eiwit dat tijdens het DNA-replicatieproces een wisselwerking heeft met het helicase is in het baculovirus replisoom het LEF3 eiwit.
   Evans *et al.*, (1997). Abstract 16th Annual meeting of the American Society for Virology.
   Carstens *et al.*, (1997). Abstract 20th Annual meeting of the Society for Invertebrate Pathology.

6. De conclusie op grond van een artikel uit 1959, dat er geen vrij manteleiwit van tymovirus kan worden verkregen onder fysiologische condities lijkt op het eerste gezicht voorbarig.
   Hellendoorn, (1998). Proefschrift Rijksuniversiteit Leiden.
   Kaper and Steere, (1959). Virology 7, 127.

7. Bij een goede omschrijving van het begrip 'potency' zouden zowel de farmaceutische industrie als de registratieautoriteiten gebaat zijn.

8. *Pestiviridae* doen hun naam eer aan.

9. Het samenvallen van het kortstondig inzakken van de financiële markten in Hong Kong en de incidentie van influenza stam H5N1 aldaar duidt erop dat dit virus ook infectieus is voor beursindexen.

10. Door de tariefsverhoging van de NS voor spitsvervoer en de invoering van het rekeningrijden, wordt het fileprobleem niet opgelost maar alleen in tijd en ruimte verplaatst.

11. Het clonen van mensen en dieren maakt DNA testen onbruikbaar voor identificatiedoeleinden.

12. Promoveren is het spelen van het spel dat wetenschap heet.

13. "Onder professoren" (W.F. Hermans, 1975) zou verplichte startliteratuur moeten zijn voor promovendi.

14. De vernieuwing die het paarse kabinet bij zijn aantreden beloofde zit 'm vooral in de naam.

Wageningen, 3 april 1998
J.G.M. Heldens

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Chapter 1

INTRODUCTION

General Introduction

Baculoviruses are found almost exclusively in insects (Granados and Federici, 1986) with a few representatives able to infect Crustacea (Summers, 1977). These viruses have a restricted host-range of one or a few related insect species, belonging mainly to the insect orders *Lepidoptera, Hymenoptera* and *Diptera* (Martignoni and Iwai, 1986; Adams and McClintock, 1991). The members of the *Baculoviridae* are characterized by the presence of rod-shaped virions (50-200 nm), often found occluded into large cuboidal proteinaceous capsules also called occlusion bodies or polyhedra. The baculoviruses, can be classified into two large genera, nucleopolyhedroviruses (NPV) and granuloviruses (GV) (Murphy *et al.*, 1995). Both types of viruses have a large, double-stranded, covalently closed DNA genome varying in size between 88-153 kilobase pairs (kbp) depending on the virus species. NPVs have either multiple nucleocapsids (M) or a single nucleocapsid (S), enveloped into a virion, which in turn is occluded into occlusion bodies. GVs occlude a single virion per occlusion body (here called granulum), which is more irregularly shaped than the occlusion body of NPVs (Blissard and Rohrmann, 1990, for review; Murphy *et al.*, 1995). The occlusion bodies range in size from 0.1 to 10 μm in diameter.

Larvae of lepidopteran insect species often cause severe feeding damage on agriculturally important crops, in ornamental plants, and on forest trees. The resistance of many of these insect species to chemical insecticides and a changing public view on the use of those environmentally hazardous chemicals has prompted the development of alternative pest control strategies. Baculovirus infections, first observed in silkworms (Benz, 1986, for historical review), regulate the size of host insect populations in nature. As a result considerable attention has been paid to their development and use as biological insecticides. Nowadays, baculoviruses are employed world-wide as ecologically safe biological control agents (Cunningham, 1995, for review). Recently, a baculovirus of the beet army worm, *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV), has been registered in The

Netherlands and the USA as biological insecticide against this pest insect (Smits and Vlak, 1994).

Due to some unique features of baculovirus replication and gene expression and the availability of suitable cell lines, baculoviruses and insect cells are also widely exploited as an expression system for the large scale production of recombinant proteins of biotechnological or pharmaceutical importance (Luckow, 1991, for review). The technological advancements associated with the generation of expression vectors, had a positive feed-back on the development of baculoviruses for improved insecticidal properties (Bonning and Hammock, 1996). An indepth analysis of the baculovirus genome organization, replication and gene expression strategy is a prerequisite for optimal exploitation of these economically important applications. This genetic analysis may also further lead to a better understanding of the principles governing baculovirus pathogenicity.

Infection cycle

Transmission of baculovirus in insect populations occurs either via oviposition of eggs contaminated with occlusion bodies or the ingestion of food or soil contaminated with these bodies by insect larvae. Upon ingestion, the occlusion body (polyhedron or granulum), predominantly composed of a 29 kDa protein called polyhedrin or granulin, dissolves in the alkaline environment of the larval midgut liberating numerous infectious virions. These virions invade the larval midgut by fusion with the microvilli of the midgut epithelial cells. The virions are uncoated in the cytoplasm and the nucleocapsids are transported to the nucleus. Here they are uncoated and the first steps of virus gene expression and genome replication take place. A characteristic feature of baculovirus infection is the existence of two infectious forms of the virus. The occlusion body-derived (ODV) form, which is infectious for insects and responsible for the spread of the infection in the population from insect to insect, and the budded virus form (BV) which is responsible for the spread of a baculovirus infection through a larval body (Federici, 1997; Williams *et al.*, 1997). Morphologically these two virus-types are largely different (Funk *et al.*, 1997). (Fig. 1.1ab) This progeny virus produced in the columnar cells of the midgut epithelium buds through the cytoplasmic membrane and basal laminae into the haemocoel of the host insect acquiring a loose fitting envelope. Alternatively, the virions are budded into the tracheal end cells (tracheoblasts) entering the tracheal system, which facilitates quick spread through the insect body (Federici, 1997; Williams *et al.*, 1997). The virus spreads into all tissues of the insect body, but predominantly targets to the fat body.

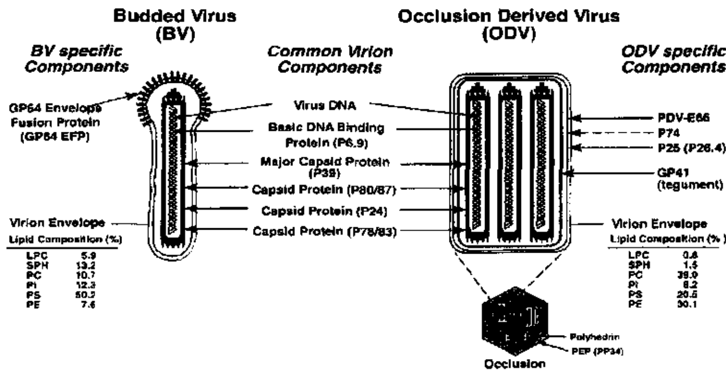

Figure 1.1a.
Structural composition of the two baculovirus phenotypes, the budded virion, BV, and the occlusion body derived virion ODV (Funk *et al.*, 1997 for review). The ODV structure represents the NPV subgroup. Proteins common to both virion types are indicated in the middle of the figure. Proteins specific to either BV or ODV are indicated on the left or right respectively. The polar nature of the baculovirus capsid is indicated in the diagram with the claw-like structure at the bottom and the ring-like nipple at the top of the capsid. The possible location of p74 is indicated by a dashed line. Lipid compositions of the BV and ODV envelopes derived from AcMNPV infected Sf-9 cells (Braunagel and Summers, 1994) are indicated (LPC, lysophosphophatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethonalamine).

The virions produced in this second round of infection (>24 h) are enveloped in the nucleus in a *de novo* formed, tight-fitting membrane, followed by occlusion into occlusion bodies. (Fig. 1.1ab) The fat body is the major infected tissue and as a result the main producer of new occlusion bodies. Eventually the infected cell disintegrates after nuclear and cellular membranes break down releasing the occlusion bodies in the environment. (Federici, 1997; Williams *et al.*, 1997, for review). Virus-encoded proteins such as fibrillins, chitinases and cathepsins, have been implicated in this process (Hawtin *et al.*, 1995; Slack *et al.*, 1995; Hill *et al.*, 1995; van Oers and Vlak, 1997).

As predicted by their size, baculoviral genomes encode about 150 average sized genes. After entrance into the celland uncoating of the virion in the nucleus, these genes are expressed sequentially, in a cascade-like fashion in which each successive phase is dependent on the previous one (Blissard and Rohrmann, 1990). Regulation of baculovirus gene expression occurs at the transcriptional level. Three separate phases, **early**, **late** and **very late**, are distinguished during a baculovirus infection. Genes expressed during the **early** phase of the infection are transcribed by a host-cell encoded RNA polymerase.

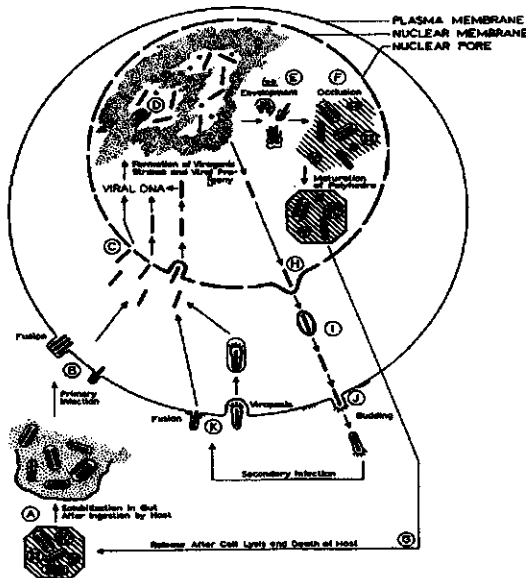

Figure 1.1b.
Schematic representation of the baculovirus infection cycle (from van der Beek, 1980; van Strien, 1997). Ingested polyhedra are solubilized in the midgut and virions are released (A). The envelopes of the virions fuse with the plasma membrane of the insect cell (B). After traversing the cytoplasm virions enter the nucleus at a nuclear pore, uncoat and the viral DNA enters the nucleus (C). Progeny viral nucleocapsids are synthesized in the virogenic stroma (D). Following envelopment in the nucleus (E), progeny nucleocapsids are initially released by budding (H,I,J). Budded virions infect adjacent cells by endocytosis (K). Nucleocapsids produced in later stages of an infection become occluded in polyhedral protein (F). Finally, the occlusion bodies are released by lysis of the infected cell.

The expression of early baculovirus genes can be blocked by typical eukaryotic RNA polymerase II inhibitors such as α-amanitine.Their promoters contain consensus transcription signals such as TATA boxes and 5'-CAGT-3' transcriptional initiation sites that are activated by the host cell transcriptional machinery. Among the important early genes are the immediate-early gene 1 (*ie-1*), *ie-2*, *ie-3*, *HE*65 and *p35*. The *ie-1*-gene product (IE1) is involved in the transactivation of many genes that are expressed during later phases of a baculovirus infection. The role of the other *ie*-genes is still unclear (Friesen, 1997). A specific role is played by P35, which has anti-apoptotic activity (Clem, *et al.*, 1991; Clem and Miller, 1994; Clem, 1997).

The transcription of early genes is enhanced *in cis* by homologous regions or *hr*s, which are found interspersed in the genomes of many baculoviruses. These *hr*s contain repeats of imperfect palindromic sequences mostly separated by AT rich stretches or short direct repeats (Kool *et al.*, 1995, for review). They increase early promoter activity and

function irrespective of their orientation towards the enhanced gene. Maximum gene expression is obtained when IE1 binds the *hr*. The **late** phase is defined by viral transcription that occurs concurrently with or immediately after the onset of viral DNA replication. Late genes are transcribed by an α-amanitine resistant RNA polymerase that is possibly virus encoded. Among the late genes are those involved in virion structure and morphogenesis. All late genes are characterized by the presence of a 5'-A/GTAAG-3' promoter element that functions as transcriptional start site (Lu and Miller, 1997). Genes that are expressed **very late** in the infection process, such as polyhedrin or p10, are mostly associated with virus packaging, polyhedra formation or cell lysis (Lu and Miller, 1997).

Application of baculoviruses as bio-insecticides and as gene expression vectors

Many lepidopteran insect species cause severe economic losses in important agricultural and horticultural crops throughout the world. Since many pest insects have become resistant to most commonly used chemical insecticides and since the use of hazardous chemicals is disputed for ecological reasons for a long time (Bohmfalk, 1986), alternative bio-control strategies such as the use of parasites, predators and microorganisms are strongly promoted. Baculoviruses represent a natural component of the ecosystem and have been successfully applied as bio-insecticides in various biological and integrated pest management programs (Gilbert and Kerkut, 1985; Black *et al.*, 1997). Unfortunately, these viruses are still unable to compete with classical chemicals due to their relatively slow speed of action, the special application technology required, their low field persistence and their limited host specificity. The latter, economically unattractive property however may also be considered as an asset from an environmental and biosafety point of view. Other disadvantages are related to the difficulty and cost aspects of large scale production in larvae or in bio-reactors. Biotechnological approaches such as the genetic modification of viruses and the use of cell culture offer opportunities to eliminate some of these drawbacks. Introduction of insect specific neurotoxin genes (Maeda *et al.*, 1991; McCutchen *et al.*, 1995; Stewart *et al.*, 1991; Tomalski and Miller, 1991; 1992) in the genome of AcMNPV or the manipulation of the insect hormone levels (O'Reilly and Miller, 1989; 1990; 1991; Bonning and Hammock, 1996), resulted in baculoviruses with an increased speed of kill or which reduced feeding damage caused by the insects. Bio-assays and contained field trials carried out with wild-type and genetically engineered baculoviruses have addressed recombinant-virus efficacy, stability and environmental safety (Bonning and Hammock, 1996; Cory *et al.*, 1994; Black *et al.*, 1997).

Development of the baculovirus expression system opened up the possibility to genetically modify baculovirus genomes for biocontrol purposes (King and Possee, 1992; O'Reilly *et al.*, 1992; Richardson, 1995, for reviews). The engineering procedure prescribes, in short, that the gene of interest is cloned in designed transfer vectors containing baculovirus sequences flanking the site of insertion. After co-transfection of the baculovirus genome and the particular transfer vector, recombination will occur between the homologous sequences of the transfer vector and the viral DNA resulting in the insertion of the foreign gene in the specific locus. Purification of the recombinant from the wild-type virus background via plaque purification assays is facilitated by the presence of selectable markers in the transfer vector such as ß-galactosidase (Vlak *et al.*, 1990; Zuidema *et al.*, 1990), luciferase (Oker-Blom *et al.*, 1993) or the green fluorescent protein from *Aequorea victoria* (Reiländer *et al.*, 1996). Strong promoters of genes that are expressed during the later stages of infection such as p10, polyhedrin and p6.9 have been successfully used to drive the high level expression of foreign genes. Most post-translational modification (phosphorylation, acetylation, amidation, etc.) occurs in insect cells normally as in any other animal cell system. The glycosylation of proteins in insect cells is slightly different from mammalian cells. Large scale production of the recombinant proteins has been established and optimized using cascades of bio-reactors (de Gooijer, 1995 ;van Lier, 1995).The baculovirus insect cell expression system is one of the systems od choice in order to obtain large amounts of near authentic proteins from an animal source.

Baculovirus DNA replication

The shift from host-encoded to virus-dependent transcription is a key phase of a baculovirus infection process. It is hypothesized that the immediate early genes transactivate an array of delayed genes encoding among others those proteins involved in DNA replication (Kool *et al.*, 1995, for review). In general DNA replication is initiated by the formation of a replisome complex involving *cis*-acting elements in the virus genome, generally known as origins of DNA replication (*ori*), and *trans*-acting elements (Kornberg and Baker, 1992; DePamphilis, 1993, for review).

These *cis*-acting elements in the AcMNPV genome have been localized using a transient DNA replication assay (Kool *et al.*, 1995 and Lu *et al.*, 1997, for review). All but one of the replicating *cis*-acting elements could be related to homologous regions, *hr*s, found dispersed in the baculovirus genome. *Hr*s contain one or more palindromic repeats, which are important for replication activity. The number of palindromes present in the *hr*s appeared to

be independent of the replication efficiency (Leisy and Rohrmann, 1993). Mutagenesis of the central core sequence of the palindrome does influence its capability to replicate (Leisy *et al.*, 1995). The role of the putative secondary structure of *hr*s (hairpins) in the replication or transcription process remains enigmatic (Rasmussen *et al.*, 1996). *Hr*s and their ability to prime replication have been identified in many more baculoviruses such as *Choristoneura fumiferana* MNPV (Xie *et al.*, 1995), *Orgyia pseudotsugata* MNPV (Theilmann and Stewart, 1992; Ahrens *et al.*, 1995a) and *Bombyx mori* NPV (Maeda and Majima, 1990; Majima *et al.*, 1993).

In addition to *hr*-like putative origins of DNA replication a non-*hr*-like sequence, active as an origin of DNA replication, has also been identified in the genomes of OpMNPV (Pearson *et al.*, 1993) and AcMNPV (Kool *et al.*, 1994b). The non-*hr* origins are devoid of any *hr*-like palindromes or repeats and their complex overall organization is unrelated to *hr*-sequences or to each other. The accumulation of non-*hr* sequences in defective interfering particle (DIP) genomes after multiple passaging of baculovirus in cell culture further suggests their putative role in baculovirus DNA replication (Lee and Krell, 1992, 1994).

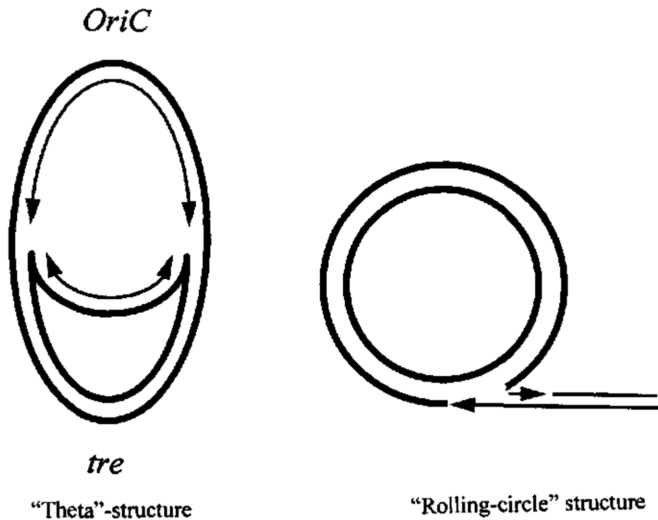

Figure 1.2.
Schematic representation of phage λ "rolling-circle" and *Escherichia coli* "theta" DNA replication mechanisms (Kornberg and Baker, 1992).

The role that each of the individual origins might play in baculovirus DNA replication, whether they are all functionally active simultaneously and whether they are interchangeable between the virus species, is still unknown. Deletion of a single *hr* from the

AcMNPV or BmNPV genome did not influence the replication of these viruses (Majima *et al.*, 1993; Rodems and Friesen, 1993). The presence of multiple replication origins dispersed in baculovirus genomes may ensure that DNA replication occurs. The mechanism by which the large baculovirus DNA genome is replicated is also unknown. Preliminary results revealed the occurrence of high molecular weight DNA, probably containing unit length viral genomes, during baculovirus infection (D. Levin, personal communication). Characterization of the replication mechanism of plasmids harboring baculovirus replication origins showed the appearance of concatameric unit length DNA molecules (Leisy and Rohrmann, 1993). This suggests a 'rolling circle' like DNA replication mechanism for baculoviruses. A 'theta'-like replication mechanism, however can not yet be excluded. The acceleration of the expression of the replication machinery exploiting the *hr*s would facilitate a fast initial amplification of circular molecules using several origins at the same time (Kool *et al.*, 1995, for review). Limitations in the concentration of replication factors would favour a 'rolling-circle' mechanism (Fig. 1.2) at later stages in the infection process.

DNA replication also involves *trans*-acting factors such as helicases, DNA polymerases, primases and a number of other factors (Kornberg and Baker, 1992). A set of *trans*-acting factors required for the transient replication of AcMNPV- (Kool *et al.*, 1994a) and OpMNPV-*hr*s (Ahrens and Rohrmann, 1995 a,b; Ahrens *et al.*, 1995 b) has been identified ;. the Late Expression Factors 1 (LEF1), LEF2, LEF3, DNA polymerase, helicase and IE1 pe38, p35 and IE2. The function of these LEFs during late gene expression and DNA replication was addressed using library-dependent transient CAT gene expression from baculovirus early and late promoters (Passarelli and Miller, 1993ab; Li *et al.*, 1993; Todd *et al.*, 1995), and DNA replication assays (Lu and Miller, 1995; Kool *et al.*, 1994; Lu *et al.*, 1997). Biochemical evidence for the exact function of the individual factors in the replication process and the assembly of the replisome is limited. LEF1 could be a primase-associated factor based on amino acid sequence similarity with Herpes simplex virus UL8 (Kool *et al.*, 1994). LEF1 interacts with LEF2, which could be the baculovirus homologue of Herpes simplex virus UL42-like DNA polymerase processivity factor (Evans *et al.*, 1997; Kool, 1994). In AcMNPV, mutation of *lef2* in the AcMNPV genome resulted in a mutant deficient in very late gene function. LEF2 therefore may have a putative double function, in late gene expression and in DNA replication (Merrington *et al.*, 1996). LEF3 has single stranded DNA binding properties (Hang *et al.*, 1995; Ahrens *et al.*, 1995b) and may be functional as a homotrimer in solution (Evans *et al.*, 1997). A putative helicase or P143 (Lu and Carstens, 1991; 1992) binds *hr*s (Laufs *et al.*, 1997) and the gene is transactivated by IE1 and pe38 (Lu and Carstens, 1993). The *p143* gene product, P143 or helicase, is required for DNA replication as evidenced by the isolation of temperature sensitive AcMNPV mutants with a defect *p143* gene that prevents DNA replication and late gene transcription at the non-

permissive temperature (Gordon and Carstens, 1984; Lu and Carstens, 1991). The putative helicase has another interesting feature since it also appears to be involved in the host range specificity of AcMNPV-BmNPV recombinants (Kondo and Maeda, 1991; Croizier *et al.*, 1994). The role of the baculovirus DNA polymerase in DNA replication is somewhat enigmatic. It has been localized in many baculoviruses (Tomalski *et al.*, 1988; Bjornson *et al.*, 1992; Cowan *et al.*, 1994; Liu and Carstens, 1995; Ahrens and Rohrmann, 1996) and found to be essential for DNA replication (Kool *et al.*, 1994). Lu and Miller (1995), however, provided evidence that its role is auxiliary and that its function can be carried out by host-cell DNA polymerase. Whether IE1 is an integral part of the replisome or whether it only functions as transactivator of the other "DNA replication genes" remains to be investigated.

Scope of the thesis

Crop damage caused by *S. exigua* larvae is a severe problem from an agricultural and economic point of view. Due to resistance of this insect to most commonly used chemical insecticides the use of wildtype SeMNPV to control this pest insect has been proposed and in some countries this virus is already registered as a bio-insecticide. SeMNPV is an attractive insecticide since it is monospecific (one host) and relatively virulent for *S. exigua* larvae (Smits, 1987). Nevertheless its speed of action is too slow to compete succesfully with chemical insecticides. The application of genetic methods developed for the baculovirus-insect cell expression system and prior experience with the introduction of insect specific neurotoxin genes in the genome of AcMNPV, promote the idea to improve the insecticidal properties of SeMNPV by genetic engineering.

With respect to virulence and host range specificity SeMNPV differs from many other baculoviruses, most of which are able to infect a number of related insect species and which need more time to cause disease in infected insect larvae. In contrast to the baculovirus type species, *Autographa californica* MNPV (AcMNPV), and to *Orgyia pseudotsugata* MNPV (OpMNPV), whose genomes are completely sequenced (Ayres *et al.*, 1994; Ahrens *et al.*, 1997), at the onset of the research described in this thesis, molecular knowledge of the SeMNPV genome was limited to some DNA restriction profiles of different geographical SeMNPV isolates (Gelernter *et al.*, 1986; Caballero *et al.*, 1992). Concomitant research, focusing on the sequence and transcriptional analysis of the genomic region between the *polyhedrin* and *p10* genes (van Strien, 1997), revealed major differences in the genetic organization of this area between AcMNPV and OpMNPV on the one hand and SeMNPV on the other. Phylogenetic analysis using for instance parsimony on a number of baculovirus

polyhedrin (Zanotto *et al.*,1993), *ecdysteroid-UDP-glucosyltransferase* (Hu *et al.*, 1997) and *ribonucleotide reductase* large subunit (van Strien *et al.*, 1997) genes revealed that SeMNPV is a member of a different clade than AcMNPV, OpMNPV, *Choristoneura fumiferana* MNPV and BmNPV, suggesting that SeMNPV diverged rather extensively from the above mentioned baculoviruses (van Strien, 1997).

Insight in to the molecular characteristics that specify the distinct biological properties of SeMNPV is important for the successful and biosafe genetic modification of this virus. Since little is known about the molecular genetics of the SeMNPV genome, the understanding of key steps in the infection process such as virus entrance, gene expression, genome replication or virion assembly, are required prior to the development and application of genetic engineering strategies. The present study aims at unravelling of the SeMNPV genome replication process and hence the identification and characterization of the components forming the SeMNPV DNA replication machinery. This information will indicate whether *Baculoviridae*, as a family, encode a universal set of DNA replication factors.

To assist in these molecular genetic studies, the construction of a plasmid and cosmid library of SeMNPV DNA and the establishment of a detailed physical map is essential (Chapter 2). Studies on gene function and genetic modification of the SeMNPV genome, via classical homologous recombination strategies require cell lines that support virus replication and maintenance. Therefore, the capacity of *S. exigua* cell lines to replicate and maintain the SeMNPV genome stably during multiple passaging is investigated (Chapter 2).

AcMNPV and OpMNPV belonging to the same phylogenetic clade, encode a replication machinery, consisting of only partially specific *cis-* and *trans*-acting DNA replication factors. Chapters 3 and 4 focus on the localization and characterization of *cis*-acting elements, non-*hr* and *hr* origins of DNA replication, respectively, in the SeMNPV genome. Their similarity and differences to *hr* and non-*hr* elements in other baculoviruses are discussed. Furthermore the specificity of these elements in baculovirus DNA replication is investigated (Chapter 3 and 4).

Subsequently, the identification of SeMNPV *trans*-acting DNA replication factors is initiated. As a first step the localization, sequence and transcriptional analysis of a *trans*-acting DNA replication factor, p143, with a putative function as helicase and in host specificity is described in Chapter 5. The role of SeMNPV p143 in the specificity of genome replication as well as the specificity of other *trans*-acting DNA replication factors in the process is discussed in this chapter.

To determine the genomic environment of the SeMNPV *p143* gene and to study the phylogenetic position of this virus, the flanking regions of this gene are sequenced and

transcriptionally analyzed. The relatedness of a genomic stretch of 11 kb of contiguous and a further 14 kb of partial sequence to corresponding sequences in the genomes of AcMNPV and OpMNPV is discussed (Chapter 6 and 7).

To study the virus-specific interaction between the individual elements of the SeMNPV replisome, the identification of all other SeMNPV *trans*-acting DNA replication factors in the viral genome is essential. To identify these, sequence analysis of the complete SeMNPV genome is initiated and the 'state-of-the-art' is discussed (Chapter 7). The availability of the complete nucleotide sequence and insight in the complete genetic organization, would facilitate the localization and gene content of the large mutation that occurs after replication of SeMNPV in cell culture (Chapter 2). Sequence information also allows phylogenetic analysis of SeMNPV based on the comparison of conserved and diverged gene clusters (Chapter 7).

Due to the inability of *S. exigua* cell lines to properly replicate and maintain SeMNPV, recombinants via homologous recombination cannot be generated and as a result alternative strategies have to be developed. Based on the physical and partial genetic map of the SeMNPV genome, the cloning and recombination of the baculovirus genome in *Saccharomyces cerevisiae* using yeast artificial chromosome (YAC) and yeast centromere plasmid (YCp) technology is investigated. Pilot experiments on the cloning and recombination of the AcMNPV and SeMNPV genomes in yeast and the recombination of the SeMNPV *in vivo* are described (Chapter 7).

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Chapter 2

SPODOPTERA EXIGUA MULTIPLE NUCLEOPOLYHEDROVIRUS DELETION MUTANTS GENERATED IN CELL CULTURE LACK VIRULENCE *IN VIVO*[1]

Summary

The baculovirus *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) has high potential for development as a bio-insecticide for control of the beet armyworm (*S. exigua*). It is highly infectious for *S. exigua* larvae and its host range is very narrow. A prerequisite for such application is the possibility of growing this virus in large quantities, e.g. in insect cell lines. It was observed, however, that polyhedra of SeMNPV plaque-purified in Se-UCR1 cells did not cause larval mortality or morbidity when fed to *S. exigua* larvae. As this phenomenon suggested a genetic alteration in *in vitro* produced SeMNPV, comparative restriction analysis of *in vitro* and *in vivo* produced SeMNPV DNA was performed. The restriction patterns of various isolated plaques showed identical differences to those of the wildtype genome and suggested that a large, single deletion had occurred in the *in vitro* produced viral genome. In order to localize this deletion more precisely a detailed physical map of the wildtype SeMNPV genome was constructed, using the restriction endonucleases *Xba*I, *Bam*HI, *Bgl*II, *Pst*I, *Sst*I, *Hin*dIII, and *Spe*I. Moreover the entire SeMNPV genome was cloned into a library containing five overlapping cosmids and a plasmid library. About 80 restriction sites have been located and the orientation of the map has been set according to the localization of the *polyhedrin* and *p10* genes. The approximate size of the viral genome was determined to be 134 kilobase pairs. Based on this map it could be established that mutant SeMNPV, obtained by passage in cell culture, contained a single deletion of approximately 25 kbp between map units 12.9 and 32.3.

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Introduction

The beet army worm (*Spodoptera exigua*; Lepidoptera, Noctuidae) is an agriculturally important pest insect in (sub)tropical regions of the Northern hemisphere and in greenhouses. The insect is resistant to many commonly used chemical insecticides. Recently a baculovirus of this insect, *S. exigua* nucleopolyhedrovirus (SeMNPV) has been registered in several countries as a biological insecticide (Smits and Vlak, 1994). SeMNPV is an attractive bioinsecticide since the virus has a narrow host-range and is relatively virulent compared to other baculoviruses (Smits *et al.*, 1988).

It may take several days after infection until the larvae stop feeding. The insecticidal properties of SeMNPV, in particular the speed of action, might be improved by genetic engineering. Successful attempts have been reported for *Autographa californica* MNPV (AcMNPV) via the introduction of insect-specific neurotoxin genes into the genome or the construction of *ecdysteroid-UDP-glucosyltransferase* (*egt*) gene deletion mutants (Stewart *et al.*, 1991; McCutchen *et al.*, 1991; Tomalski and Miller, 1991; O'Reilly and Miller, 1991). A prerequisite for successful genetic modification of SeMNPV is the availability of a physical and genetic map. Furthermore, a cell line able to support viral replication would facilitate the engineering of SeMNPV and the structural and functional analysis of its genome.

SeMNPV has a circular double stranded DNA genome of about 130 kilobase pairs (kbp) (Caballero *et al.*, 1992). Restriction fragment length polymorphism in several SeMNPV isolates has been reported (Caballero *et al.*, 1992), but a detailed physical map of the viral genome is not yet available. Three SeMNPV genes have been characterized and provisionally localized on genome fragments: *polyhedrin* (van Strien *et al.*, 1992), *p10* (Zuidema *et al.* 1993) and *ubiquitin* (van Strien *et al.*, 1996). Two insect cell lines derived from *S. exigua* have been described that support SeMNPV replication (Gelernter and Federici, 1986b; Hara *et al.*, 1993; Hara *et al.*, 1994). Preliminary experiments on the replication of SeMNPV in the cell line Se-UCR1 indicated that the budded virus (BV) was highly infectious for these cells, but that the polyhedra produced lacked infectivity for insects. This phenomenon could be correlated with a deletion of about 25 kbp from the SeMNPV genome. In this report a physical map of the SeMNPV genome for seven restriction endonucleases is described allowing the genetic analysis of *in vivo* and *in vitro* produced SeMNPV and the localization of the deletion in the latter.

Materials and methods

Virus, insects and cells.

Spodoptera exigua MNPV (SeMNPV/US) (Gelernter and Federici, 1986a) was obtained from Dr. B.A. Federici, Department of Entomology, University of California at Riverside, Riverside CA, USA, in the form of polyhedra. The virus was propagated in fourth instar larvae of *S. exigua* (Smits *et al.*, 1988). Larvae were infected by contamination of artificial diet with polyhedra. Hemolymph from SeMNPV-infected insects was used as a source of budded virus (BV) for the infection of cultured *Spodoptera exigua* cells (Gelernter and Federici, 1986b). This cell line (Se-UCR1), obtained from Dr. B.A. Federici, was maintained in plastic cell culture flasks in TNM-FH medium (Hink, 1970) supplemented with 10% foetal calf serum. *S. exigua* fourth instar larvae were infected with a dose of 10^7 SeMNPV polyhedra per ml, sufficient to kill 99% of the larvae. Four days post infection (p.i.) hemolymph was obtained from a cut proleg and used to infect 10^6 Se-UCR1 cells. Plaque purification of SeMNPV BV was carried out according to the procedures described by Summers and Smith (1987).

DNA isolation, Southern blot hybridization and molecular cloning.

Wild-type SeMNPV DNA was obtained from alkali-liberated virions purified after alkaline treatment of polyhedra followed by sucrose gradient centrifugation (SeMNPV PD-DNA) (Caballero *et al.*, 1992). Alternatively, viral DNA was isolated from SeMNPV BV (SeMNPV BV-DNA) according to the procedures described by Summers and Smith (1987). SeMNPV (BV- or PD- derived) or cosmid DNA thereof was digested with restriction endonucleases and electrophoresed in 0.8% agarose gels, transferred to Hybond N^+ nylon membranes (Amersham) and hybridized with ^{32}P-labelled DNA fragments of SeMNPV according to procedures described by Sambrook *et al.* (1989). *Xba*I-digested SeMNPV fragments (C through R) were isolated from agarose gels by the freeze-squeeze method (Sambrook *et al.*, 1989) and cloned into pUC vectors.

SeMNPV PD-DNA was partially digested with the restriction enzyme *Sau*3A-I to generate fragments of about 35 kbp. These fragments were ligated into *Bam*HI digested and dephosphorylated pWE15 (Stratagene, La Jolla, CA). Cosmid ligation mixes were packaged *in vitro* into lambda phage heads and transduced into *Escherichia coli* DH5α cells according to the protocols of the manufacturer (Stratagene, La Jolla, CA). Ampicillin resistant colonies were selected and cosmid DNA was isolated by a rapid miniscreen procedure (Sambrook *et al.*, 1989). Maxipreparation of cosmid DNA was carried out according to the Qiagen-tip 100 protocol (QIAGEN Inc., USA).

Dot blot analysis.

Cosmid and plasmid DNA was isolated according to the minipreparation method described and 1/10 of the yield of DNA was denatured in 200 μl of 200 mM NaOH for 10 min. The dot blot apparatus (Biorad) was assembled and the wells were washed with 400 μl 2 M NaCl before the denatured cosmid DNA was applied to the filter (Hybond N^+, Amersham). The membrane was removed and air-dried prior to baking for 2 h at 80 °C. Hybridization was performed with ^{32}P-labelled DNA fragments according to Sambrook *et al.* (1989).

Results

Infectivity of in vivo and in vitro produced polyhedra

Fourth instar *S. exigua* larvae were orally infected with polyhedra produced in insects (*in vivo*) or derived from plaque-purified, *in vitro* produced SeMNPV. Larvae infected with *in vivo* produced SeMNPV polyhedra died and decayed normally. However, larvae that were infected with SeMNPV polyhedra derived from *in vitro* produced, plaque-purified SeMNPV pupated normally and did not die. BV from plaque-purified SeMNPV was highly infectious for Se-UCR1 cells. When injected into the hemolymph this BV did not cause morbidity or mortality. Haemolymph isolated from larvae infected with *in vitro* produced, plaque-purified SeMNPV polyhedra, was highly infectious for Se-UCR1 cells indicating that the mutant virus does pass the larval midgut cells and that it at least undergoes one round of replication. These results suggest that the *in vitro* produced SeMNPV is genetically altered resulting in the loss of *in vivo* mortality and morbidity. Therefore, the DNA of SeMNPV produced *in vitro* and *in vivo* was subjected to restriction enzyme analysis to futher study this phenomenon.

Comparison of in vivo and in vitro produced SeMNPV DNA

The DNA of *in vivo* and *in vitro* produced SeMNPV was analyzed with restriction enzymes *Xba*I and *Pst*I (Fig. 2.1) and others (not shown). *In vitro* produced SeMNPV fragment *Xba*I-A had disappeared and a new smaller fragment between *Xba*I-B and C appeared (Fig. 2.1, lanes 3 and 4). Likewise, fragments *Pst*I C and D were no longer present in the *in vitro* produced SeMNPV DNA (Fig. 2.1, lanes 1 and 2). Various independently isolated plaques from repeated *in vivo* infections showed identical restriction profiles. When BVs were analyzed from Se-UCR1 cells infected with haemolymph-derived BVs (first *in vitro* passage) and compared to *in vivo* produced SeMNPV, several submolar fragments were detected in all the restriction profiles (data not shown). When these BVs were further average

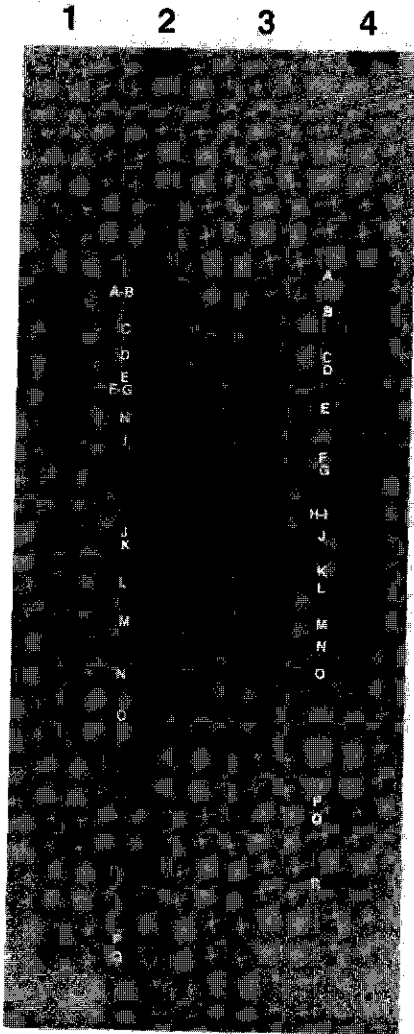

Figure 2.1.
Autoradiograph of PD-DNA from *in vitro* (lanes 1 and 3) and *in vivo* (lanes 2 and 4) produced SeMNPV, digested with *Pst*I (lanes 1 and 2) and *Xba*I (lanes 3 and 4), separated in 0.8% agarose, Southern blotted to Hybond N^{+} membrane and hybridized with ^{32}P-labelled *Xba*I digested SeMNPV *in vivo* produced PD-DNA. Restriction fragments are lettered in order of size.

size of the entire SeMNPV passaged over Se-UCR1 cells for two more times and plaque-purified, submolar bands were no longer observed even by Southern blot hybridization (Fig. 2.1). These results suggest that a large deletion had occurred in the passaging of hemolymph-derived BV in cell culture and that genes present in the deletion are essential for virus virulence *in vivo* but not *in vitro*.

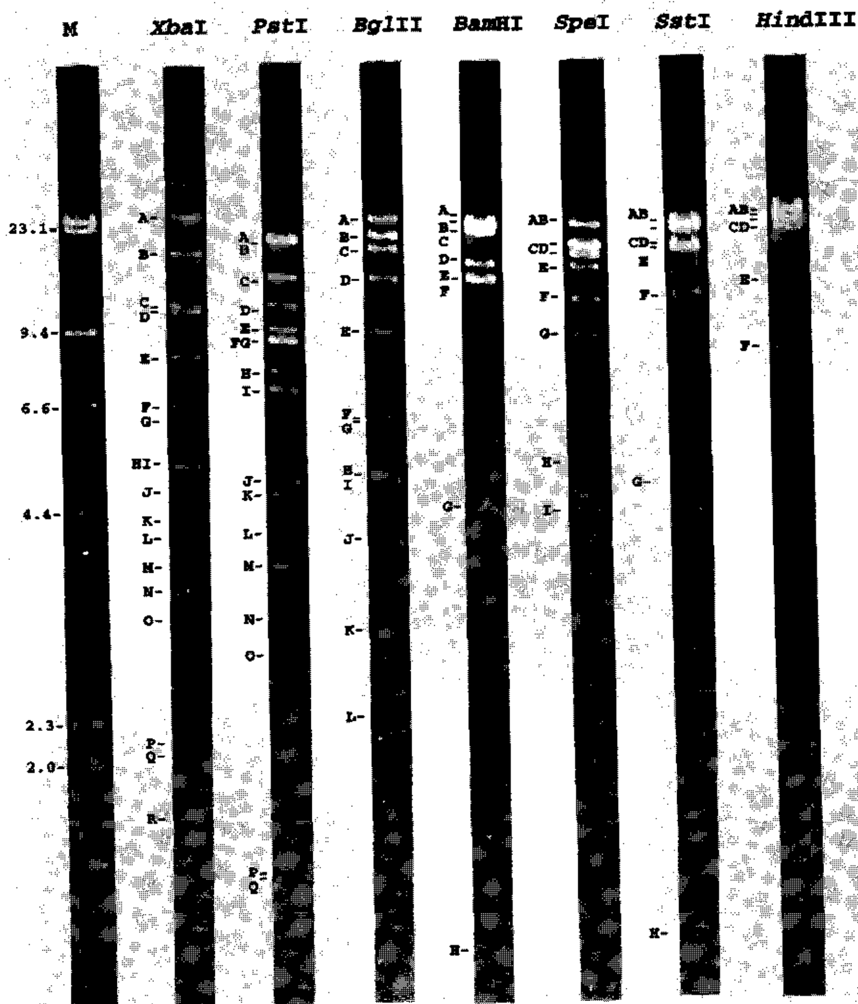

Figure 2.2.
Electrophoresis of SeMNPV PD-DNA produced *in vivo* digested with *Xba*I (lane 2), *Pst*I (lane 3), *Bgl*II (lane 4), *Bam*HI (lane 5), *Spe*I (lane 6), *Sst*I (lane 7), *Hind*III (lane 8) in a 0.8% agarose gel. Digested lambda DNA (lane 1) served as size standard. Restriction fragments are lettered in order of size.

Physical mapping of the SeMNPV genome

To locate the deletion in the SeMNPV genome, a physical map was constructed. Polyhedra derived (PD) SeMNPV DNA was digested with the restriction enzymes *Xba*I,

*Bam*HI, *Bgl*II, *Pst*I, *Sst*I, *Hin*dIII and *Spe*I, and separated by agarose gel electrophoresis (Fig. 2.2). Fragment sizes were estimated by comparison with size markers in adjacent lanes and ranged from approximately 36 kbp for the *Xba*I-A fragment to 1.1 kbp for the *Sst*I-H fragment (Table 2.1). Smaller fragments for these enzymes were not detected with this technique or by Southern blot hybridization (data not shown). By totalling the sizes of the fragments generated by the respective restriction enzymes thegenome was calculated to be 134.1 kbp. This is about the size of the genome of AcMNPV (Ayres *et al.*, 1994).

fragment	*Xba*I	*Pst*I	*Bam*HI	*Hin*dIII	*Spe*I	*Bgl*II	*Sst*I
A	35.6(13.6)	22.0	29.6(25.2*)	30.7	27.6(18.0*)	36.1(11.7)	29.2
B	17.5	21.0	28.6(23.0*)	29.2 (17.0*)	22.4	21.0	26.3
C	11.5	13.8 (d)	25.8	27.3 (14.8*)	19.8	19.5	21.8
C	10.7	11.5 (d)	16.4	26.0	16.5	15.8	20.0
E	8.0	9.0	14.2	13.0	15.8 6.2*)	10.5	18.6 4.5*)
F	6.2	8.7	14.0 (d)	8.5	12.5	6.4	12.5 2.2*)
G	5.8	8.7	4.5		9.3	6.2	4.5
H	5.0	7.6	1.2 (d)		5.3 (d)	5.3	1.1
I	4.6	6.8			4.4	5.0	
J	4.0	4.8				4.1	
K	3.9	4.6				3.1	
L	3.5	3.9				2.4	
M	3.3	3.6					
N	3.0	3.1					
O	2.6	2.6					
P	2.1	1.4					
Q	2.0	1.3					
R	1.7						
Σ	133.6	134.4	133.7	134.7	133.6	135.4	134.0

Table 2.1.

Fragment sizes of occlusion body derived SeMNPV-DNA generated by the restriction enzymes *Bam*HI, *Bgl*II, *Pst*I, *Xba*I, *Spe*I, *Hin*dIII and *Sst*I. Fragment sizes are indicated in kbp. Restriction fragments that are altered in mutant SeMNPV DNA are either deleted (d) or truncated (*). Truncated fragments and their sizes need to summated to obtain the actual fragment size in the deletion mutant. The truncated fragments are displayed in the row of the fragment from which they originate.

SeMNPV PD-DNA was digested to completion with *Xba*I and fragments C through R (11.5 through 1.7 kbp) were cloned into plasmid pUC19. The fragments *Xba*I-A and *Xba*I-B (35.6 and 17.5 kbp, respectively) were too large to be inserted into pUC plasmid vectors. In

order to clone the entire viral genome, a cosmid library was constructed. A set of five cosmid clones from the library spanning the entire viral genome except a small region located in the *Xba*I-D fragment (Fig. 2.3b) was selected from the library by dot blotting cosmids and Southern hybridization with ^{32}P-labeled *Xba*I fragments (A through R) of SeMNPV-DNA. Cross-blot hybridizations provided data about overlapping cosmid inserts. The occurrence of contiguous overlapping fragments in these cosmids confirmed the circularity of the SeMNPV genome.

Physical maps of the five cosmid clones were constructed by single and double-digestions using the seven restriction enzymes mentioned above and Southern hybridizations. By compilation of the physical maps of the five cosmid clones a complete physical map of the viral genome containing about 80 restriction endonuclease sites was derived (Fig. 2.3). This map was further validated by hybridizing individual ^{32}P-labeled *Xba*I fragments to Southern blots of SeMNPV PD-DNA digested (single and double) with the seven restriction enzymes. The adenine residue at the translational initiation codon of the polyhedrin gene, upstream of the junction between the *Xba*I-D and R fragments (van Strien *et al.*, 1992) was designated as zero point of the physical map (Fig. 2.3a and c). The orientation of the map was set by the location of the p10 gene of SeMNPV (Zuidema *et al.*, 1993) on the *Xba*I-H fragment at the right side of the map and is in agreement with the convention for linearized baculovirus maps (Vlak and Smith, 1982).

Mapping of the deletion in DNA of SeMNPV produced in vitro

When the sizes of the DNA fragments of SeMNPV produced *in vitro* were summated, the genome size was calculated to be about 110 kbp. This is approximately 25 kbp shorter than the DNA of the *in vivo* produced SeMNPV. The fact that only *Xba*I-A (35.6 kbp) disappeared and a new fragment of about 14 kbp was identified suggests that the deletion is contiguous. Based on the physical map of wild-type SeMNPV-DNA the deletion in the DNA of the *in vitro* produced SeMNPV could be approximately located. This was done by hybridization of the latter DNA with plasmids overlapping the deletion. Fragments *Pst*I-C and -D, *Bam*HI-F and -H and *Spe*I-H were absent, whereas fragments *Xba*I-A, *Bam*HI-A and -B, *Bgl*II-A, *Spe*I-A and-E, *Sst*I-E and -F and *Hin*dIII-A and -D hybridized to new (junction) fragments (Table 2.1). Since the size of these junction fragments could be determined and compared, the deletion could be approximately located between map units 12.9 and 32.3 (Fig. 2.3).

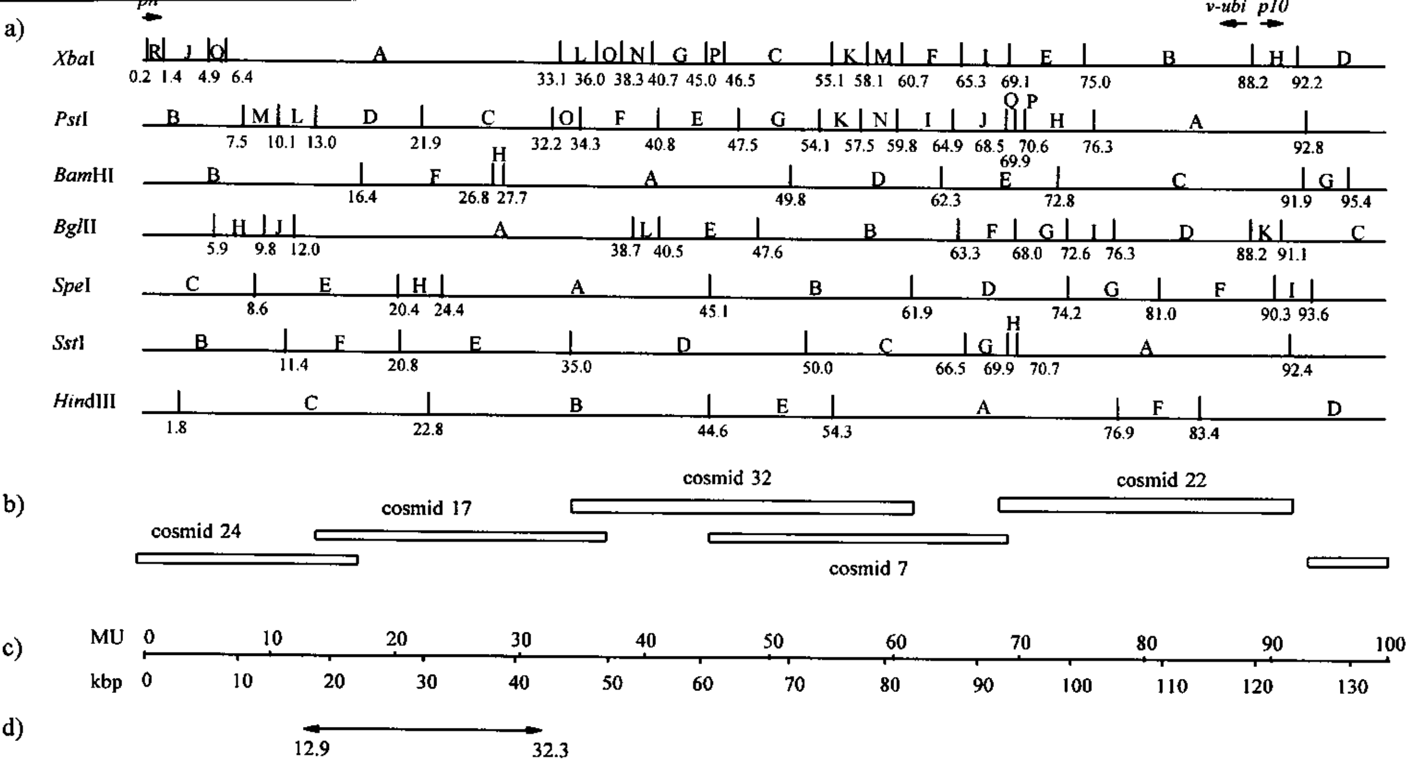

Figure 2.3.

Physical map of SeMNPV PD-DNA.

a) Physical map of the SeMNPV-PD genome for the restriction endonucleases *Bam*HI, *Bgl*II, *Pst*I, *Xba*I, *Spe*I, *Hind*III and *Sst*I with the position of the site in map units.

b) Location of the set of five cosmids (7, 17, 22, 24 and 32) on the SeMNPV genome.

c) Setting of the map units and kilobasepairs scale. The zero point is at the A of the ATG of the *polyhedrin* gene which is located upstream of the junction between the *Xba*I-D and R fragments..

d) The bidirectional arrow spans the deletion in the genome of *in vitro* produced SeMNPV (between map units 12.9 and 32.3).

Discussion

Upon serial passaging of AcMNPV in *Spodoptera frugiperda* cells at high multiplicity defective interfering viruses arise with deletions up to 50 kbp (Kool *et al.*, 1991). However, a deletion of about 25 kbp of the viral genome within the first passage, as is the case for SeMNPV, has not been observed previously. Moreover, it is not reported that these early arising defective interfering particles of AcMNPV are defective in larval infection. Studies are currently underway using *in vitro* produced SeMNPV polyhedra of passage one, five and ten to determine virulence, LD_{50} and LT_{50} in *S. exigua* larvae (E.M. Colbers and J.M. Vlak, personal communication).

The question is whether the SeMNPV deletion mutant is defective and needs the co-infection of a wild type helper virus in a very low concentration to complement the functions of the deleted genes, or whether it is indeed a viable virus, which could be obtained by plaque purification. If a helper virus is involved, its concentration is below the level of detection by Southern hybridizations. The presence of a helper virus is unlikely since *in vitro* produced SeMNPV polyhedra do not cause any effect *in vivo* nor does the injection of BV into the hemolymph. The assumption that no helper virus is involved implies that no genes involved in viral encapsidation or replication are located on the deleted fragment. Plaque purification of wild-type virus is therefore impossible since upon multiple passaging in the Se-UCR1 cell culture the deletion of the viral genome apparently starts immediately and processes continuously until the final stable deletion mutant is generated. Since plaque purified SeMNPV polyhedra did not cause disease in the insect anymore, it could be concluded that the 25 kbp deletion contains information that is important for virus virulence *in vivo*. However, it cannot be excluded that mutations elsewhere in the genome and not altering the restriction enzyme profiles are contributing to or causing the loss of *in vivo* activity of mutant SeMNPV. *In vivo* rescue of the deletion mutant virus using cosmids 24 and 17 (Fig. 2.3) should answer this question as this would restore virulence for insects.

Limited nucleotide sequence analysis of the segment, deleted in the defective viral genome, revealed that it encodes genes which are involved in the larval decay, such as *cathepsin* (Ohkawa *et al.*, 1994) and *chitinase* (Hawtin *et al.*, 1995) or insect hormone regulation, such as *egt* (Zuidema *et al.*, in preparation). In cell culture apparently no selection pressure exists on the presence of either type of the above described genes in the viral genome. Therefore these genes may be deleted spontaneously out of the SeMNPV genome when the virus is maintained in cell culture. Lack of infectivity of *in vitro* produced SeMNPV

is a major limitation towards the application of cell culture for large scale operations.

Hara *et al.* (1993) reported that SeMNPV produced in the Se-301 cell line was still infectious to larvae. Rearrangements present in plaque-purified SeMNPV-DNA as compared to wild-type were not noted. This may suggest that a 'cell factor' may be involved in the generation of defective or mutant viruses. Alternatively the field isolate used may be less susceptible for large deletions under in cell culture conditions. These hypotheses will be further explored by the isolation and testing of novel *S. exigua* cell lines and detailed comparative restriction mapping of the genomes of SeMNPV isolates and *in vivo* infectivity studies.

When the genetic organization of SeMNPV is compared with those of OpMNPV and AcMNPV with respect to the location of the *polyhedrin* and *p10* genes, the SeMNPV *polyhedrin* gene (van Strien *et al.*, 1992) is transcribed in the same direction as the AcMNPV *polyhedrin* gene (Ayres *et al.*, 1994), but in the opposite direction of the OpMNPV *polyhedrin* gene (Leisy *et al.*, 1986a). The distances between these two genes in the various viruses are also different, being 19.3 kbp for AcMNPV (Ayres *et al.*, 1994), 22.3 kbp for OpMNPV (Leisy *et al.*, 1986ab) and 11.6 kbp for SeMNPV, respectively (van Strien *et al.*, 1992; Zuidema *et al.*, 1993). This suggests that between these baculoviruses the genetic organization is not entirely conserved despite the similar length of their genomes (132 kbp). The genome size of the SeMNPV-US isolate (134 kbp) described here is in agreement with previously reported estimated genome sizes of SeMNPV-US and several SeMNPV Spanish field isolates (132 kbp) (Caballero *et al.*, 1992), but not with the size Hara *et al.* (1995) reported recently for Japanese plaque-purified and field isolates of SeMNPV (122 and 105 kbp, respectively).

Southern blot hybridizations with homologous fragments did not show any cross-hybridization with any fragment in the SeMNPV genome. However, it cannot be ruled out that small and/or imperfect homologous regions are interspersed throughout the SeMNPV genome, which could not be detected with the methods that were used in this study. Recently, small *hr*-like sequences were detected by sequence analysis of SeMNPV (R. Broer *et al.*, in preparation).

The availability of a cosmid library and a physical map of SeMNPV-DNA constructed for several restriction enzymes now allows a detailed study of the SeMNPV genome, its replication mechanism, the characterization of genes located on the deleted segment. Furthermore, the physical map of SeMNPV-DNA facilitates the study of the genetic relatedness of this virus with other baculoviruses.

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Chapter 3

SPECIFICITY OF MULTIPLE HOMOLOGOUS GENOMIC REGIONS IN *SPODOPTERA EXIGUA* NUCLEOPOLYHEDROVIRUS DNA REPLICATION[2]

Summary

Sequence analysis of the region upstream of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) ubiquitin gene (van Strien *et al.*, 1996) revealed the presence of four near-identical 68 base pair (bp)-long palindromic repeats. This region, named Se*hr*6 and located at map unit (m.u.) 88 of the SeMNPV genome on pSe*Eco*RI-2.2 showed structural homology to previously identified homologous regions (*hr*s) in a number of other baculoviruses. *Hr*s function as enhancers of transcription and as putative origins (*ori*s) of baculovirus DNA replication. Five additional *hr*s (Se*hr*1-Se*hr*5) were identified on the SeMNPV genome by Southern blot hybridization with an 18 bp-long oligonucleotide, complementary to a sequence conserved within the arms of the four palindromic repeats of Se*hr*6. Se*hr*1 to Se*hr*6 were found dispersed on the SeMNPV genome at m.u. 8.0, 30.0, 38.5, 51.0, 77.0, and 88.0, respectively. Sequence analysis of these *hr*s confirmed the presence of palindromic repeats, highly similar to those found in p*SeEco*RI-2.2. The number of palindromes varied from one (Se*hr*4) to nine (Se*hr*1) per *hr*. The Se*hr*s are all present in non-coding regions of the SeMNPV genome and also contain multiple putative transcription recognition sequences. Plasmids containing either of the Se*hr*s replicated in a SeMNPV-dependent DNA replication assay. The Se*hr*s were unable to replicate in an AcMNPV-dependent DNA replication assay. This was in contrast to the previously observed SeMNPV non-*hr* type *ori*, which replicated in the presence of both AcMNPV and SeMNPV. These data suggest that the replication of SeMNPV and the role of *hr*s in this process is highly specific.

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Introduction

DNA replication is a key process in the multiplication of DNA viruses which involves *cis-* and *trans*-acting elements (Kornberg and Baker, 1992). Baculovirus DNA replication has been predominantly studied for *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) and *Orgyia pseudotsugata* MNPV (OpMNPV) (see Lu *et al.*, 1997, for review). Within the AcMNPV genome two types of *cis*-acting elements have been identified as putative origins of DNA replication (*ori*). These *ori*s were able to replicate transiently when transfected into insect cells in the presence of AcMNPV, which provided the six essential *trans*-acting factors, IE-1, DNA polymerase, helicase, LEF-1, LEF-2, LEF-3, and the anti-apoptotic factor P35 (Kool *et al.*, 1994).

The first type of *ori* is represented by the homologous regions (*hr*s), which are found dispersed over the AcMNPV genome (Cochran & Faulkner, 1983; Pearson *et al.*, 1992; Kool *et al.*, 1993). AcMNPV *hr*s are characterized by the presence of two to eight repeats of a 72 bp-long sequence with an internal 28 bp-long imperfect palindrome with an *Eco*RI site at its center. *Hr*s have been identified in the genomes of a number of baculoviruses such as *Bombyx mori* NPV (BmNPV) (Majima *et al.*, 1993), *Orgyia pseudotsugata* MNPV (OpMNPV) (Ahrens *et al.*, 1995), *Lymantria dispar* MNPV (LdMNPV) (Pearson and Rohrmann, 1995), *Choristoneura fumiferana* MNPV (CfMNPV) (Xie *et al.*, 1995) and *Anticarsia gemmatalis* MNPV (AgMNPV) (Garcia-Canedo *et al.*, 1996). Therefore, the presence of *hr*s can be considered a characteristic feature of baculoviruses.

In AcMNPV and OpMNPV, *hr*s also act as enhancers of early gene expression when placed in *cis* to immediate-early and delayed-early promoters. The efficiency of enhancement depends on the presence of the baculovirus regulatory immediate-early gene product IE1 (Guarino and Summers, 1986; Theilmann and Stewart, 1993). AcMNPV IE1 binds as a dimer to the palindromic sequences of an *hr* (Rodems and Friesen, 1995). Interaction of IE1 with these sequences is essential for *hr*s to function as transcriptional enhancers (Leisy *et al.*, 1995). The 28 bp core of the palindrome acts as *ori*, whereas additional flanking sequences are required for enhancer activity (Leisy *et al.*, 1995; Habib *et al.*, 1996).

The second type of putative baculovirus *ori* does not contain *hr*-related sequences (non-*hr ori*), but direct repeats and AT-rich regions resembling eukaryotic *ori*'s (Kool *et al.*, 1993; De Pamphilis, 1993). Only one copy of such a non-*hr ori* was found in the AcMNPV genome (Kool *et al.*, 1994; Lee & Krell, 1994). Non-*hr ori*'s have also been identified in the genomes of OpMNPV and *Spodoptera exigua* MNPV (SeMNPV) (Pearson *et al.*, 1993; Heldens *et al.*, 1997). Enhancing activity of non-*hr*s has not been demonstrated yet. Up to this point it is unclear what the role or relative contribution of either of these two types of putative

oris is in baculovirus DNA replication *in vivo*. The mechanism of baculovirus DNA replication is enigmatic, although a rolling circle model has been proposed (Leisy and Rohrmann, 1993).

SeMNPV is a member of the Baculoviridae family and has a double stranded circular DNA genome of approximately 130 kb (Murphy *et al.*, 1995; Heldens *et al.*, 1996). SeMNPV infects only a single host insect, the beet army worm *S. exigua*, and is successfully applied as a biological insecticide against this pest insect (Smits and Vlak, 1994). A detailed restriction map and an overlapping cosmid library of SeMNPV DNA have recently become available (Heldens *et al.*, 1996). Several SeMNPV genes have been identified such as those encoding polyhedrin, *p10*, *rr1* (ribonucleotide reductase) and *ubiquitin* (Van Strien *et al.*, 1992; Zuidema *et al.*, 1993; Van Strien *et al.*, 1996; Van Strien *et al.*, 1997). The genetic organization of the SeMNPV genome appeared to be considerably different from that of AcMNPV and OpMNPV (Van Strien, 1997; Ayres *et al.*, 1994; Ahrens *et al.*, 1997). In this report the identification and characterization of *hr*s in the genome of SeMNPV is described and their replication competence in SeMNPV and AcMNPV-infected insect cells is investigated. The specificity of *hr*s may be one factor involved in the specificity of SeMNPV DNA replication.

Materials and methods

Cells, virus, plasmids and cosmids

S. frugiperda (*Sf*-AE-21) (Vaughn *et al.*, 1977) and *S. exigua* (Se-IZD-2109) cells (a gift from B. Möckel) were cultured in TNM-FH medium (Hink, 1970) supplemented with 10% fetal calf serum (FCS). The SeMNPV-US isolate (Gelernter and Federici, 1986) and the AcMNPV E2 strain (Smith and Summers, 1982) were produced using *S. exigua* fourth instar larvae. Routine cell culture maintenance and AcMNPV and SeMNPV infection procedures were carried out according to published procedures (Summers and Smith, 1987; van Strien *et al.*, 1996; Heldens *et al.*, 1996). The SeMNPV plasmid and cosmid libraries were described previously (Heldens *et al.*, 1996).

Southern blot hybridization

SeMNPV DNA, which was isolated from occlusion body-derived (ODV) viral DNA was digested with various restriction enzymes, separated in a 0.7% agarose gel and transferred to Hybond-N nylon membrane (Southern, 1975). An 18 bp-long oligonucleotide, RB-33 (5'-TAC ACG ATC TTT GCT TTC-3'), was made based on a conserved sequence

within the P repeats within Se*hr6* (see below, Fig. 3.6A). The membrane was hybridized overnight in Church buffer (0.25 M sodium phosphate, pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA) at 50° C with RB-33, which was end-labelled with [γ-^{32}P]dATP using T4-kinase (Gibco BRL). The blot was washed once at room temperature followed by an incubation step for 30 min at 50° C in the Church buffer to remove unbound label and primer. The blot was exposed to Kodak XAR film.

Construction of hr-containing plasmid clones

SeMNPV DNA fragments were cloned into either *p*UC19, *p*TZ19 or *p*Bluescript-KS+, and transformed into *Escherichia coli* DH5α using standard techniques (Sambrook *et al.*, 1989). DNA isolation, purification, digestions with restriction enzymes (Gibco BRL), agarose gel electrophoresis and Southern blotting were carried out according to standard procedures (Sambrook *et al.*, 1989)

Plasmid pSe*Eco*RI-2.2 containing the SeMNPV ubiquitin gene, was described previously by van Strien *et al.* (1996). The fragments that hybridized to the RB-33 were cloned and analyzed. Clone pSeCHK-5.7 was obtained after digestion of SeMNPV fragment *Xba*I-C with *Hin*dIII and *Kpn*I, and subsequent isolation of the 5.7 kb *Hin*dIII-*Kpn*I restriction fragment and cloning into pUC19. Plasmids pSe*Xba*I-H and pSe*Xba*I-N were taken from the *Xba*I-library of SeMNPV in pUC19 (Heldens *et al.*, 1996). Clone pSeB*Pst*I-5.6 was obtained after digestion of cosmid 17 DNA (Heldens *et al.*, 1996) with *Bam*HI and *Pst*I. Plasmid pSe*Pst*I-M was obtained by digestion of SeMNPV ODV DNA with *Pst*I and insertion of the *Pst*I-M fragment into *Pst*I-digested pUC19. Clone pSeP*Spe*I-6.3 was obtained after digestion of cosmid 22 DNA (Heldens *et al.*, 1996) with *Pst*I and *Spe*I, and insertion of a 6.3 kbp fragment into *Pst*I and *Xba*I-digested pUC19. Ac*hr5* was present on pAc*Hin*dIII-L (Kool *et al.*, 1993). All plasmids were amplified in *E.coli* DH5α and JM101 (*Dam*$^+$) strains.

Replication assay

The assay to test the replicative ability of the Se*hr* and Ac*hr* plasmids was as previously described by Heldens *et al.* (1997) for the *Se*MNPV non-*hr*. In short 10^6 Sf-AE-21 cells were transfected with 1 μg SeMNPV or AcMNPV *hr*-containing plasmids and infected 16 h later with the respective MNPVs with a multiplicity of infection of 1 $TCID_{50}$ unit per cell. Plasmid pUC19 was added (1 μg) as control for the amount of plasmid DNA retrieved after extraction. The cells were harvested 48 h post infection (hpi) (AcMNPV) or 72 hpi (SeMNPV). Total DNA was isolated from 10^6 infected cells (Summers & Smith, 1987) and resuspended in 60 μl H_2O. One aliquot (10 μl) was digested with *Hin*dIII to linearize the plasmid DNA. A second aliquot (10 μl) was digested with *Hin*dIII and *Dpn*I. The use of *Dpn*I allows the discrimination of input (*Dpn*I-sensitive) and replicated plasmid DNA (*Dpn*I-

insensitive) (Kool *et al.*, 1993). After agarose gel electrophoresis, the DNA was transferred to a nylon membrane filter (Hybond-N) and hybridized to α^{32}P-labelled pUC19 to detect plasmid sequences (Sambrook *et al.*, 1989).

Nucleotide sequencing

The nucleotide sequence of the cloned fragments was obtained by sequencing overlapping subclones of the fragments and/or by a primer walking strategy using standard and custom-designed oligonucleotide sequence primers. Sequencing was carried out at the Core Facility for protein and DNA Chemistry at Queens University in Canada using the dideoxy chain termination based protocol (Sanger *et al.*, 1977). Sequence analyses were carried out using UWGCG computer programs (Devereux *et al.*, 1984) and MEGALINE for Windows (DNASTAR Inc., 1995). The relevant nucleotide sequence data are available in the Genbank nucleotide sequence database under accession number XXXXXXX.

Results

Sequence analysis of pSeEcoRI-2.2

Analysis of sequences upstream of the SeMNPV *ubi* gene region on fragment pSe*Eco*RI-2.2 (m.u. 87.2 to 89.0) revealed the presence of four homologous repeats located in a non-coding region of about 900 bp (Fig. 3.1b). The four homologous repeats (P1-P4) contained a near-perfect palindrome of 68 bp in length, of which each of the last three repeats (P2 to P4) were centred around a *Bgl*II site. The *Bgl*II site in P1 was imperfect. Further analysis of the region between palindromes P3 and P4 showed the presence of two direct repeats (DR1a and DR1b) of 47 bp in length (Fig. 3.1c). The organization of this region is comparable to that of other baculovirus *hr*s (Lu *et al.*, 1997). This suggests that the identified palindromic repeats might represent a SeMNPV *hr*-type *ori* (Se*hr*).

Within eukaryotic *oris* the processes of transcription and replication are often tightly linked (Heintz *et al.*, 1992). This is reflected by the presence of multiple transcription factor binding sites and transcription initiation sites near or within eukaryotic *oris*. Several such sequences were identified in the region encompassing the putative Se*hr* (Fig. 3.1c; Fig. 3.2). A CGTGC motif is found, which is an important early transcription initiation site in AcMNPV DNA polymerase (Tomalski *et al.*, 1988) and helicase (*p143*) genes (Lu and Carstens, 1992). This motif, or its inverse, was present four times in this Se*hr*. Two CGTGC motifs were clustered in a 50 bp segment located 350 bp upstream of the 5' end of the P1 (not shown). One CGTGC motif was located 20 bp upstream of the 5' end of P4. Three

consensuspolyadenylation signals (AATAAA) were present near the 3 end of P2, P3 and P4. Those at the 3' end of P2 and P4 were in an antigenomic orientation. Six copies of the MLTF/USF motif (CANNTG) (Carthew *et al.*, 1985) and two copies of a CCAAT-motif (Benoist *et al.*, 1980) were present within the 1300 bp *Sal*I to *Eco*RI region. Ten copies of a less well characterized motif, 5'-CGATT-3' or its inverse (Lee & Krell, 1994), were also present within a 800 bp region downstream of P3 (Fig. 1c and Fig. 3.2, Se*hr*6).

Identification and sequence analysis of additional SeMNPV hrs

The presence and dispersed occurrence of *hr*s appears to be a common feature of baculovirus genomes. An 18 bp-long oligonucleotide fragment, RB-33, was designed based on a conserved region in palindromes P1 to P4 in pSe*Eco*RI-2.2 and hybridized to SeMNPV DNA restriction fragments to identify other *hr* regions on the SeMNPV genome. Several

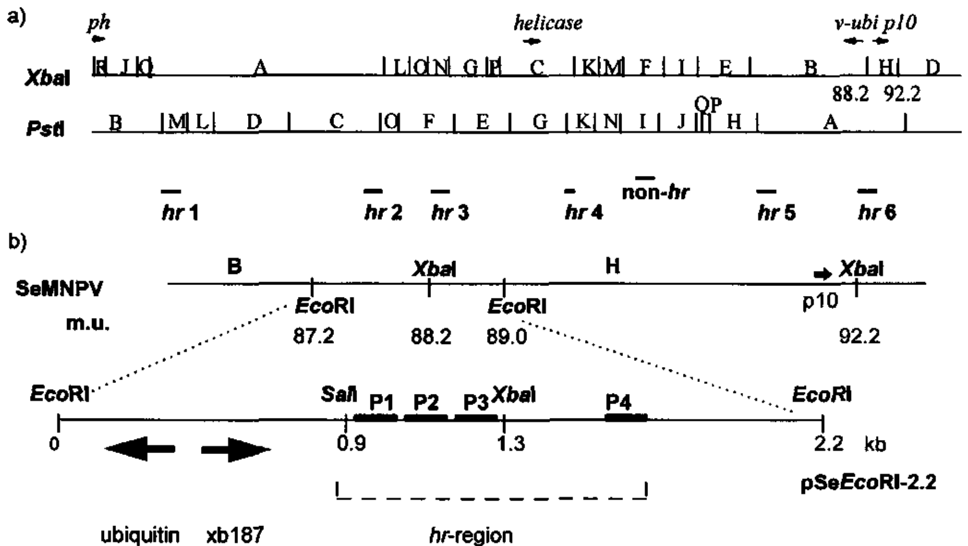

Figure 3.1.

a) Genomic location of *hr*s on the genome of SeMNPV with *Xba*I and *Pst*I restriction sites. The location and direction of transcription (arrows) of the SeMNPV *polyhedrin* (*ph*), *helicase*, *ubi* and *p10* gene, and the location of the SeMNPV non-*hr* type origin, are shown.

b) The location of the pSe*Eco*RI-2.2 fragment between map units 87.2 and 89.0 on the *Xba*I-restriction map of SeMNPV DNA. The black boxes represent the position of the palindromic repeats P1 - P4 within the *hr* on the pSe*Eco*RI-2.2 fragment.

c) Nucleotide sequence of the 1.3 kb *Sal*I-*Eco*RI fragment within fragment pSe*Eco*RI-2.2. Restriction sites are indicated in italics letters. Palindromic repeats P1 to P4 are underlined. An asterisk (*) represents a mutation in P1 that disrupts the *Bgl*II-site. The direct repeats DR1a and DR1b are doubly underlined. The CGATT-motif is denoted in bold and marked with a ♦, above or below the sequence depending on whether the motif is present on the forward or complementary strand. Putative poly-A signals are in bold. The CANNTG (MLTF/USF)-motif are marked as •. The stopcodons of ORF xh135 and xb187 (van Strien, 1997) are boxed.

Figure 3.1c

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                                                     Stop ORFxb187
      SalI
1     GTCGACACCGTACCTTACGAAACTATGGATTGTGAATAAATCATATGATGATTAAACTTGTTTTTGATATTGCATCATCC

                                                       *
81    AATAATTTTTCATAGACGATCTTTGCTTTCGTCCAAAATCTTCGATGAAAGCAAAGATCGTGTACTAAAATTCAATATGC
                                                       P1

                                                                              BglII
161   TCCTCTTTAAAGAATTGACTTTAATTTTAGTACACGATCTTTGCTTTCGTCCAAGATCTTCGATGAAAGCAAAGATCATG
                                                                               P2

241   TACTAAAATTTATTATTGCGCCATATTTTTGTAATTACGTCATTATGACTCATTTGTTTGTCACGAGTTTATCAACCTCA

                                                                                  BglII
321   CACATCCTGTTGACCTCTTTTTTAATTGCGTCATTCTGTCGCTATTTTCAGTACACGATCTTTGCTTTCGTCGAAGATCT
                                                                                   P3

                                                                ClaI♦
401   TGGACGAAAGCAAAGATCGTGTACCAAAGTTAAAAGATGACATCATCATATCGATTATTGCATCATCCTAATAAAGTTTC

                XbaI                         ♦
481   CATTTAATTCTAGATTATACACAATTTTCAAGTACGATTCTGAAACGTGAATCAATTTCGACGACATGCACAATTGCACG

                                          ♦
561   ATCTCGAAAATGACGTTAATTTCAATGAATAACGATTTTTACTCACAATCTTGAAATTGACGTTGGTTTCGATCGACATT
                   DR1a                                                  DR1b

641   GACATTTATATTTTGAGTTTACGATCTCCACACGTATGCCCACTTCGTCGTTGAAGTTAAACTCTGCGCGCATTTACTTA

721   CAGTTTCAAGAACACGAAATCTTCATCGAATCGTACATGTGTTTCGAGATCGGTGCACATATGCACGAGTGTGTAAAAAA
                                     ♦

                                             BglII
801   TTGATTTTAGTACACGATCTTTGCTTTCGTCCAAGATCTTCGACGAAAGCAAAGATCGTGTATTACAATTTTTATTTATG
                                              P4

                          Stop ORFxh135♦
881   AAAAGATGATGCAAAATTATTCTTTGACGATTGTGATGGTATTTGGTGATGGCGATGAAGAGGACGCAATCGTCGTAGTC
                                                                                 ♦

961   GTCGTGTCGTTTGCGCTAACATTAAAAATTAGATTGTCGTTGAACGATGAAAAATCTACAGAAATGGTTCCGATGACTGT

1041  TTTATCGGCAACGTTGACGACGTATATGGTGTCGGGCTTGCCGTCTTTGATGTACATGTCGTAGTCGAAAATGTTTGTAA

                                                                      ClaI
1121  CGTAATCGGTCAACGGATCGACGATCAAATTGAGTTTTTCCAATTGCTTGACCGAATCGATGTTGATGTTGGCGCTGACG
          ♦                                                            ♦

                                                       ♦
1201  ACGCCCTTGCAAAGATTTAGCAGAGTCATGTAGTGGTCAAAGCGATTATACAGCTTTGACGAGAACGACGCGTTTTTTAG

                      EcoRI
1281  AAATCGCTGGTAGTGAATTC 1301
        ♦
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oligonucleotide-specific hybridization signals were observed, which were subsequently mapped to restriction fragments *Xba*I-B, *Xba*I-C *Xba*I-H, *Xba*I-N, *Pst*I-M (Fig. 3.1a), *Spe*I-G and *Sst*I-E (not indicated). Two fragments, *Xba*I-B and *Xba*I-H, are adjacent fragments on the physical map of the SeMNPV genome and represent pSe*Eco*RI-2.2. These hybridization data and comparison with the location of the respective fragments suggest that *hr*-like sequences occur dispersed on the SeMNPV genome as well.

Further subcloning of fragments *Xba*I-C, *Spe*I-G and *Sst*I-E and hybridization with RB-33 revealed (data not shown) that putative SeMNPV *hr*s are present on fragments pSeB*Pst*I-5.6, pSeCHK-5.7, pSeP*Spe*I-6.3, derived from *Pst*I-C, *Xba*I-C and *Xba*I-B, respectively. Further *hr*s were found on pSe*Pst*I-M and pSe*Xba*I-N. The *hr*s are numbered Se*hr*1 to Se*hr*6 according to their relative map position on the physical map of the SeMNPV genome (Fig. 3.1a).

Sequence analysis of the fragments containing Se*hr*1 to Se*hr*5 confirmed the presence of clusters of P repeats, which were highly homologous to those found in Se*hr*6. The number of P repeats present varied per *hr* ranging from one in Se*hr*4 to nine in Se*hr*1 (Fig. 3.2, Table 3.1). The sequences of Se*hr*1 to Se*hr*5 were examined for the presence of the putative transcriptional motifs and DRs found in Se*hr*6 (Fig. 3.1c). A size limit of 500 bp was

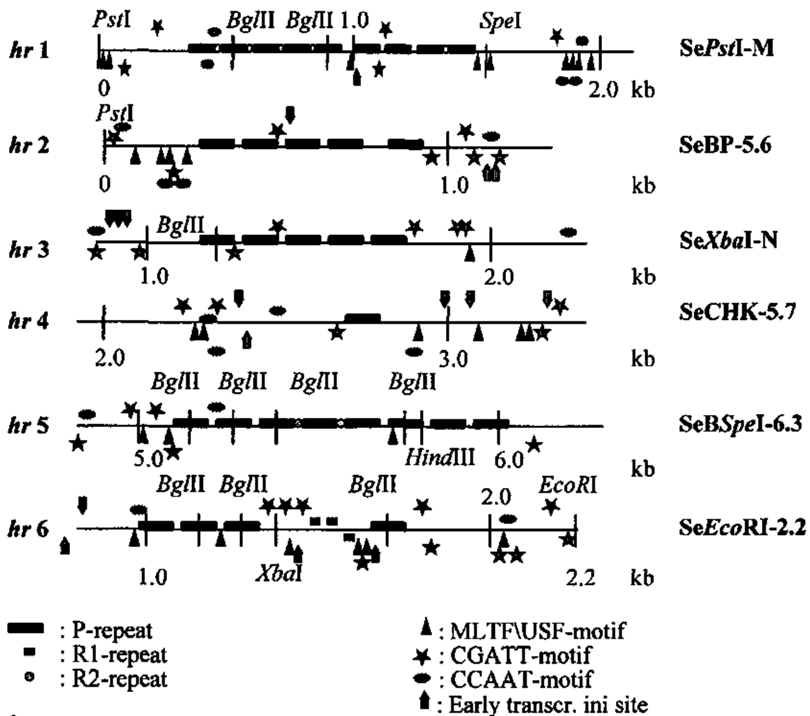

Figure 3.2.
Organization of palindromic repeats (P), direct repeats (DR) and other motifs within Se*hr*1 to *Se-hr*6.

set to either side of each *hr* in the analysis of sequence motifs. A novel direct repeat sequence element (DR2), 37 bp in length, was found in front of five out of the nine P repeats observed in Se*hr*1, and in front of two out of the seven P repeats in Se*hr*5. The DR2 motif, characterized by the consensus TCATcGCtAAAaATAGATTTGACgCAATacaAAACT, was not present in the other *hr*s. The DR1 motif identified in Se*hr*6 was not present in Se*hr*1 to Se*hr*5.

Alignment of Sehr repeats

When all thirty-two P repeats in Se*hr*1 to Se*hr*6 are aligned, a consensus sequence could be derived which forms a perfect hairpin (Fig. 3.3a and b). None of the individual P repeats contains the consensus sequence. Twenty-six nucleotides are absolutely conserved, of which an AAAGCAAA stretch (right arm sequence) is most notable. The SeMNPV P repeats are further characterized by the presence of a *Bgl*II site at the core/loop region of the palindrome. The *Bgl*II site is sometimes imperfect. Variations occur in the top and the bottom of the hairpin. Alignment of the consensus sequence or each of the individual SeMNPV P repeats failed to show sequence homology with *hr*s of AcMNPV, OpMNPV, LdMNPV, SpliNPV, CfMNPV or AgMNPV, but the arrowed G and C (Fig. 3.3b) are absolutely conserved at that position in all *hr*s (not shown).

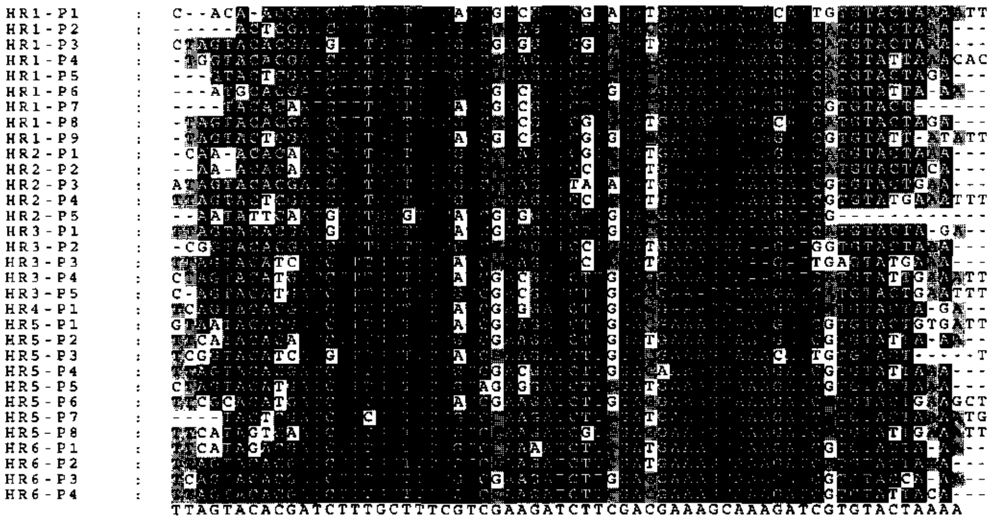

Figure 3.3.

a) Alignment of all 32 palindromic repeats from Se*hr*1 to Se*hr*6 and deduced Se*hr* consensus sequence. Sequences are presented in the forward orientation. The differences in gray shading above the consensus sequence indicate the level of conservation, from black (completely conserved) to light (less conserved) shading.

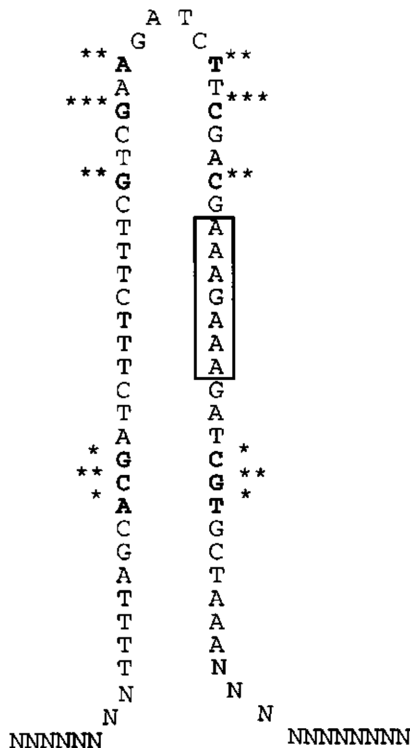

Figure 3.3. (Continued)

b) Potential hairpin structure of individual P repeats of the SeMNPV *hrs*. *, ** and *** represents sequence variation between 30 and 60%, 60 and 90% and > 90% respectively. The boxed sequence and the bold nucleotides are conserved in all repeats.

Replicative ability of Sehrs

The ability of all Se*hr*-containing fragments to replicate was tested in a SeMNPV-dependent DNA replication assay (Fig. 3.4). All Se*hrs* were able to replicate in the presence of SeMNPV as helper-virus (Fig. 3.4, lanes 2 to 7). The replication was *hr*-specific, since plasmid pUC19 (Fig. 3.4, lane 8) and a SeMNPV fragment without a putative *ori*, pSe*Xba*I-F1 (Fig 3.4, lane 10) (Heldens *et al.*, 1997, did not replicate in the assay. Sf-AE-21 cells transfected with pSe*Eco*RI-2.2 (Fig. 3.5, lane 1) and pUC19 (Fig. 3.5, all lanes) alone were included in the assay in order to identify the background of input DNA for *Dpn*I-digestions. All *hr*-containing SeMNPV fragments (Fig. 3.5, lanes 2 to 7) replicated at a lower level than the non-*hr* type *ori* (Heldens *et al.*, 1997) present on pSe*Xba*I-F2 (Fig. 3.5, lane 11). Based on the replicative ability of pSeCHK-5.7 (Se*hr*4; Fig. 3.5, lane 4) and of pSe*Xba*I-H (Fig. 3.1a; results not shown), both containing a single P repeat, it was concluded that the replication assay was highly sensitive and that presence of a single palindromic sequence was sufficient for replication activity.

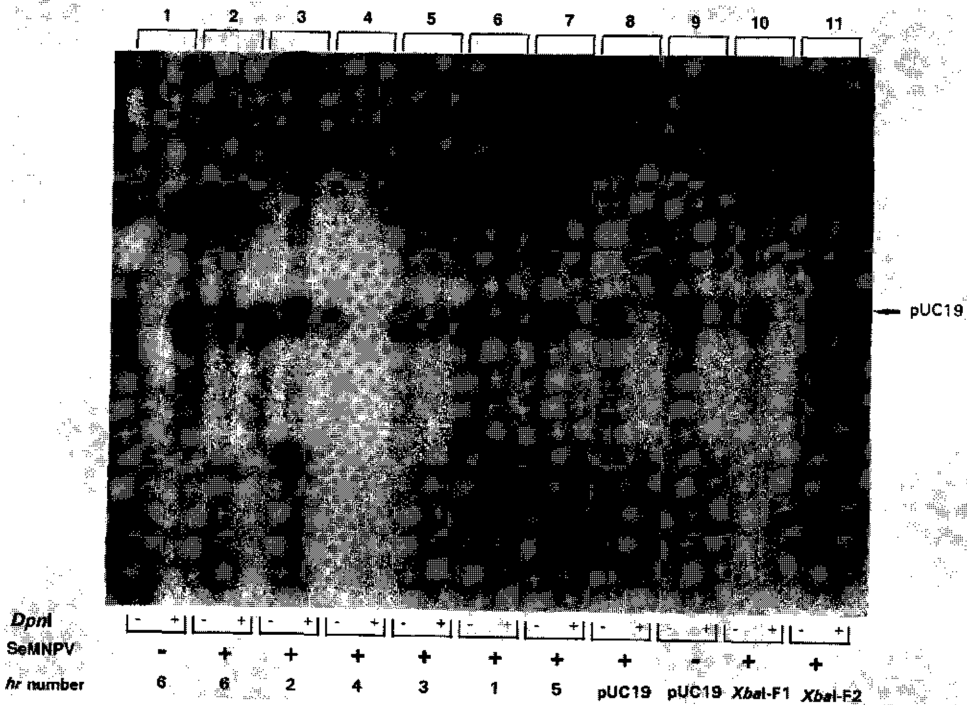

Figure 3.4.
Transient replication assay of putative *hr*-containing SeMNPV fragments in Sf-AE-21 cells infected with SeMNPV. Sf-AE-21 cells (5 X 10^4) were transfected with 0.5 μg of SeMNPV plasmid and pUC19 as a internal control. Plasmid pSe*Eco*RI-2.2 (Se*hr6*) (lane 2), pSeB*Pst*I-5.6 (Se*hr2*) (lane 3), pSeCHK-5.7 (Se*hr4*) (lane 4), pSe*Xba*I-N (Se*hr3*) (lane 5), pSe*Pst*I-M (Se*hr1*) (lane 6), pSeP*Spe*I-6.3 (Se*hr5*) (lane 7), pUC19 alone (lane 8), pSe*Xba*I-F1 (lane 10) and pSe*Xba*I-F2 (Se non-*hr*) (lane 11) transfected Sf-AE-21 cells followed by SeMNPV infection. Plasmids pSe*Eco*RI-2.2 (Se*hr6*) (lane 1) and pUC19 (lane 9) were without SeMNPV infection. The arrow indicates the position of pUC19, which served as an internal control for DNA yields obtained and for the efficiency of *Dpn*I-digestions. The + or - sign above the lanes indicate whether the sample was digested with *Hin*dIII (-) or with both *Hin*dIII and *Dpn*I (+). Southern hybridization was carried out using ^{32}P-labelled pUC19 as a probe.

Specificity of Sehrs in DNA replication

To test the specificity of the *hr*s for SeMNPV DNA replication factors, it was investigated whether AcMNPV could recognize and replicate SeMNPV *hr ori*s and SeMNPV the AcMNPV *hr ori*s. *Sf*-AE-21 cells were thus transfected with Se*hr6* or Ac*hr5* and infected with AcMNPV or SeMNPV, respectively. The results show that Se*hr6* only replicates in the

presence SeMNPV (Fig. 3.5, lane 2) and not in the presence of AcMNPV (Fig. 3.5, lane 1). Conversely, Ac*hr*5 did not replicate in the presence of SeMNPV (Fig. 3.5, lane 4) suggesting that the DNA replication is *hr*-specific. These results contrast with the observation that the SeMNPV non-*hr ori* was able to replicate to a significant level in the presence of AcMNPV as helper virus (Heldens *et al.*, 1997).

	AcMNPV-*hr*	OpMNPV-*hr*	CfMNPV-*hr*	SeMNPV-*hr*	SpliMNPV-*hr*
AcMNPV	+	-	-	-	+
OpMNPV	+	+	nd	nd	nd
CfMNPV	+	nd	+	nd	nd
SeMNPV	-	nd	nd	+	-
SpliNPV	+	nd	nd	nd	+

Table 3.1.
Number and types of sequence motifs found within Se*hr*1 to Se*hr*6. Palindromes with TACACGATCTTTCTTTC, DR1 with TGAACGTTAATTTC and DR2 with TAGATTTGAC consensus repeat. AcMNPV early transcription signal CGTGC (Tomalski *et al.*, 1988),. MLTF/USF motif CANNTG (Carthew *et al.*, 1985).

Discussion

Analysis of a 1.5 kb region flanking the SeMNPV ubiquitin gene (Van Strien *et al.*, 1996), revealed the presence of sequence motifs structurally reminiscent of homologous regions (*hr*s) in other baculoviruses. This region (Se*hr*6) contained four palindromic repeats (P1-4), two direct repeats (DR) and a number of other motifs which could be involved in regulation of transcription. Se*hr*6 also replicated in a SeMNPV-dependent DNA replication assay. This strongly suggests that this SeMNPV sequence is an *hr* (Lu *et al.*, 1997).

Hybridization of a conserved oligonucleotide sequence of these P repeats of Se*hr*6 with the SeMNPV genome led to the identification of five additional *hr*-regions in the genome, numbered Se*hr*1 to Se*hr*5 according to their relative position on the SeMNPV physical map (Heldens *et al.*, 1996). The Se*hr*s described here are interspersed throughout the genome in a similar fashion as in other baculoviruses. Even an *hr* with only a single P repeat (Se*hr*4) was identified by Southern hybridization making the presence of additional *hr*s of this type unlikely. The presence of multiple *hr*s in baculoviruses may provide redundancy of *ori*s to ensure DNA replication.

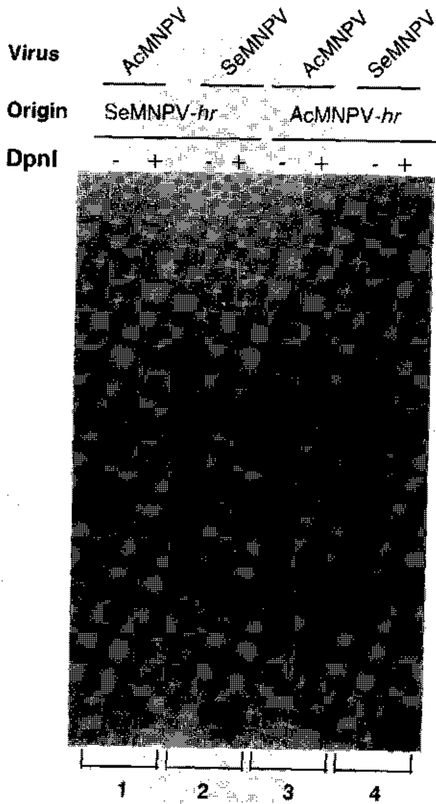

Figure 3.5.

Specificity of SeMNPV *hr* activity in transient replication assays. Sf-AE-21 cells were transfected with pAc*Hin*dIII-L (Ac*hr*5) followed by SeMNPV infection (lane 4), pAc*Hin*dIII-L (Ac*hr*5) followed by AcMNPV infection (lane 3), pSe*Eco*RI-2.2 (Se*hr*6) followed by SeMNPV infection (lane 2), pSe*Eco*RI-2.2 (Se*hr*6) followed by AcMNPV infection (lane 1). The + or - sign above the lanes indicate whether the sample was digested with *Hin*dIII (-) or with both *Hin*dIII and *Dpn*I (+).

A single repeat is a minimal requirement for plasmid-dependent DNA replication (Leisy *et al.*, 1995). All plasmids containing an Se*hr* were replication competent in transient replication assays (Fig. 3.4), whereas numerous SeMNPV DNA-containing plasmids were found to be negative in the assay with the exception of SeMNPV *Xba*I-F which contained a non-*hr ori* (Heldens *et al.*, 1997). It is possible that other *hr*-like sequences occur, but these have not yet been discovered in the 70 kb sequence presently available for SeMNPV (R. Broer, unpublished results). Comparison of the replicative activity of the six Se*hr*s did not reveal a positive correlation between the number of P repeats and the replicational signals observed.

Motifs DR1 and DR2 may be candidates for modulation of DNA replication and/or transcription since they occur in Se*hr*1, Se*hr*5 and Se*hr*6, but not in Se*hr*2 to 4. The Se*hr* regions also contained a number of other motifs and repeats which may be involved in either DNA replication and/or enhancement of transcription by Se*hr*s. Preliminary experiments indicate that Se*hr*6 acts as an enhancer of SeMNPV *ie-1* expression (D.A. Theilmann and E.A. van Strien, personal communication). Mutational analysis of these motifs could provide more insight into the role that these sequences might play in DNA replication and enhancement of transcription.

All Se*hr*s contained a near-identical 68 bp-long palindromic repeat with no sequence homology with other known baculovirus *hr*s (Fig. 3.3a). The palindrome is much larger than that of AcMNPV (28 base pair) and CfMNPV (36 bp), but the structure with a central core sequence resembling a restriction enzyme recognition site is highly similar to *hr*s of other baculoviruses. Both the length of the repeat and the unique sequence may contribute to the specificity in replication competence of the Se*hr*s for SeMNPV. Sequence alignment of all SeMNPV *hr*s indicate that there is a highly conserved octamer sequence in one of the arms of each repeat. Although sequence homology between baculovirus *hr*s is lacking, a juxtaposed GC bp (Fig. 3.3b) is highly conserved at a fixed position in each repeat. The functional significance of this putative G-C pair as well as the octamer box is unclear.

	hr-1	*hr-2*	*hr-3*	*hr-4*	*hr-5*	*hr-6*
P-repeat	9	5	5	1	8	4
DR1-repeat	0	0	0	0	0	2
DR2-repeat	5	0	0	0	2	0
CCAAT	5(3)	4(2)	2	4(2)	2	2
Early	2(2)	3(2)	3	5(1)	0	4(3)
MTL/USF	14	5	1	6	3	6
Poly-A	5(3)	3(1)	5(2)	6(5)	6(4)	3(2)
CGATT	6(2)	7(4)	7(3)	5(20	5(3)	10(5)

Table 3.2.
Transient replication assays of baculovirus *hr*s in baculovirus-infected insect cells. +, positive signal; -, negative signal, nd, not determined.

Individual P repeats showed mismatches in conserved locations in the sequence (Fig. 3.3a) and they are characterized by the presence of a (degenerated) restriction site, *Bgl*II, at the putative bulge region of the stem-loop (Fig. 3.3b). A similar situation exists in AcMNPV where a degenerated *Eco*RI site is present in the *hr* (Cochran and Faulkner, 1983).

Mismatches are found in all identified palindromic repeat-containing baculovirus *hr*s. Rasmussen *et al.* (1996) investigated whether these mismatches allowed a palindromic repeat to form a cruciform structure, which could enable the binding of the baculovirus transactivator IE-1. However, it was found that IE-1 did not bind to a 42 bp perfect and imperfect hairpin structure derived from Ac*hr*1 under cruciform forming conditions suggesting that the cruciform formation does not have a direct role in IE-1 binding. IE-1 did however bind to the 42 bp perfect and imperfect oligonucleotides with equal affinity under normal non-cruciform forming conditions, indicating that the conserved AcMNPV palindromic mismatches do not affect IE-1 binding. Maybe the binding of IE-1 to the palindrome is insufficient to enhance transcription, but is required for replication underscoring the bifunctionality of baculovirus *hr*s (Habib *et al.*, 1996).

In contrast to the Se*Xba*I-F2 non-*hr ori* (Heldens *et al.*, 1997) the replication of Se*hrs* is highly specific. This is unlike the situation with AcMNPV *hr*s when tested with other baculoviruses (Table 3.2). Plasmids containing Ac*hr*5, for example, replicate when transfected into OpMNPV-infected Ld652-Y cells (Ahrens *et al.*, 1995) or CfMNPV-infected Cf-124-T cells (Xie *et al.*, 1995). However, Cf*hr*1-containing plasmids could not replicate in the presence of AcMNPV in Sf21 cells (Xie *et al.*, 1995) as is the case for Se*hrs*. *Spodoptera litoralis* MNPV *hr* (Faktor *et al.*, 1997) does replicate in AcMNPV-infected cells, but not in SeMNPV-infected cells (J.G.M. Heldens, unpublished results). These data suggest that AcMNPV *hr*s are often more promiscuous than *hr*s of other viruses except when they are transfected into SeMNPV-infected cells. These data provide evidence that SeMNPV DNA replication is a highly specific process.

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Chapter 4

IDENTIFICATION AND FUNCTIONAL ANALYSIS OF A NON-*HR* ORIGIN OF DNA REPLICATION IN THE GENOME OF *SPODOPTERA EXIGUA* MULTICAPSID NUCLEOPOLYHEDROVIRUS[3]

Summary

The genome of *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) was screened for the presence of putative origins of DNA replication (*ori*). Using a transient DNA replication assay, several fragments were identified that underwent SeMNPV-dependent DNA replication in *Spodoptera frugiperda* cells (Sf-AE-21). Preliminary sequence data revealed the presence of multiple copies of homologous repeats (*hr*). Restriction fragment *Xba*I-F2 showed a distinct sequence reminiscent of *Autographa californica* and *Orgyia pseudotsugata* MNPV non-*hr oris*. Deletion analysis of this fragment indicated that the essential sequences of this putative non-*hr ori* mapped within a region of 800 bp. Sequence analysis of this region showed a unique distribution of six different (im)perfect palindromes, several poly-adenylation motifs and the occurrence of multiple direct repeats. No sequence homology or similarities to other reported baculovirus DNA replication origins were detected. The spatial and modular distribution of these motifs are similar to those of the non-*hr oris* of AcMNPV and OpMNPV. Alignment of baculovirus non-*hr* and consensus eukaryotic *oris* revealed no consensus baculovirus non-*hr ori* but indicated that each of the non-*hrs* studied sofar is unique. From the structural similarity, however, it was concluded that the SeMNPV *Xba*I-F2 *ori* represents a baculovirus non-*hr* type *ori*. In addition evidence is provided that SeMNPV renders more specificity to baculovirus DNA replication than AcMNPV.

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Introduction

Damage by the beet army worm, *Spodoptera exigua*, causes severe economic losses in important agricultural crops in (sub)tropical regions and in greenhouse cultivations in more moderate climate zones. Recently *S. exigua* MNPV (SeMNPV) has been registered in several countries as a biocontrol agent for the beet army worm (Smits and Vlak, 1994). Unfortunately, as for all baculoviruses, the speed of action of SeMNPV is relatively slow compared to chemical insecticides. It may take several days before virus infection reduces the feeding activity of the insects (Payne, 1988; Vlak, 1993). Nevertheless, SeMNPV is an attractive biocontrol agent, since it is relatively virulent for *S. exigua* larvae as compared to other baculoviruses for this insect (Gelernter and Federici, 1986a; Smits *et al.*, 1988).

Further improvement of the insecticidal properties of SeMNPV might be achieved by genetic engineering. Introduction of insect specific neurotoxin genes (McCutchen *et al.*, 1991; Stewart *et al.*, 1991; Tomalski and Miller, 1991) into and the deletion of the *ecdysteroid-UDP-glucosyltransferase* gene (O'Reilly and Miller, 1991) from the genome of *Autographa californica* MNPV (AcMNPV) resulted in recombinant viruses which showed an increased speed of action and reduced feeding damage. In contrast to AcMNPV (Ayres *et al.*, 1994) little is known about the genetic organization of the SeMNPV genome. Genetic modification of SeMNPV requires more detailed knowledge about the molecular genetics and replication mechanism of the viral genome. A detailed physical map has recently been constructed (Heldens *et al.*, 1996b) and a few genes e.g. coding for polyhedrin (van Strien *et al.*, 1992), p10 (Zuidema *et al.*, 1993) and ubiquitin (van Strien *et al.*, 1996) have been identified and characterized. Information about the identity and genetic location of *cis-* and *trans*-acting factors involved in the replication of SeMNPV DNA is lacking.

In AcMNPV (Pearson *et al.*, 1992; Kool *et al.*, 1993ab, 1994ab; Leisy and Rohrmann, 1993; Lee and Krell, 1994), *Orgyia pseudotsugata* MNPV (OpMNPV) (Pearson *et al.*, 1993; Ahrens and Rohrmann, 1995a), *Choristoneura fumiferana* MNPV (CfMNPV) (Xie *et al.*, 1995) and *Lymantria dispar* MNPV (LdMNPV) (Pearson and Rohrmann, 1995) origins of DNA replication (*ori*s) have been identified and characterized using an infection-dependent plasmid DNA replication assay. This assay is based on the resistance of *ori* containing plasmids that have been able to replicate in animal cells, to digestion with the restriction enzyme *Dpn*I, in contrast to plasmids amplified in bacterial cells. Two types of *ori*s have so far been recognized, designated as *hr* and non-*hr* (Kool *et al.*, 1995). *Hr*s contain 1 to 8 discrete 30 bp imperfect palindromic homologous repeats, which are interspersed along the genome (Cochran and Faulkner, 1983) and which also serve as enhancers of transcription (Guarino and Summers, 1986; Guarino *et al.*, 1986). The non-*hr* *ori*s of AcMNPV (Kool *et*

al., 1994a) and OpMNPV (Pearson *et al.*, 1993) are more complex and unique. They contain AT-rich regions, direct repeats and *hr* unrelated palindromes. The non-*hr* *ori* of AcMNPV showed some distant structural homology to consensus eukaryotic origins of DNA replication (DePamphilis, 1993; Kool *et al.*, 1994a). AcMNPV and OpMNPV *trans*-acting factors, required for *ori* activity in transient DNA replication assays have been determined (Kool *et al.*, 1994c; Ahrens and Rohrmann, 1995bc; Ahrens *et al.*, 1995). Among these factors are a helicase, DNA polymerase, LEF1, LEF2, LEF3 and IE-1.

The genome of SeMNPV has been explored for the presence of origins of DNA replication. Two types of replicative motifs have been identified, *hr* and non-*hr* (Heldens *et al.*, 1996a). The identification and analysis of the *hr* origins are described elsewhere (Chapter 3). In this chapter we describe the characterization of a putative non-*hr* origin of DNA replication in the genome of SeMNPV and we discuss its relatedness to similar *ori*s found in AcMNPV and OpMNPV.

Materials ans methods

Cells and Virus

S. frugiperda (Sf-AE-21) cells (Vaughn *et al.*, 1977), *S. exigua* (Se-UCR1) cells (Gelernter and Federici, 1986*b*) and *S. exigua* (Se-IZD2109) cells (B. Möckel and H.G. Miltenburger, unpublished results) were cultured in TNM-FH medium (Hink, 1970), supplemented with 10% fetal calf serum (FCS). The *S. exigua* multicapsid nucleopolyhedrovirus US-isolate (SeMNPV) (Gelernter and Federici, 1986a) was used as wild type (wt) virus. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Summers and Smith, 1987; King and Possee, 1992).

Plasmid constructions

SeMNPV subgenomic fragments were cloned into pUC19 and transformed into *Escherichia coli* DH5α using standard techniques (Sambrook *et al.*, 1989). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis and Southern blotting were carried out according to standard protocols (Sambrook *et al.*, 1989).

DNA Replication assay

The infection-dependent DNA replication assay was based on transfection of Sf-AE-21 cells with plasmids containing SeMNPV DNA sequences, harboring putative origins of

DNA replication followed by infection with SeMNPV to provide the viral *trans*-acting factors necessary for plasmid replication. Plasmid DNA was subsequently isolated from the insect cells and assayed for resistance to *Dpn*I digestion. Plasmid DNA amplified in *E. coli* DH5α (dam$^+$), are methylated at adenine residues. *Dpn*I cleaves only DNA molecules that contain a methylated adenine residue in its recognition site (GATC). DNA that is amplified in insect cells does not contain methylated adenine residues and will therefore be *Dpn*I resistant.

The DNA replication assay was performed essentially as described previously for AcMNPV replication (Kool *et al.*, 1993ab) with slight modifications. In brief, Sf-AE-21 cells were plated onto 35-mm-diameter tissue culture dishes at a density of $2*10^6$ cells per dish 24 h before transfection. Approximately 2 h prior to transfection the medium was removed from the cells and the cells were washed with TNM-FH medium without BSA and FCS. Cells were transfected with 1 μg of plasmid DNA using the calcium phosphate method (Graham and van der Eb, 1973; Summers and Smith, 1987). After 4 h of incubation at 27°C the medium was replaced with 2 ml TNM-FH medium supplemented with 10% FCS. Sixteen h after the transfection the cells were superinfected with SeMNPV (passage 1) at a multiplicity of infection of 2 $TCID_{50}$ units per cell.

DNA analysis

SeMNPV-infected cells were harvested 72 h post infection (p.i.) and total DNA was isolated as described by Summers and Smith (1987). Half of the DNA was digested with restriction enzyme *Hin*dIII to linearize the plasmid, the other half was digested with *Hin*dIII plus *Dpn*I to determine if plasmid replication had occurred. After agarose gel electrophoresis the DNA was transferred to Hybond N^+ nylon membrane (Southern, 1975) and hybridized with ^{32}P-labeled pUC19 DNA (Sambrook *et al.*, 1989).

Sequencing

Both strands of overlapping DNA fragments were sequenced using an automated DNA sequencer (Applied Biosystems) using the dideoxy chain-termination protocol (Sanger *et al.*, 1977). Sequence analyses were carried out using the UWGCG computer programs (Devereux *et al.*, 1984).

Results

Insect cell line performance in virus-dependent DNA replication assay

To identify SeMNPV DNA sequences that can function as origins of DNA replication recombinant plasmids containing these sequences have to be transfected efficiently into cell lines which support SeMNPV replication. Two *S. exigua* cell lines, Se-UCR1 (Gelernter and Federici, 1986b) and Se-IZD2109 (B. Möckel and .H.G. Miltenburger, unpublished results) are permissive for SeMNPV replication, whereas Sf-AE-21 cells are semi-permissive. These cell lines were tested for their transfection efficiency using the plasmid pAcDZ1, which contains the lacZ gene under control of a constitutive promoter (*hsp*70) as a reporter. Plasmid DNA was transfected into the insect cells using both the lipofectin (Life Technologies) and calcium phosphate precipitation method. The transfection efficiency, defined as percentage of blue cells per μg DNA in the presence of X-gal, for the *S. exigua* cell line Se-UCR1 was very low (< 0.1%) compared to Sf-AE-21 cells (100%). The cell line *Se*-IZD2109 could not be accurately tested since the cells clump as soon as the transfection reagents were added. In addition this cell type has endogenous ß-galactosidase activity which made assessment of the transfection difficult. The clumping of Se-IZD2109 cells during transfection, most likely prevents the efficient infection of the cells nested in the interior of the clump by the virus which is applied the next day. Hence, the *S. exigua* cell lines were considered to be unsuitable for use in transient DNA replication assays. It was therefore decided to perform all subsequent analyses in Sf-AE-21 cells.

Although *S. frugiperda* is a non-susceptible host for SeMNPV, budded virus (BV) derived from heamolymph of SeMNPV-infected *S. exigua* larvae or generated in one of the *S. exigua* cell lines was able to infect Sf-AE-21 cells (Heldens *et al.*, 1996b). Polyhedra were observed in 50% of the cells at 72 h post infection (p.i.). However, SeMNPV passaged twice in Sf-AE-21 cells was not able to infect new Sf-AE-21 cells (Heldens *et al.*, 1996b). Besides all this the transfection of Sf-AE-21 cells is well documented (King and Possee, 1992). Therefore, the DNA replication assays were performed in Sf-AE-21 cells with budded virus (BV) derived from haemolymph of SeMNPV-infected 4th instar larvae or from SeMNPV passaged once in Se-IZD2109 cells.

Identification of an SeMNPV origin of DNA replication

To identify regions in the viral genome that can function as origins of DNA replication, a plasmid library harboring fragments of the SeMNPV genome was tested for the ability of individual plasmids to replicate in SeMNPV-infected Sf-AE-21 cells. This plasmid library contained 16 out of 18 possible *Xba*I subclones. Fragments *Xba*I-A and -B were not

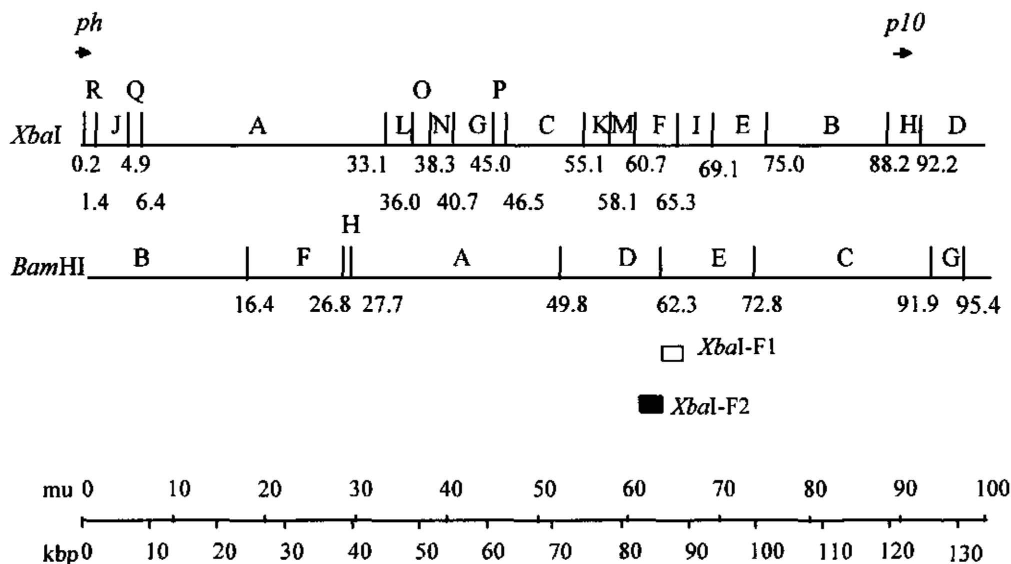

Figure 4.1.
Physical map of SeMNPV DNA for restriction enzymes *Bam*HI and *Xba*I and a reference scale in map units (mu) and kilobase pairs (kbp). Subfragments *Xba*I-F1 and -F2 are indicated by an open and black bar, respectively.

tested since they are too big, 35.6 and 17.5 kb respectively, to be cloned in pUC19 (Fig. 4.1). These DNA replication studies demonstrated clearly that several *Xba*I fragments gave rise to *Dpn*I-resistant DNA (Heldens *et al.*, 1996a), indicative for DNA replication. Preliminary nucleotide sequence analysis (Chapter 3) suggested that all replicative fragments, except for *Xba*I-F, contained identical palindromic motifs reminiscent of AcMNPV *hr*s. This fragment (Se-*Xba*I-F) had quite distinct sequence motifs compared to other *Xba*I fragments. Fragment *Xba*I-F was subcloned as two *Xba*I-*Bam*HI subfragments using the single *Bam*HI site within *Xba*I-F and these subclones were designated *Xba*I-F1 and *Xba*I-F2 (Heldens *et al.*, 1996b) (Fig. 4.1). Of these two subclones only *Xba*I-F2 (m.u. 60.7 to mu. 62.3) replicated in an infection dependent manner (data not shown).

To further investigate and better map the *ori*-active region in fragment *Xba*I-F2 (3.2 kbp), a more detailed physical map of the fragment was constructed and several subclones were generated using internally located restriction enzyme sites (Fig. 4.2a). The *Xba*I-F2

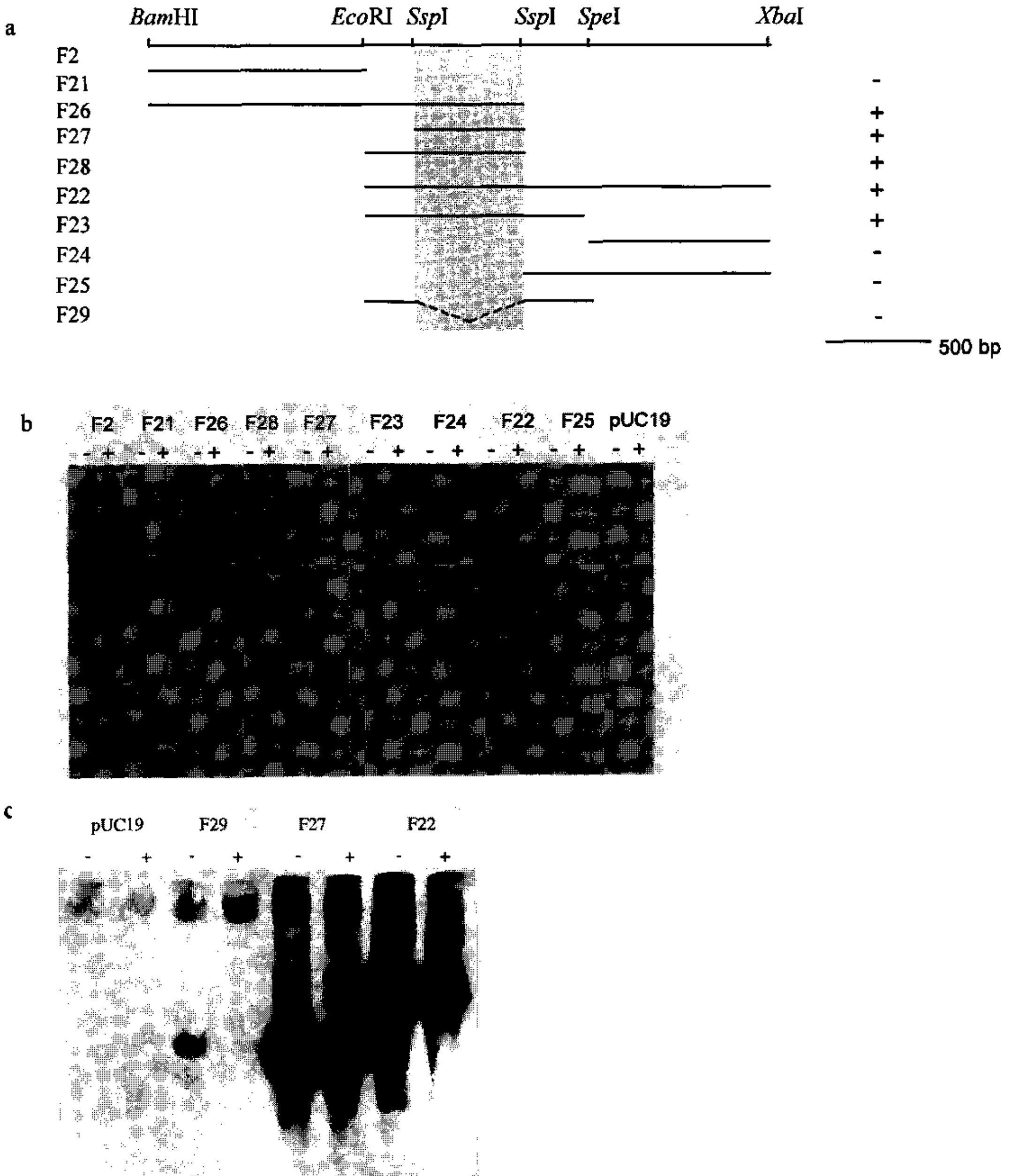

Figure 4.2.

Replication activity of several *Xba*I-F sublones. a) Restriction map of fragment *Xba*I-F2 and a schematic representation of the subclones tested for replication activity. The bar indicates 0.5 kbp. +/- indicative for replication activity of the subclones. b and c) Replication assay. Sf-AE-21 cells were transfected with 1 μg of plasmid DNA (*Xba*I-F subclones and pUC19) and infected with SeMNPV (MOI=2 $TCID_{50}$ units/cell), 24 h post transfection or mock infected. Cellular DNA was isolated 48 h p.i. and digested with *Hin*dIII with (+) or without (-) *Dpn*I. Southern blotting and hybridization was carried out using ^{32}P-labeled pUC19 as probe.

subclone and subclones thereof were tested for their replication ability along with pUC19 as a negative control (Fig. 4.2b). *Xba*I-F2 and the subclones F22, F23, F26, F27 and F28 replicated in SeMNPV-infected Sf-AE-21 cells, while the subclones F21, F24 and F25 did not replicate in the virus-dependent DNA replication assay. This suggested that the *ori* active sequences were located between the two *Ssp*I sites around m.u. 61.4, spanning a region of approximately 800 bp.

To confirm that the *ori* active sequence of *Xba*I-F2 was indeed located in the internal 800 bp *Ssp*I fragment, this subfragment was deleted from the *Xba*I-F2 fragment generating clone *Xba*I-F29. Subsequently, subclones *Xba*I-F22, *Xba*I-F27, the *Xba*I-F29 were tested for their ability to replicate (Fig. 4.2c). Plasmid *Xba*I-F29 was unable to replicate indicating that the internal 800 bp *Ssp*I fragment harbors essential *ori* active sequences. These results do not rule out the existence of *ori* auxiliary sequences outside of this *Ssp*I fragment, since subfragment *Xba*I-F22 appeared to replicate as efficiently as the entire *Xba*I-F2 subclone (Fig. 4.2bc). To prove that the *ori* active fragment was not only functional in Sf-AE-21 cells but also in cells derived from the natural host of SeMNPV, fragment Se-*Xba*I-F27 was tested for replication activity in SeMNPV-infected Se-IZD2109 cells. Fragment Se-*Xba*I-F27 did replicate in these cells although at a lower efficiency compared to its replication in Sf-AE-21 cells (data not shown). This is probably due to the inefficient transfection and infection process for these cells as described above.

Sequence analysis of the ori region of fragment XbaI-F2

The 800 bp internal *Ssp*I fragment from Se-*Xba*I-F2 and about 200 bp up- and downstream from this fragment was sequenced (Fig. 4.3). The *Ssp*I fragment contains two small perfect palindromes (P1 and P5) of 10 and 12 nt in size and 4 imperfect palindromes (P2, P3, P4, P6) varying in size between 11 and 33 nt (Table 4.1). Palindrome P2 appears twice in the sequence (P2a and P2b). Palindromes P3 and P2b are located in the left and right arm of palindrome P6 respectively. Several direct repeats were also observed (R1-R4), each appearing twice and varying in size from 9 to 19 nt. An internal fragment of repeat R2 (R2-int) appears twice in the sequence. Repeat R3 covers also the left arm of palindrome P6 and repeat R4 is present once on the plus strand and once on the minus strand of the DNA. This complex structure of palindromes and direct repeats strongly resembles the non-*hr* structure identified in the AcMNPV *Hin*dIII-K and OpMNPV *Hin*dIII-N fragments (Kool *et al.*, 1994a; Pearson *et al.*, 1993). The absence of multiple palindromic repeats suggest that this *ori* is distinct from an *hr*-like origin.

No open reading frame could be identified in any of the six reading frames in the SeMNPV non-*hr*. Clusters of transcription factor binding sites are, however, often associated with eukaryotic and viral origins of DNA replication (Heintz *et al.*, 1992). A number of these

*Acc*I

1 TATGTCTCGA CGGGTTC*GTA TAC*GTTACCG ATCCCCAGGA CAAACTCACA

51 TACCACACGA TACAAATCGT TCCCCGCATC GATGCGTACG TGACTTTCGA

101 CAAAAAGAAC GTGTACGTCG ATGTAATATA CAATGGTGAA ACTATTTCAA

*Ssp*I

151 AAATTAGAAT CAAAACCCAA TTTGCCGGCA ACGTTTT*AAT ATT*GTAACTG

P1

201 TGAATCAAAT GTGAATCTCC ACAAAGTCGT CGTGCTATCG AATTTAATAT

251 AGATTTCGGC GAAAGACGCC TATAATATCG ATGAAGCGGT CCGAGTCGAA

301 TGTTTGCGCC AAAAATTTTT CAATTTCATT TCAAGCGAAA CAAGGGCCAC

351 TTTCGACAAC GAGTGAGGTT GGCTAAATTT TTAATAAACG TTTCATAAAG

R1

401 TCGGTTTCGA CGAAAAATGC GTTATCGTCG AGTAAGTCTA AGAATCTTTG

P2a

451 AGTAAACCAT TTTTTTTTTC AATGCGTTTT CGAAGAAACA GACGGTATTG

501 TCGGTGAAAT TAACTTGTAA AACGAAACCA ATGACATTTC ATCGAAATGT

R3

P3

551 AATTTACGTT TCGACGAAAA ACATAGCCTT TTCGACAAAA TACACATATT

P6

P2b

R1

601 GTTTTCATCG AAATGTATCC AATTGTCGAA GAAGTGATGT AAAATCTATT

R3

651 GCGCAGTTAG AAAATTGCGC AGTTACAAAA TCTATTGCGC AGTTAATTTA

R2 R2-int R2

701 TGATGATATC ATATCACCTA TTGCATCATC TTTGTCAAAA CAATATGATA

P4 R4

751 TCGATGAAAG GGATGTGTAT TTCGCAGAAA CCATATGACG CAAATCATAC

*Ssp*I

801 ATGTCGAAAC ATGTTAAAGA TGATGCAATA AAAACAG*AAT ATT*GCGTCAT

R4

851 TTATTTTGAC GAAGTGTACA GTGTTTTCGA GTAAATGCAT TTTTTTAATT

*Spe*I

901 TCAACGAA*AC TAGT*GCCAGT TTCGACGACA AAAAGTCAAT AAAAAAACAT

951 TTAAATGTAA TACAAAATTT TATTGAAAGC CGGCCACCAA AGGAACGCAA

P5

1001 GGTTTAAACG CAAAAGTGTA CATTATGACT TGTTTGCATT GAGGACACTT

Figure 4.3.

Nucleotide sequence of the *Xba*I-F non-*hr* origin. The sequence is from left to right on the physical map of fig. 4.1. Head-to-head arrows represent the (im)perfect palindromic sequences designated P; solid lines indicate direct repeats (R); dashed lines indicate putative poly-adenylation signals; restriction sites are in italics; putative transcription factor binding sites are indicated by an asterisk (*); the sequence designations correspond to those in table 4.1.

transcriptional motifs are present in the 800 bp *Ssp*I segment of *Xba*I-F2 (Fig. 4.3). A CCAAT motif (Benoist *et al.*, 1980), a modified AP-1 binding motif GTGACT(AA)C (Murakami *et al.*, 1991) and several TATA boxes were also present (Table 4.1). Consensus Sp1 (Briggs *et al.*, 1986), CRE (Montminy and Bilezkijan, 1987) or MLTF/USF (Carthew *et al.*, 1985) motifs, that have been identified in the non-*hr ori* of OpMNPV (Pearson *et al.*, 1993) are absent in the *Xba*I-F2 fragment of SeMNPV. However, sequences which resemble the recognition site (-CANNTG-) for MLTF/USF and the immunoglobulin M heavy chain enhancer μE3 (Gregor *et al.*, 1990) -GTCA---G- (opposite strand) are present at positions 206, 620 and 535, 782 respectively, as was found in BmNPV *hr*, AcMNPV *hr* and non-*hr* and OpMNPV *hr* and non-*hr* sequences. All the described motifs are interspersed with multiple direct 5'-CGA-3' (61 times) and 5'-GTT-3' (43 times) repeats. These repeats are present on both DNA strands and appear at a much higher frequency than can be predicted on a random basis.

Origin replication by heterologous viruses

Baculovirus non-*hr* origins show low sequence homology and distant structural similarity. Because of the similarities in structure but differences in the primary sequence between the non-*hr* replication origins of SeMNPV and AcMNPV, the ability of the AcMNPV *Hin*dIII-K (non-*hr*) *ori* (Kool *et al.*, 1994a) to replicate in SeMNPV-infected Sf-AE-21 cells and conversely the ability of the SeMNPV *Xba*I-F non-*hr ori* to replicate in AcMNPV-infected Sf-AE-21 cells (Fig. 4.4) was examined. The SeMNPV *Xba*I-F fragment showed a low level of replication when transfected into Sf-AE-21 cells infected with AcMNPV and high levels of replication in Sf-AE-21 cells infected with SeMNPV (Fig. 4.4). In contrast, the AcMNPV *Hin*dIII-K *ori* replicates at high levels in Sf-AE-21 cells infected with AcMNPV but it does not replicate in Sf-AE-21 cells infected with SeMNPV (Fig. 4.4). The absence of replication of pUC19-transfected Sf-AE-21 cells infected with AcMNPV or SeMNPV (data not shown) confirms the specificity of the replication reaction.

Discussion

Data presented in this paper demonstrate that a putative origin of DNA replication (*ori*) is located on the *Xba*I-F fragment of SeMNPV. Deletion analysis of this fragment showed that essential elements of this *ori* are contained in an internal *Ssp*I fragment of 800 bp (m.u. 61.4). This *ori* did not show any sequence homology to known origins of DNA replication of either the *hr* or the non-*hr* type identified in OpMNPV (Pearson *et al.*, 1993;

Ahrens *et al.*, 1995a), AcMNPV (Pearson *et al.*, 1992; Kool *et al.*, 1993ab; Kool *et al.*, 1994a), LdMNPV (Pearson and Rohrmann, 1995) or CfMNPV (Xie *et al.*, 1995). However there is sufficient structural similarity to the non-*hr ori*s of AcMNPV (Kool *et al.*, 1994a) and OpMNPV (Pearson *et al.*, 1993) to suggest that the SeMNPV *ori* identified in this paper is a non-*hr* type *ori*.

Distinct structural motifs such as perfect and imperfect palindromes, a number of short and long repeats, AT-rich stretches or putative polyadenylation signals and modified transcription factor binding sites were observed in the non-*hr ori*s present in the genomes of OpMNPV (Pearson *et al.*, 1993) and AcMNPV (Kool *et al.*, 1994a). The length of these

Motif	Sequence	Position (nt)	Length
P1	TTCCGGCAA	171-181	10
P2A-P2B	TTTCGaCGAAA	405-415; 559-569	11
P3	ACATTTCatcGAAATGT	534-550	17
P4	TGATATcATATCA	704-716	13
P5	ACATTTAAATGT	947-958	12
P6	TTTCaTCGAAAtGTAAtTTACgTTTCGAc GAA	537-569	33
A1A	AATAAA	383-388; 827-832; 938-942	6
A1B	TTTATT	850-855; 969-974	6
A2A	AAATTAA	507-513	7
A2B	TTAATTT	693-699	7
R1	TTTCGACAA	351--359; 580-588	9
R2	AAAATCTATTGCGCAGTTA	641-659; 677-695	19
R3	TTTCATCGAAATGTA	537-551; 603-617	15
R2-int	ATTGCGCAGTTA	664-675	12
R4	TATTGCATCATCTTT	719-733; 830-816	15
AP1	GTGACTTTC	90-98	9
TATA	TATA	19, 127, 248, 271	4
CTF	CCAAT	167, 528, 619	5

Table 4.1.
Structural motifs of the non-*hr ori* within the SeMNPV *Xba*I-F2 region.
P1-P6 represent (im)perfect palindromes; A1A/A2A and A1B/A2B represent putative poly-adenylation signals on the plus-strand and minus strand, respectively; repeats are indicated with R1-R4; putative transcription factor binding sites are indicated by TATA, CTF and AP1. The positions refer to the nucleotide sequence in Fig. 4.3.

putative origins varies in size from 1300 bp in AcMNPV *Hin*dIII-K (Kool *et al.*, 1994a) to 4000 bp in OpMNPV (Pearson *et al.*, 1993) and the complexity with respect to the number of different structural motifs present and the frequency with which they appear also differs

considerably (Fig. 4.5). In addition, the absence of an ORF in the SeMNPV *Xba*I-F non-*hr* further suggests that it may have a regulatory function in transcription as well. It would also be of interest to see whether the SeMNPV *Xba*I-F non-*hr* as well as the AcMNPV and OpMNPV non-*hr*s have enhancer activity in transcription.

Comparison of the non-*hr ori*s of AcMNPV (Kool *et al.*, 1994a) and OpMNPV (Pearson *et al.*, 1993) with the consensus origin of DNA replication in eukaryotes as proposed by DePamphilis (1993) and the *ori* identified in the SeMNPV-*Xba*I-F fragment revealed some striking similarities (Fig. 4.5). The primary sequence not showing any direct homology, the occurrence of multiple direct repeats, AT-rich stretches and palindromic structures found in the non-*hr ori* sequences and the absence of multiple palindromic repeats indicative of *hr*s supports the conclusion that the putative *ori* of DNA replication located on SeMNPV *Xba*I-F is of the non-*hr* type.

The complexity and size of the non-*hr ori*s and the relative position of the various motifs differs considerably among the baculo virusesdescribed here (Fig. 4.5). Unlike the *hr*s

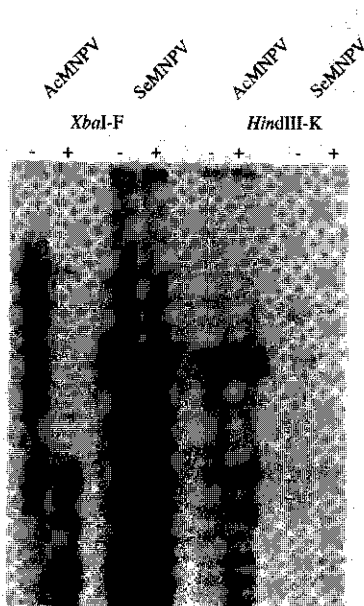

Figure 4.4.

Replication ability of non-*hr ori*s by heterologous viruses. Sf-AE-21 cells were transfected with 1 µg SeMNPV *Xba*I-F or 1 µg AcMNPV *Hin*dIII-K and then infected with either SeMNPV or AcMNPV (MOI=2 $TCID_{50}$ units/cell). Lanes marked - are digested with *Hin*dIII, and the lanes marked + are digested with *Hin*dIII and *Dpn*I except the lanes representing AcMNPV-infected, SeMNPV *Xba*I-F transfected insect cells, lanes marked - are digested with *Xba*I and the lanes marked + are digested with *Xba*I and *Dpn*I. Analysis was performed as described in the legend of fig. 4.2.

where a core 30-bp palindromic repeat is sufficient for replication activity (Pearson *et al.*, 1992; Leisy and Rohrmann, 1993), in the non-*hr*s the situation is much more complex (Kool *et al.*, 1994b). The relative importance of the various motifs in the replicative ability of AcMNPV and OpMNPV non-*hr ori* is unclear and needs further refinement. It is unlikely though that a consensus *ori* with respect to essential and auxiliary motifs can easily be derived. Deletion mutagenesis of the AcMNPV non-*hr ori* (*Hin*dIII-K) already indicated that none of the individual motifs found were essential for replication activity (Kool *et al.*, 1994a). The variation in the non-*hr*s may represent differences in the specificity of the replication process (Fig. 4.4).

The presence and distribution of structural motifs within *ori*-active sequences is most likely more important for *ori* activity than the primary sequence (DePamphilis, 1993). The overall features of eukaryotic and viral *ori*-containing sequences suggest that they are able to form multiple hairpin structures, whereas the AT-rich sequences may be involved in DNA unwinding, strand separation and replication initiation (Umek and Kowalski, 1988; DePamphilis, 1993). AcMNPV was able to replicate the SeMNPV *Xba*I-F2 non-*hr ori* (Fig. 4.4) and similar results were reported for a non-*hr ori* of OpMNPV (*Hin*dIII-N) when it was transfected into AcMNPV-infected Sf9 cells (Pearson *et al.*, 1993). Possibly, AcMNPV recognizes any sequence with structural homology with non-*hr ori*s. However, the *ori* sequence itself is certainly not the only factor contributing to the replication activity of non-*hr ori*s. It would be of interest to test for example an SV40 or herpes virus *ori*s in AcMNPV-infected Sf-AE-21 cells.

The AcMNPV non-*hr ori* (*Hin*dIII-K) did not replicate in Sf21 cells superinfected with SeMNPV, whereas the SeMNPV non-*hr ori* in *Xba*I-F2 did (Fig. 4.4). This hypothesis is supported by the observation that OpMNPV non-*hr ori* lost 50% of its replicative ability in the presence of the AcMNPV helicase instead of the OpMNPV helicase. These results are based on the outcome of virus-independent replication assays, in which the OpMNPV *trans*-acting factors, DNA polymerase, helicase, LEF1, LEF2, LEF3 and IE1, involved in DNA replication, are supplied on plasmids (Ahrens *et al.*, 1996). This result may be explained by the fact that AcMNPV and OpMNPV are more closely related to each other than to SeMNPV and that the *trans*-acting factors are mutually recognized. On this basis it can be predicted that the AcMNPV non-*hr ori* (*Hin*dIII-K) will replicate in OpMNPV-infected *L. dispar* cells. If so, non-*hr ori*s would therefore be specific only when the *cis*- and *trans*-acting factors match.

This and other reports suggest that non-*hr*s may be wide-spread among baculoviruses. It remains to be seen, however, whether they actually serve as *ori*s *in vivo*. The only evidence suggesting a functional role for the non-*hr ori*s comes from the analysis of defective viruses (AcMNPV) isolated upon serial passage, where the non-*hr* sequences seem to accumulate (Lee and Krell, 1994). Detailed fine-mapping of the *ori* activity, mutagenesis

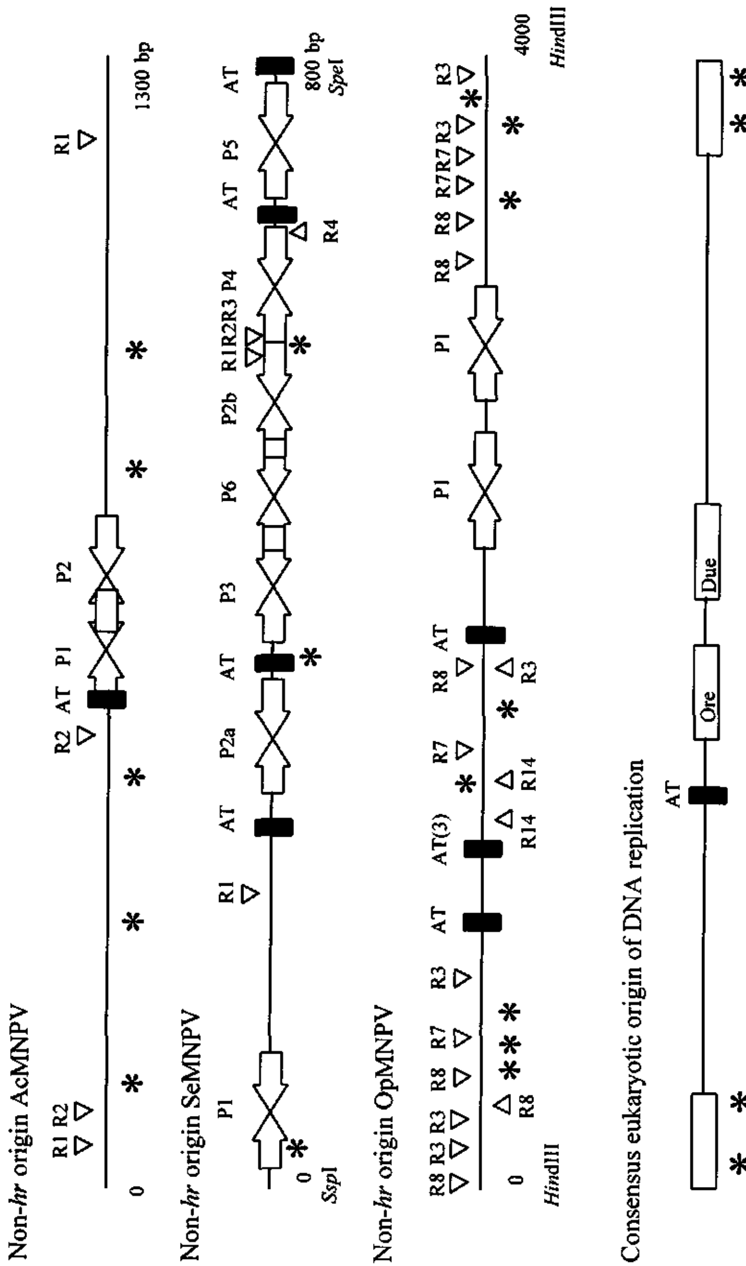

Figure 4.5.
Comparison of the arrangement of AcMNPV (Kool *et al.*, 1994a), OpMNPV Pearson *et al.*, 1993 and SeMNPV (this chapter) non-*hr* like *oris*. Arrows (P1-P6) represent palindromic sequences, black boxes (AT) represent putative poly-adenylation signals, small triangles (R1-R4 represent repeated sequences and asterisks represent putative transcriptional factor binding sites. Open boxes indicate the *ori* auxillary sequences, boxes marked ore and due represent origin recognition element and double stranded unwinding element respectively

Figure 4.5.
Comparison of the arrangement of AcMNPV (Kool *et al.*, 1994*a*), OpMNPV (Pearson *et al.*, 1993) and SeMNPV (this paper) non-*hr* like *oris*. Arrows (P1-P6) represent palindromic sequences, black boxes (AT) represent putative poly-adenylation signals, small triangles (R1-R4) represent repeated sequences and asterisks represent putative transcription factor binding sites. Open boxes indicate the *ori* auxilliary sequences, boxes marked ore and due represent origin recognition element and double stranded unwinding element respectively.

and DNA-protein binding studies need to be performed, to determine the function of the various motifs in the non-*hr* origins of baculovirus DNA replication.

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Chapter 5

CHARACTERIZATION OF A PUTATIVE *SPODOPTERA EXIGUA* MULTICAPSID NUCLEOPOLYHEDROVIRUS *HELICASE* GENE[4]

Summary

Putative baculovirus helicases have been implicated to play an important role in viral DNA replication and host specificity. The *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) helicase is therefore of interest since the virus only infects the beet army worm. Sequence analysis of the SeMNPV *lef5-p39* (mu 46.5-55.1) region, which is colinear with the *39K-lef5* area in *Autographa californica* MNPV (Ayres *et al.*, 1994), revealed an open reading frame (ORF) of 3,666 bp potentially encoding a protein with a molecular weight of 143 kDa. This protein had considerable amino acid sequence similarity (58%) to AcMNPV p143, including seven conserved motifs characteristic for helicases. In cultured insect cells this SeMNPV ORF is expressed from 4 to 12 h p.i. and its major transcript of 4 kb starts 11-12 nt upstream of the putative translational initiation site (ATG). To study their possible role in the specificity of baculovirus DNA replication, the putative AcMNPV and SeMNPV *helicase* genes were tested for their ability to replicate *hr*s (putative origins of DNA replication) in a transient DNA replication assay in insect cells. All viral *cis*- and *trans*-acting factors were provided as plasmids using either Ac*hr*2 or Se*hr*1 as DNA replication origin. SeMNPV p143 could not subsitute AcMNPV p143 in the transient assays supplemented with either *hr*. Similar results were obtained when the SeMNPV and AcMNPV *ie1* genes were exchanged. None of the essential AcMNPV *trans*-acting factors could be complemented by SeMNPV infections to support DNA replication of *hr*s. These data suggest a specific interaction between the baculovirus DNA replication factors to form the replisome and/or between the replisome and the origin of DNA replication.

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Introduction

Helicases play a key role in biological processes such as replication, repair, recombination, conjugation and transcription of DNA. They catalyse the unwinding of duplex DNA, RNA or DNA-RNA hybrids by disrupting the hydrogen bonds between the complementary base pairs (bp) in the double-stranded nucleotide filaments. The most commonly accepted mechanism for helicase functioning requires the enzyme to possess multiple DNA binding sites in order to bind reaction intermediates at the unwinding junction. This requirement seems to be met since most helicases, having one DNA binding site, appear to be active as oligomers (for review, Lohman and Bjornson, 1996). The unwinding reaction is driven by the hydrolysis of primarily ATP, although hydrolysis of other nucleotides has been reported as well (Lahue and Matson, 1988; Goetz *et al.*, 1988; Morris *et al.*, 1979). The processive unwinding of DNA also requires translocation of the helicase complex along the DNA filament, in either 3' to 5' or 5' to 3' direction, depending on the type of helicase. How DNA unwinding and translocation of the complex along the DNA is coupled to the hydrolysis of ATP is not yet understood (West, 1996).

Helicases are a diverse group of proteins, varying in size from 37 kDa for *Escherichia coli Ruv*B (West, 1996) to 143 kDa for the putative *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) helicase (Lu and Carstens, 1991) and 170 kDa for the *E. coli* long helicase related protein (Reuven *et al.,* 1996). Most organisms encode multiple helicases. *E. coli* for instances encodes 12 different helicases (Matson *et al.* 1994) and *Saccharomyces cerevisiae* at least 6 (Li *et al.*, 1992). Viruses can encode multiple proteins with helicase functions as well. Both subunit UL5 of the helicase-primase complex (Crute *et al.*, 1988, 1989) and the origin binding protein UL9 of herpes simplex virus 1 (Bruckner *et al.*, 1991) display helicase activity.

Several common amino acid sequence motifs have been identified in helicases originating from organisms as diverse as *E. coli*, bacteriophages, herpesviruses and man (Linder *et al.*, 1989; Gorbalenya and Koonin, 1988; Gorbalenya *et al.*, 1988; Hodgman, 1988a,b; Lu and Carstens, 1991). These sequence motifs are referred to as I, Ia, II through VI and the D-E-A-D box, a special version of motif II. Motifs I and II form the A and B loop of a conserved NTP binding site (Walker *et al.*, 1988). Motif Ia and a conserved tyrosine residue in motif VI are thought to be involved in the association with presumed DNA binding proteins (Hodgman, 1988). No defined function has yet been assigned to common motifs III through VI (Matson and Kaiser-Rogers, 1990).

The *p143* gene of AcMNPV has been identified as an essential gene in the baculovirus infection and DNA replication cascade through the studies of a temperature

sensitive mutant with a mutation in this gene (Gordon and Carstens, 1984). Sequence analysis of this gene revealed a high degree of similarity within the seven motifs characteristic for DNA helicases (Lu and Carstens, 1991). In the genome of *Orgyia pseudotsugata* MNPV an AcMNPV *p143* homolog has recently been identified and sequenced (Ahrens and Rohrmann, 1995a; Ahrens and Rohrmann, 1996). The importance of the p143 product in AcMNPV and OpMNPV DNA replication has also been established via transient DNA replication assays using origin-containing plasmids as reporters of DNA replication and a subset of a cosmid and plasmid library encompassing the entire viral genome (Kool *et al.*, 1994a; Ahrens and Rohrmann, 1995 ab; Ahrens *et al.*, 1995). DNA polymerase, p143, late expression factors 1 (LEF1), 2 (LEF2) and 3 (LEF3) and immediate early protein 1 (IE1) have been assigned as essential trans-acting factors required for AcMNPV and OpMNPV DNA replication (Kool *et al.*, 1994b; Ahrens and Rohrmann, 1995ab; Ahrens *et al.*, 1995). In contrast to the situation in herpes simplex virus 1 (Liptak *et al.*, 1996), little is known about the assembly of the baculovirus DNA replication complex and the interaction between the individual proteins of this complex with each other and with putative origins of DNA replication (*hr*s).

The *p143* gene of baculoviruses is not only involved in DNA replication, but possibly also in host range specificity. The host range of the various baculoviruses differs considerably. AcMNPV infections have been reported in over 40 insect species, whereas *Bombyx mori* NPV and SeMNPV infect to date only one single host, the silk worm and the beet army worm, respectively. At the cellular level, BmNPV for instance replicates in BmN cells but not in Sf-AE-21 cells, whereas AcMNPV replicates in Sf-AE-21 cells but not in BmN cells. Mixed infections of AcMNPV and BmMNPV in the Sf-AE-21 cells followed by screening in BmN cells yielded an AcMNPV recombinant capable of replicating in both cells lines (Kondo and Maeda, 1991). Detailed analysis of the recombinant virus revealed that a few amino acid changes in a 140-long amino acid stretch of the AcMNPV *p143* gene were responsible for this host range expansion (Croizier *et al.*, 1994).

The baculovirus SeMNPV has high potential for development as a bio-insecticide because of its host specificity, its high speed of action and virulence (Smits and Vlak, 1994). Phylogenetic analysis using parsimony of several SeMNPV genes suggested that this virus is a member of a different clade than AcMNPV, OpMNPV and BmNPV (Zanotto *et al.*, 1993; Cowan *et al.*, 1994; Hu *et al.*, 1997). In this paper we describe the genomic location, sequence and transcription of the SeMNPV homolog of the AcMNPV *p143* gene. The specificity of the baculovirus *p143* gene in helper virus-independent DNA replication assays has been investigated using both SeMNPV and AcMNPV homologous region (*hr*-)-like origins of DNA replication and SeMNPV and AcMNPV *p143* genes. Finally, the role of the *p143* gene products in DNA replication and host specificity is discussed.

Materials and methods

Cells and Virus

S. frugiperda (Sf-AE-21) cells (Vaughn *et al.*, 1977) and *S. exigua* (Se-IZD2109) cells (B. Möckel & H.G. Miltenburger, unpublished results) were cultured in TNM-FH medium (Hink, 1970), supplemented with 10% fetal calf serum (FCS). The US-isolate of SeMNPV (Gelernter and Federici, 1986) and the E2 strain of AcMNPV (Summers and Smith, 1987) were used as wild type (wt) viruses. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Summers and Smith, 1987; King and Possee, 1992). Budded virus (BV) used in time course infection experiments was obtained from the supernatant of Se-IZD2109 cells infected with hemolymph obtained from SeMNPV-infected fourth instar *S. exigua* larvae. AcMNPV BVs were obtained from the supernatant of Sf-AE-21-infected cells. BV titers were determined by the end point dilution method (Vlak, 1979) and expressed as $TCID_{50}$ units per ml.

Plasmid constructions

SeMNPV subgenomic fragments were cloned into pUC19, pBluescript KS^+ (Stratagene) or pGEM7zf$^+$ (Promega) and transformed into *E. coli* DH5α using standard techniques (Sambrook *et al.*, 1989). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis and Southern blotting were carried out according to standard protocols (Sambrook *et al.*, 1989). Unidirectional deletions were performed using the *Exo*III-based 'Erase-a-Base kit' according to the protocols of the manufacturer (Promega).

DNA replication assay

The infection-independent DNA replication assay was based on transfection of Sf-AE-21 cells with plasmids containing SeMNPV or AcMNPV DNA sequences, harboring the putative origin of DNA replication SeMNPV-*hr*1 (Heldens *et al.*, 1995) and AcMNPV-*hr*2 (Kool *et al.*, 1993) respectively, and the viral *trans*-acting factors necessary for plasmid DNA replication (Kool *et al.*, 1994b). AcMNPV-*lef1* (mu 7.5-8.7) was cloned as a 1.6 kb *Nru*I-*Eco*RI fragment in pUC19, AcMNPV-*lef2* (mu 1.9-2.6) as a 0.94 kb *Mlu*I fragment into the *Bam*HI site of pUC19 using *Mlu*I-*Bgl*II linkers, AcMNPV-*DNA pol* (mu 38.9-41.6) as a 2.3 kb *Sst*I-*Eco*RV into pBluescript KS^- and AcMNPV-*lef3* (mu 42.8-44.5) as a 2.3 kb *Eco*RI-*Apa*I fragment into pJDH119 (Hoheisel, 1989). The AcMNPV *helicase* gene (mu 59.9-63.5) was cloned as a 4.8 kb *Eco*RI-*Ssp*I fragment into pBluescript KS^-. The AcMNPV-*p35* was provided as fragment *Eco*RI-S. AcMNPV-*ie1* (mu 94.7-96.9), *ie2* (mu 96.9-98.9) and *pe38* (mu 98.9-100.0) were cloned as a 2.9 kb *Cla*I-*Hin*dIII fragment in pUC8, as an 1.3 kb *Pst*I-

*Pst*I and as an 1.5 kb *Pst*I-*Eco*RI fragments in pUC19, respectively. The map positions of the AcMNPV fragments were taken from Ayres *et al.* (1994). SeMNPV-*ie1* (mu 92.2-95.4) was cloned as a 4.2 kb *Xba*I-*Bam*HI fragment in pUC19, whereas SeMNPV *helicase* was provided as the entire SeMNPV-*Xba*I-C fragment.

The DNA replication assay was performed essentially as described previously for AcMNPV and SeMNPV DNA replication (Kool *et al.*, 1994ab; Heldens *et al.*, 1997) with slight modifications. In brief, Sf-AE-21 cells were plated onto 35-mm-diameter Petri dishes at a density of $2X10^6$ cells per dish 24 h before transfection. Approximately 2 h prior to transfection the medium was removed from the cells and the cells were washed with TNM-FH medium without BSA and FCS. Cells were transfected with 1 μg of either SeMNPV or AcMNPV *hr*-containing plasmid and plasmids containing the replication genes in equimolar amounts taking 0.5 μg DNA of a 5 kb plasmid as standard. The DNAs were mixed with 35 μl H_2O and 15 μl lipofectin (Gibco-BRL) in 1 ml of TMN-FH medium without BSA and FCS. After 4 h of incubation at 27 °C the medium was replaced with 2 ml TNM-FH medium supplemented with 10% FCS.

Infection-dependent DNA replication assays were based on transfection of Sf-AE-21 cells with plasmids containing SeMNPV or AcMNPV DNA sequences, harboring putative origins of DNA replication (SeMNPV-*hr*1 and AcMNPV-*hr*2, respectively) and/or the viral *trans*-acting factors necessary for plasmid replication (Kool *et al.*, 1994b) as described. Between 16 and 24 h post transfection the cells were infected with SeMNPV or AcMNPV BV at a multiplicity of infection of 2 $TCID_{50}$ units per cell.

DNA analysis

Transfected cells were harvested 72 h post transfection (48 h p.i.) and total DNA was isolated from the insect cells (Kool *et al.*, 1994a). Half of the DNA was digested with the restriction enzyme *Hin*dIII to linearize the plasmid, the other half was digested with *Hin*dIII plus *Dpn*I to determine if plasmid replication had occurred. After agarose gel electrophoresis the DNA was transferred to nylon membrane (Hybond N, Amersham; Southern, 1975) and hybridized with ^{32}P-labeled pUC19 DNA (Sambrook *et al.*, 1989).

Isolation of total RNA and Northern blotting

Total RNA for Northern blot and primer extension analysis was isolated from SeMNPV-infected Se-IZD2109 cells at several time points p.i., as described by Xie and Rothblum (1991). Total RNA was denatured, electrophoresed and blotted to Hybond N nylon membrane (van Strien *et al.*, 1992). To identify *p143* transcripts, the blot was hybridized for 16 hours at 65 °C with [α^{32}P]dCTP-labeled riboprobes. Riboprobes were generated by *in vitro* transcription (Sambrook *et al.*, 1989) using T7 or T3 RNA polymerase (Gibco-BRL) of

cloned DNA fragments containing the putative SeMNPV helicase gene in pBluescript KS$^+$ (Stratagene). After hybridization (65 °C (overnight) in Church buffer (0.25 M Sodium phosphate pH 7.2, 7% SDS, 1% BSA, 1mM EDTA)), the filters were washed for 5 min with 2*SSC, 0.5% SDS at room temperature, 30 min with 2*SSC, 0.1% SDS at 65 °C and 30 min with 0.1* SSC, 0.1% SDS at 65 °C. The filters were exposed to Kodak XAR film.

Primer extension

To identify the transcriptional start site(s) of the SeMNPV *p143* gene, 15 ng of an oligonucleotide (5'-CATTCTTGTCCACGGCCTCG-3') complementary to nucleotides +50-+70 relative to the translational initiation site of the *p143* mRNA, was labeled at the 5' end with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Gibco-BRL) in 50 mM Tris-HCl, pH 9.5, 10 mM $MgCl_2$, 5 mM DDT, 5% glycerol for 45 min at 65 °C followed by heat denaturation at 90 °C for 10 min. The labeled oligonucleotide was purified on an 1 ml Sephadex-G25 column and added to 10 µg of total RNA, isolated from infected cells. The mixture was denatured at 90 °C for 5 min and annealed at 55 °C for 15 min. Reverse transcription was carried out at 48 °C for 1 h in a volume of 15 µl, containing 5 mM of each of the dNTPs and 1 µl of Superscript reverse transcriptase (Gibco-BRL) in a buffer supplied by the manufacturer. The reaction was stopped by the addition of 5 µl 'stop' buffer (95% v/v formamide, 0.01% xylene cyanol and 0.01% bromophenol blue). Six µl of the reaction mixture was analyzed in a 6% polyacrylamide sequence gel, which was then dried and exposed to Kodak XAR films.

Sequencing

Both strands of overlapping DNA fragments of the SeMNPV helicase gene and its flanking regions were sequenced from fragments generated with the 'Erase-a-Base' system (Promega) using an automated DNA sequencer (Applied Biosystems) using the dideoxy chain-termination protocol (Sanger *et al.*, 1977). Sequence analyses were carried out using the UWGCG computer programs (Devereux *et al.*, 1984) and deduced amino acid sequences were compared with the updated GenBank/EMBL, SWISSPROT and PIR data libraries using BLAST and FASTA programs latest releases.

Localization of the SeMNPV p143 gene

To identify the AcMNPV *p143* gene homolog in the genome of SeMNPV several radioactive DNA probes were constructed from different domains in the AcMNPV *p143* gene. These probes were hybridized at various stringencies to Southern blots with SeMNPV DNA isolated from polyhedra derived virions (ODV) and digested with a number of restriction enzymes (Heldens *et al.*, 1996a). None of these probes showed unambiguous hybridization signals, that could be related to one or a few subgenomic fragments (results not shown). This suggested that the nucleotide sequence of the *p143* gene of SeMNPV displayed limited nucleotide sequence homology to its AcMNPV counterpart.

A partial genetic map of the SeMNPV genome was constructed based on sequence analysis of the termini of a *Xba*I plasmid library described by Heldens *et al.* (1996a) and homology searches in data banks. Sequences similar to AcMNPV-*lef5* (Passarelli and Miller, 1993) and *p39* (Thiem and Miller, 1989) were identified at the termini of fragment *Xba*I-C (Fig. 5.1a). The distance (ca 11.5 kb) between these two genes appeared to be similar in AcMNPV and SeMNPV. The AcMNPV *p143*, a putative DNA helicase, is located in between the *lef5* and *p39* gene (Lu and Carstens, 1991; Ayres *et al.*, 1994). Assuming colinearity between the AcMNPV and SeMNPV genomes in this region, the putative helicase gene of SeMNPV should be found in the middle of fragment *Xba*I-C.

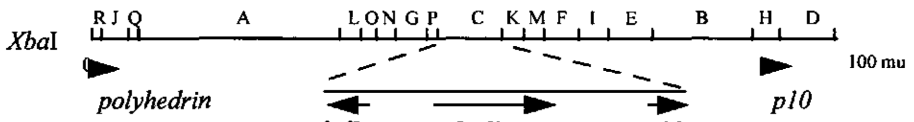
A detailed physical map of *Xba*I-C was constructed using several restriction enzymes (Fig. 5.1b) and numerous subfragments were cloned into pGEM7zf^{+} and their termini were sequenced. Two subclones, pCHK and pCSK, did have a considerable degree of amino acid sequence similarty to AcMNPV and OpMNPV p143 (Lu and Carstens, 1991; Ahrens and Rohrmann, 1996) and were further analyzed.

Sequence analysis of the SeMNPV p143 gene

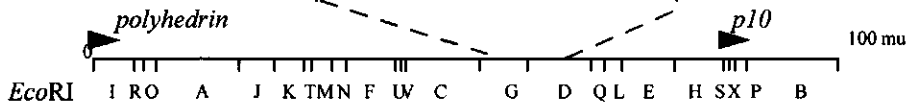
Sequence analysis of SeMNPV fragment *Xba*I-C revealed a large open reading frame (ORF) encompassing 3,666 basepairs potentially encoding a polypeptide with a predicted molecular weight of 143 kDa. This putative protein showed an overall identity of about 42% and a similarity of about 60% to the p143 amino acid sequences of AcMNPV and OpMNPV (Fig. 5.2; Table 5.1). The size of the polypeptide was also in agreement with the sizes of the p143 polypeptides of AcMNPV and OpMNPV (1221 amino acids; 143 kDa and 1223 amino acids; 140 kDa respectively) (Lu and Carstens, 1991; Ahrens and Rohrmann, 1996). The SeMNPV p143 ORF was located between mu 47.0 and 49.7 of the physical map of the viral DNA (Heldens *et al.*, 1996a).

The conserved motifs (N terminus-I-Ia-II-III-IV-V-VI-C terminus) that characterize pro- and eukaryotic helicases are present in the C-terminal part (amino acids 917-1221) of the SeMNPV p143 and their spatial order is identical to that found in other members of the helicase superfamily (Gorbalenya and Koonin, 1988; Gorbalenya *et al.*, 1988; Hodgman, 1988ab) (Fig. 5.2). For SeMNPV p143 the identity within the seven conserved motifs differed

a SeMNPV



b AcMNPV



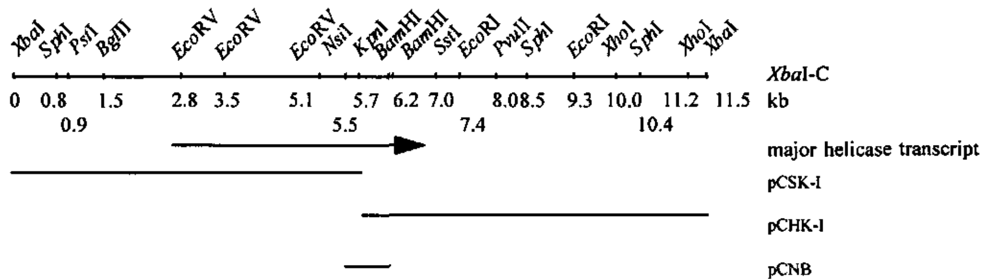


Figure 5.1.
a) Physical map of the SeMNPV and AcMNPV genomes for the restriction enzymes *Xba*I and *Eco*RI, respectively, and location of various genes.
b) Detailed physical map of the SeMNPV *Xba*I-C fragment for various restriction enzymes. The positions of the restriction sites are indicated relative to the position of the 5' *Xba*I site (mu 46.5). The major helicase transcript is represented by an arrow. The fragments subcloned for sequencing (pCSK-1 and pCHK-1) and riboprobe (pCNB) generation are indicated by horizontal lines.

from more than 70% identity for motif I to less than 25% identity for motif VI with the other baculovirus p143s (Fig. 5.3). These data are in agreement with the identities found for the conserved motifs in helicases of other organisms (Fig. 5.3; general consensus).

Achel : [illegible] : 75
Bmhel : [illegible] : 75
Ophel : [illegible] : 74
Sehel : [illegible] : 80

L-zipper

Achel : [illegible] : 154
Bmhel : [illegible] : 155
Ophel : [illegible] : 152
Sehel : [illegible] : 145

1 2 3 4 5 B1

Achel : [illegible] : 227
Bmhel : [illegible] : 228
Ophel : [illegible] : 232
Sehel : [illegible] : 216

Achel : [illegible] : 306
Bmhel : [illegible] : 307
Ophel : [illegible] : 309
Sehel : [illegible] : 291

Achel : [illegible] : 386
Bmhel : [illegible] : 387
Ophel : [illegible] : 389
Sehel : [illegible] : 370

Achel : [illegible] : 466
Bmhel : [illegible] : 467
Ophel : [illegible] : 468
Sehel : [illegible] : 447

Achel : [illegible] : 545
Bmhel : [illegible] : 546
Ophel : [illegible] : 547
Sehel : [illegible] : 519

B2

host range factors

Achel : [illegible] : 625
Bmhel : [illegible] : 626
Ophel : [illegible] : 627
Sehel : [illegible] : 599

NLS NLS

Achel : [illegible] : 704
Bmhel : [illegible] : 705
Ophel : [illegible] : 704
Sehel : [illegible] : 679

NLS

Achel : [illegible] : 781
Bmhel : [illegible] : 782
Ophel : [illegible] : 782
Sehel : [illegible] : 758

Achel : [illegible] : 825
Bmhel : [illegible] : 826
Ophel : [illegible] : 826
Sehel : [illegible] : 838

NLS

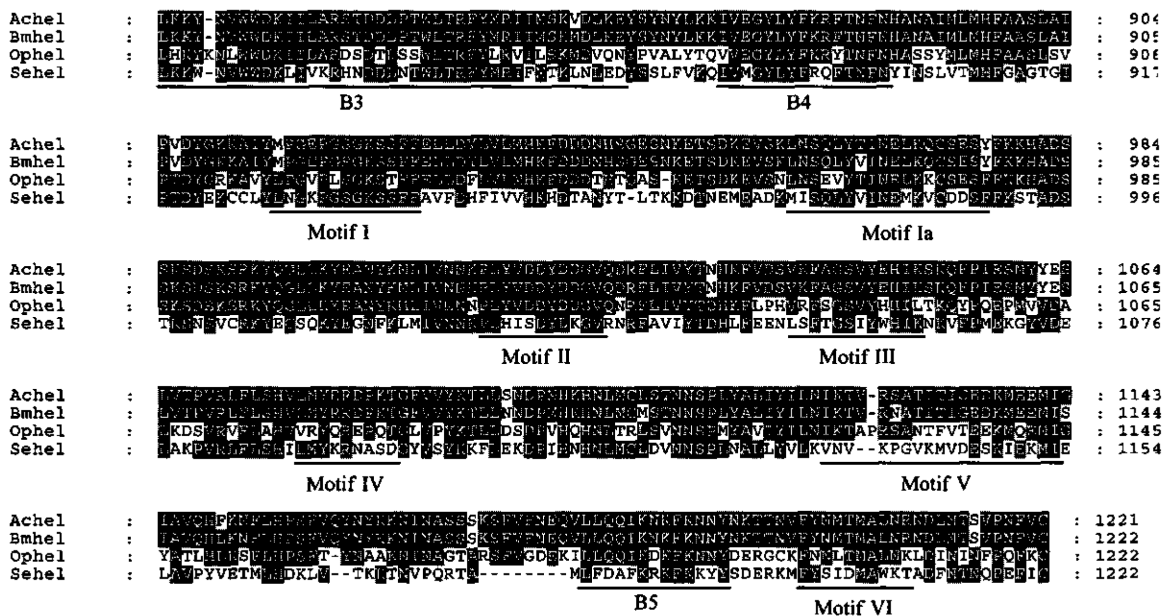


Figure 5.2.
Alignment of the deduced amino acid sequences of AcMNPV, BmNPV, OpMNPV and SeMNPV *p143* genes. Amino acids identical in two of the four polypeptides are shaded. The seven conserved helicase motifs are underlined and denoted I through VI; the nuclear localization signals are indicated by -*NLS*-; the amino acids involved in host range expansion are indicated by asterisks superscribed by "host range factors" and the regions in the four baculovirus helicases that display a higher degree of identity are underlined and denoted B1 through B5.

Additional motifs that have been described for a subgroup of helicases related to transcription factor eIF-4a (Linder *et al.*, 1989) including D-E-A-D, S-A-T and H-G-I-G-R motifs, were not present in SeMNPV p143. One or more amino acids in the p143 protein (H^{551}, V^{556}, S^{564}, F^{577} of AcMNPV; Y^{552}, L^{557}, N^{565}, L^{578} in BmNPV) are involved in the extension of the host range of AcMNPV-BmMNPV recombinants from Sf-AE-21 cells to BmN cells (Kondo and Maeda, 1991; Croizier *et al.*, 1994) (Fig. 5.2). At the homologous position in SeMNPV (L^{530} and L^{551}) only partial homology with BmNPV and AcMNPV is found (Croizier *et al.*, 1994). SeMNPV p143 encodes an additional 36-long amino acid aspartate-rich domain between residues 781 and 817 (Fig. 5.2). Such a domain is not present in the OpMNPV, AcMNPV and BmNPV p143 polypeptides. Two putative nuclear localization signals, K**K/R, are present in all four baculovirus p143s (in the AcMNPV sequence at position 612 and 618). The nuclear localization signal identified by Lu and

Carstens (1991) is not conserved in SeMNPV and OpMNPV p143. A putative signal may be located in the 36 amino acid long insertion (aa 781-817), unique to SeMNPV. The SeMNPV p143 contains a V^{83}-V^{90}-M^{97}-D^{104}-V^{111} motif that resembles the modified zipper motif of AcMNPV and BmNPV. In OpMNPV a similar motif can be observed in this area (F^{86}-L^{93}-V^{100}-L^{107}-I^{104}) (Fig. 5.2).

helicase	BmNPV	OpMNPV	SeMNPV
AcMNPV	**96.0**	**58.0**	**43.3**
	97.9	73.2	65.8
BmNPV		**58.1**	**44.0**
		72.8	65.8
OpMNPV			**39.2**
			61.9

Table 5.1.
Amino acid homology of p143 polypeptides of AcMNPV, BmNPV, OpMNPV and SeMNPV. Similarity, normal typeface; identity, bold typeface

Transcription analysis

Transcriptional activity of the SeMNPV *p143* gene in insect cells was determined by Northern blot analysis of total RNA, isolated at various times after infection, using a ^{32}P-labeled strand-specific probe of the *p143* gene. Using a *p143* mRNA-specific riboprobe generated from linearized plasmid pCNB (Fig. 5.1b) two transcripts were detected. A specific transcript of approximately 4 kb was observed between 4 and 12 h p.i. and a non-specific transcript of 1.9 kb was present from 0 to 48 h p.i. (Fig. 5.4a). The latter transcript is probably due to aspecific hybridization of the probe to rRNA. The specific 4kb transcript accumulated until 8 h p.i. and decreased shortly thereafter. After 12 h p.i. no *p143* specific transcripts of this size could be detected (Fig. 5.4a). However, larger transcripts of 4.1 kb, 5.3 kb and 6.5 kb were present in RNAs isolated at 48 h p.i. (Fig. 5.4a).

RNA primer extension analysis was performed on total infected-cell RNA isolated at various times p.i. to determine the transcriptional start site of the *p143* gene (Fig. 5.4b). This assay revealed two major starts at A residues, at position -11 and -12 relative to the putative translational initiation codon ATG (Fig. 5.4c). These starts could already be detected in RNAs isolated 4 h p.i. The presence of a specific *p143* transcript at 4 h p.i. is in accordance with the Northern analysis (Fig. 5.4a). The SeMNPV *p143* start site does not show any homology to consensus sequence of baculovirus early or late promoter elements or to the major transcriptional start site 5'-GCGTGC-3' that has been determined for AcMNPV *p143*

```
              Motif I           Motif II       Motif III      Motif IV
AcMNPV  915-MPGEPGSGKSSFFE-87-PLYVDDYDDGV-16-VKFAGSVYEHIK-25-LMYRRDPKT-
OpMNPV  917-LPGVPLSGKSTFFE-86-PLYVDDYDDGV-16-VRFSGSVYHHIL-25-VRYQREPQT-
BmNPV   916-MPGEPGSGKSSFFE-86-PLYVDDYDDGV-16-VKFAGSVYEHIK-25-LMYRRDPKT-
SeMNPV  928-LNGKPGSGKSSFFA-86-PLHISDYDKGV-16-LSFTGSIYWHIK-25-LMYKRNASD-

Baculovirus MPG PGSGKSSFFE    PLYVDDYDDGV    V F GSVY HIK    LMYRR P T
consensus   L    L    T  A      HI    K      L     I    L

general     V G PG GKS           VDE             GD  Q           R
consensus   I A A    T           I                S

                Motif V              Motif VI             Motif Ia
AcMNPV  -37-IKTV-RSATITIGEDKMEEMI-58-YNMTMALNR  -32-LNSQLYTINELKQCSESY
OpMNPV  -37-IKTAPRSANTFVTEEKMQEMI-58-NNLTMALNK  -31-LNSEVYTINELKKCSESF
BmNPV   -37-IKTV-RNATITIGEDKMEEMI-58-YNMTMALNR  -32-LNSQLYVINELKQCSESY
SeMNPV  -37-VNV  KPGVKMVDESKIEKMI-48-YSIDMAWKT  -31-MISQLYVINEMKVCDDSF

Baculovirus IKT  R A   I E KM EMI    YNMTMALN       LNSQLYTINELK CSESY
consensus              V               I                  V          F

General       T    AQG E    V            VALSR        VT  T  AA N    L
consensus     L    SK  S    A             LVT         IA      T E    I
                   VH  T                  GM          A         R    V
                       R                  SI
```

Figure 5.3.

Comparison of seven conserved helicase motifs between the four baculovirus p143 polypeptides

and *DNA polymerase* (Lu and Carstens, 1992; Tomalski and Miller, 1988). No late transcriptional start site could be indentified (Fig. 5.4b), although a putuative start (ATAAG) is present upstream of the early start site.

Comparison of the three baculovirus *p143* promoters revealed that the promoter elements in AcMNPV and OpMNPV *p143* genes are similar, but that the promoter of SeMNPV is organized differently (Fig. 5.4c). It should be noted however that detailed transcription analysis has only been performed on the AcMNPV *p143* gene (Lu and Carstens, 1992). The AcMNPV *p143* gene has a leader sequence of more than 150 nucleotides upstream of the putative translational start site. The OpMNPV *p143* gene has a similar leader structure (Ahrens and Rohrmann, 1996). These leaders do contain a similar minicistron of 5

short SeMNPV leader sequence, but upstream of the transcriptional start site between positions -82 and -61 (6 amino acids) (Fig. 5.4c).

A classical poly(A)signal (AATAAA) (Birnstiel *et al.*, 1985) was found immediately downstream of the translational stop codon of the SeMNPV *p143* gene (Fig. 5.4d). The predicted size of the helicase mRNA is therefore 4.0 kb, assuming that the *p143* mRNA (ORF 3.7 kb) contains a poly (A) tail of approximately 300 adenine residues. This is in agreement with the value of 4.0 kb deduced from Northern analysis (Fig. 5.4a). Another poly(A) signal was found in the opposite strand of the DNA at position 3709 (Fig. 5.4d), belonging to an adjacent ORF (ORF p25; J.G.M. Heldens, unpublished results) located on the opposite DNA strand. The first deduced amino acid sequences of an ORF overlapping with the SeMNPV *p143* promoter region which extended into the *p143* coding sequence (30 nt) show high homology to AcMNPV *p19* (Ayres *et al.*, 1994).

Specificity of SeMNPV and AcMNPV p143 genes in DNA replication

To study the cross-activity of the putative SeMNPV and AcMNPV *p143* genes in baculovirus DNA replication, a transient DNA replication assay was performed in which the AcMNPV *p143* gene was substituted by its SeMNPV homologue. In this assay all AcMNPV *trans*-acting factors were provided as plasmids (Kool *et al.*, 1994b) (Fig. 5.5). When the AcMNPV *p143* gene was replaced by the SeMNPV *Xba*I-C fragment, which contains the SeMNPV *p143* gene, no *Dpn*I-resistant bands could be detected (Fig. 5.5; lane 1 and 2). As helicases require, by definition, DNA binding to be able to unwind the double-stranded DNA, it was hypothesized that the SeMNPV p143 may be unable to recognize specifically motifs in the AcMNPV-*hr*2 origin. However, when AcMNPV-*hr*2 was replaced by SeMNPV-*hr*1, no *Dpn*I-resistant DNA bands could be detected (Table 5.2) in any case, suggesting that the AcMNPV replisome or one of its components is unable to activate the SeMNPV origin. This could mean, firstly that the origin replisome complex interaction is very specific, and secondly that either the formation of the replisome is highly specific i.e. SeMNPV p143 cannot substitute AcMNPV p143, or SeMNPV p143 is not or hardly transactivated by the AcMNPV replication gene products, in particular by IE1.

Preliminary data indicated that both SeMNPV and AcMNPV IE1 can transactivate either an AcMNPV-*hr*2-Ac39K promoter-CAT construct as well as a SeMNPV-*hr*1-Ac39K promoter-CAT construct (D.R. Theilmann and E.A. van Strien, unpublished results), suggesting that AcMNPV and SeMNPV IE1's are able to transactivate heterologous genes. However, when the AcMNPV *ie1* gene was replaced by the SeMNPV *ie1* gene (plasmid XDB1), no *Dpn*I resistant DNA was detected (Fig. 5.5, lane 3 and Table 5.2) suggesting that either the IE1 protein can only act in homologous replication events or is not able to transactivate AcMNPV replication genes.

Complementation of AcMNPV plasmid-dependent DNA replication by infection with SeMNPV.

SeMNPV is able to replicate plasmids containing fragments harboring SeMNPV non-*hr* or *hr oris* in Sf-AE-21 cells (Heldens *et al.*, 1997; Heldens *et al.*, 1996b), indicating that all necessary SeMNPV *trans*-acting DNA replication factors are expressed. To test whether the negative result of the substitution of AcMNPV-*p143* with SeMNPV-*p143* obtained in the described assay above was due to limited expression of SeMNPV-*p143*, a "complementation" assay was designed. All but one of the AcMNPV *trans*-acting factors were transfected together with AcMNPV-*hr*2, into Sf-AE-21 cells. One day post transfection the omitted factor was complemented by SeMNPV through an infection (Table 5.3). Via this assay it was also posible to test whether any of the other AcMNPV DNA replication genes could be replaced by their respective SeMNPV counterparts. However, the complementation assays did not show any positive DNA replication signal, suggesting a high degree of specificity in the formation of the replisome complex in AcMNPV and SeMNPV DNA replication. AcMNPV-*hr*2 plus the complete set of AcMNPV essential replication genes gave rise to a positive DNA replication signal (positive control) confirming the specificity of the respective replication mechanism. Sf-AE-21 cells transfected with AcMNPV-*hr*2 did not show a DNA replication signal upon infection with wild type SeMNPV (negative control) (Table 5.3).

Figure 5.4.

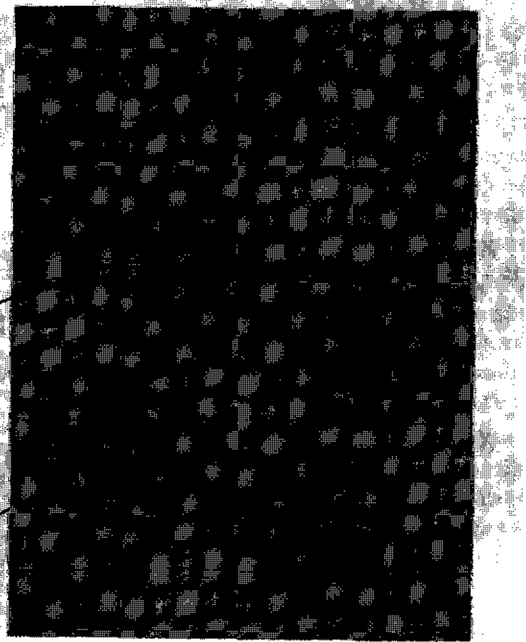
Transcriptional analysis of SeMNPV *helicase* gene.

a) Northern analysis of total RNA extracted from uninfected (lane 0) and SeMNPV-infected Se-IZD2109 cells 2, 4, 8, 10, 12, 24, 48 h p.i. The specific helicase transcript of 4 kb, identified with the strand-specific riboprobe (Fig. 5.1b) is indicated by an arrow.

b) Primer extension analysis of helicase transcripts performed with an oligonucleotide primer complementary to the nucleotides between +50 and +70 downstream of the translational initiation site, ^{32}P-labeled at the 5' end. The oligonucleotide was annealed to total RNA from uninfected (lane 0) and SeMNPV-infected cells isolated 4, 8, 24 h p.i. and elongated by reverse transcription. The sizes of the extension products were determined by comparison with a sequence ladder run alongside (lanes C, T, A and G) obtained from a SeMNPV *helicase* containing plasmid and the oligonucleotide as sequence primer.

c) Promoter sequence comparison of AcMNPV, OpMNPV and SeMNPV *helicase*. The translational initiation sites (ATG) are in bold, early (E) or late (L) transcriptional start sites are indicated with arrows. The SeMNPV late promoter element ATAAG from which no transcript could be detected in underlined (dashed). Putative minicistrons are boxed.

d) Nucleotide sequence of the 3' end of the SeMNPV *helicase* gene. Canonical poly(A) signals and the stop codon (TAA) of the helicase gene are underlined, and dashed, respectively.



c

```
SeMNPV   1  AAATTGTATA GCAATACGTT CTCGGGTCTT ATCAGGAATA AACGTTCCAC ACGGTCCGGG GGAACCGATA GATCGTACAC GTCGACGTAC GCTCCGAATT
                                                                    E
AcMNPV   1  AAATTATACA ACACCACATT TTCGGGCGCA ATAATAAATA ATCGTTCGGT GCGGGCGGGC GTGCTTAAAT CGTAAATCTG TACATACGCG CCAAACAACA

OpMNPV   1  TGTCAAAATT GTACAGCACT ACGTTTTCGG GCGCAATAAT AAACAGTCGC TCGGTGCGCG CGGGTGTGCT GAGGTCGTAG ATCCGCACGT GCGTCCCGTA
                                                     (L)                                   E
SeMNPV 101  GTAAAGTGTT TTGATGGTCG TGTATCAATT TTAATACTTG TGTTCGATAA GGATTTAAAT AGAGGAGCGC GATCAAAAAA ATCAATAGAC ACAAAATGGC AACAGCGCCG ATAACTGTCG TCGACGTCAT
                                                L
AcMNPV 101  ATGTGTTGTT GAAATCTTGT ATTAGCGTTT CTATTCTATG TAAGAATGGA TGGTGATTTT TGATCGAAAT TATTAAATAA ATTAACACGA AAAGTACAAA CACGATAGCC AACATGATTG ACAACAT
                                                  L
OpMNPV 101  CAGCAGCGTG TTGTCGAAAT CGCGCAGCAG CGCCTGAACC CTGTTTAAGA ATGGATGGTG GTCTCGCAGC GCCACCGCGA CGTACATAAA CAGCAACACC GCGAGCGCGA TCGCTGCCAT CATGAACAGC A
```

d

```
SeMNPV 3656  GTTTATATGT TAAATAATAA TAATAAAAAA AATTGTTGTT AACCAAATTG  TATTTTTATT   <——  p25
             helicase
```

Origin	helicase	ie1	replication
AcMNPV-*hr*	Ac	Ac	+
	Ac	***Se***	-
	Se	Ac	-
	Se	***Se***	-
SeMNPV-*hr*	Ac	***Se***	-
	Se	Ac	-
	Se	***Se***	-
AcMNPV-*hr*	AcMNPV		+
	SeMNPV		-
SeMNPV-*hr*	SeMNPV		+
	AcMNPV		-

Table 5.2.
Transient DNA replication assay using AcMNPV-*hr*2 and SeMNPV-*hr*1 as origins of DNA replication. *Trans*-acting factors were provided by the virus (AcMNPV or SeMNPV) or as plasmids. *Lef1*, *lef2*, *lef2*, *lef3*, *DNA pol*, *pe38*, *ie2* and *p35* originate from AcMNPV. *Helicase* and *ie1* originate either from SeMNPV, indicated by ***Se***, or from AcMNPV, indicated by ***Ac***. Positive (+) or negative (-) DNA replication signals are indicated.

Discussion

In this paper we describe the localization, nucleotide sequence and transcriptional analysis of an ORF in the SeMNPV-*Xba*I-C fragment encoding a putative helicase. The deduced amino acid sequence showed a high degree of similarity to AcMNPV, OpMNPV and BmNPV p143 polypeptides. The SeMNPV p143 amino acid sequence is more distantly related to the other three polypeptides (Fig. 5.2 and Table 5.1), which is consistant with the phylogeny of other genes of these viruses (Cowan *et al.*, 1994; Hu *et al.*, 1997). The SeMNPV *p143* ORF contains seven conserved motifs that are characteristic for DNA and RNA-dependent eukaryotic helicases (Gorbalenya and Koonin, 1988; Gorbalenya *et al.*, 1988). These motifs are very well conserved both in sequence and spatial order in baculovirus p143 proteins.

Biochemical evidence for the hypothesis that p143 is a helicase, i.e. the determination of *in vitro* helicase and NTP hydrolysis activity (Abdel-Monem *et al.*, 1976; Kuhn *et al.*, 1978), is still lacking. Overexpression of the AcMNPV *p143* gene in baculovirus and bacterial expression systems has been established recently and the purified baculovirus p143 protein may allow the further study of these functions. The ability of the AcMNPV p143 to bind AcMNPV *hr5* showed its DNA binding properties (Laufs *et al.*, 1997).

Motif I (amino acid 928-940) and II (amino acid 1025-1035) contain the common consensus sequences for ATP and GTP binding sites (Walker *et al.*, 1988; Seraste *et al.*, 1990). The consensus DD/E motif in the B loop of the NTP binding site (motif II, amino acid 1025 to 1035) is altered into a SD motif in SeMNPV p143. This may result in the formation of an altered interaction between the A-loop, the magnesium ion and the aspartic acid in the B-loop (Walker *et al.*, 1988; Hodgman, 1988ab; Seraste *et al.*, 1990). A conserved tyrosine residue (Y^{1202}), thought to be important for DNA binding activity, is present in motif VI (amino acid 1202 to 1210) of SeMNPV (Fig. 5.2). Defined functions of the motifs III (amino acid 1052 to 1063), IV (amino acid 1089 to 1096) and V (amino acid 1135 to 1153) have not yet been assigned.

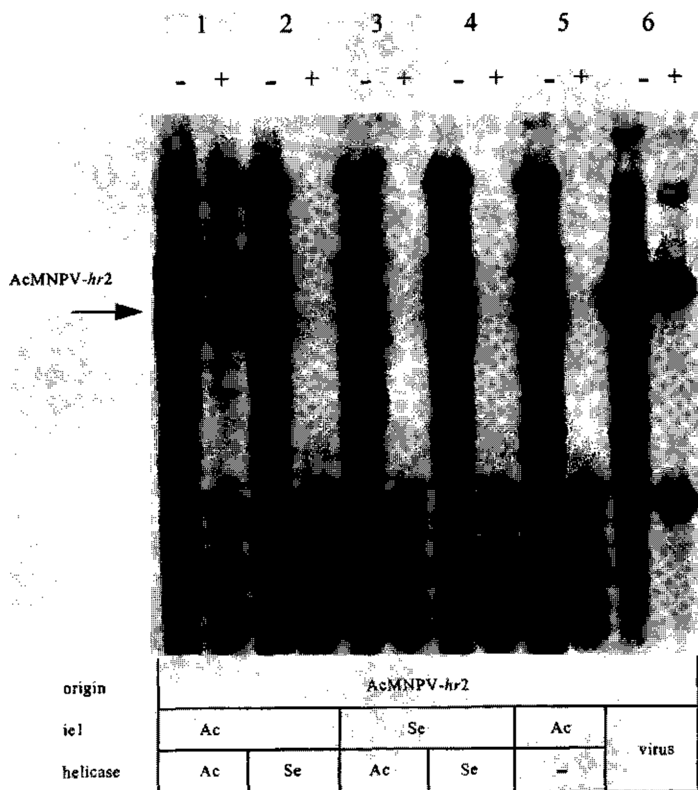


Figure 5.5.
Transient DNA replication assay using AcMNPV-*hr2* as a DNA replication origin. *Trans*-acting factors were provided by virus infections (AcMNPV; lane 6) or as plasmids (lanes 1 -5). Plasmids containing *lef1*, *lef2*, *lef2*, *lef3*, *DNA pol*, *pe38*, *ie2* and *p35*, originate from AcMNPV, were provided in lanes 1-5. *Helicase* and *ie1* originate either from SeMNPV, indicated by **Se**, or from AcMNPV, indicated by **Ac**. DNA in the lanes indicated + are digested with *DpnI*/*Hin*dIII, whereas DNA in the lanes indicated - is digested with *Hin*dIII only. Transient DNA replication gives rise to *DpnI* resistant bands of the size of AcMNPV-*hr2* (indicated by an arrow).

Besides the seven conserved helicase motifs the four baculovirus p143 polypeptides contain additional regions that display an even higher degree of identity. These regions are indicated in figure 5.2 as B1 (amino acid 135 to 148), B2 (538 to 545), B3 (826 to 866), B4 (875 to 889) and B5 (1181 to 1193) of the AcMNPV p143 sequence and may be specific for baculovirus helicases. Some of these regions e.g. B1 and B2 show almost 100% identity. The consensus sequence L****K*KFY*Y of motif B5 is striking since it is located in the C terminal part of the polypeptide which is the least conserved. The B5 motif is even better conserved than motif VI.

Most helicases are biologically active as oligomers (Matson and Kaiser-Rogers, 1990). The putative L-zipper motifs found in all four baculovirus p143s might be involved in this oligomerization. In the *p143* gene of SeMNPV, however, this putative hydrophobic interaction site may be affected by the presence of an aspartic acid (D^{104}) at position 4 of the zipper motif (Fig. 5.2).

Four amino acid changes in a small region (amino acids 550-578) of the AcMNPV p143 expanded the host range of this virus to *B. mori* cells (Kondo and Maeda, 1991; Croizier *et al.*, 1994). In SeMNPV amino acids at the same position do not have any identity to the corresponding amino acids in OpMNPV and only partly to those in BmNPV. Specific mutagenesis of the pertinent amino acids in the SeMNPV helicase gene might shed some light on the role in host range determination. However, the generation of specific SeMNPV mutants is rather difficult when this process is dependent on cell culture. Upon one passage in cell culture this virus quickly generates defective interfering viruses (Heldens *et al.*, 1996a).

Analysis of SeMNPV *p143* transcription revealed one major transcript starting at a promoter element 11/12 nucleotides upstream relative to the translational start codon. This promoter element (5' ATCAATA 3') does not show any homology to consensus baculovirus early or late transcriptional start sites or to elements of the AcMNPV *DNA polymerase* and *p143* gene promoters (5'-GCGTGC-3'). In contrast to the transcription of AcMNPV *p143*, no transcript originating from a late promoter element (TAAG) could be detected in SeMNPV-infected IZD2109 cells. Compared to the length of the untranslated leader of the AcMNPV *p143* gene the untranslated leader of SeMNPV *p143* is relatively short. Such short leader sequences are not uncommon as they have been reported for SeMNPV *ubiquitin* (van Strien *et al.*, 1996) and *Heliothis armigera* granulovirus *enhancin* (Roelvink *et al.*, 1995). Unusual transcriptional start sites with limited homology to consensus baculovirus early or late start sites have also been reported for the large subunit of the SeMNPV *ribonucleotide reductase* gene (van Strien *et al.*, 1997). Since another ORF partly overlaps with the 5' end of the SeMNPV *p143* gene and since a putative poly(A) signal is located on the opposite DNA strand close to the 5' end of the gene, it seems that the SeMNPV genome is tightly organized at this locus.

A putative minicistron, believed to have a regulatory function in gene expression (Chang and Blissard, 1996) is present upstream of the translational start codons of AcMNPV, OpMNPV and SeMNPV *p143*. In AcMNPV and OpMNPV this minicistron is present in the untranslated leader of early and late transcripts, whereas in SeMNPV a minicistron is located upstream of the transcriptional initiation site. The exact function of minicistrons in baculovirus gene expression remains enigmatic.

Northern blot analysis revealed that the length of the major *p143* transcript is 4.0 kb and that it accumulates from 4 h p.i. until 8 h p.i. After 12 h p.i. no specific 4.0 kb transcripts could be detected until 48 h p.i. The size of the SeMNPV *p143* transcript is in agreement with the size of the AcMNPV *p143* transcript (Lu and Carstens, 1992). Larger transcripts (5.2 and 6.8 kb) were also detected late in the infection process of AcMNPV infected Sf-AE-21 cells (Lu and Carstens, 1992). These transcripts are similar in size compared to the larger transcripts detected in SeMNPV-infected IZD2109 cells (5.3 and 6.5 kb, respectively). The 6.5 kb transcript in SeMNPV infected cells might therefore be the transcript to be spliced as has been suggested for the 6.8 kb transcript in AcMNPV-infected *S. frugiperda* cells (Lu and Carstens, 1992). The origin of the late 4.1 kb transcript (48 h p.i.) is unknown.

Hr containing plasmids from SeMNPV or AcMNPV could not be replicated by the heterologous virus (Heldens *et al.*, 1996b), indicating that the interaction between origins of DNA replication and *trans*-acting factors is virus specific. Swapping of the AcMNPV *p143* or *ie1* genes for the SeMNPV homologues in the transient DNA replication assay did not result in any DNA replication signal. Moreover, complementation of each of the essential AcMNPV replication genes by an SeMNPV infection did not result in transient DNA replication. Since SeMNPV replicates in the semi-permissive Sf-AE-21-cells, the SeMNPV DNA replication genes must be functionally active (Heldens *et al.*, 1997). The negative results from swapping the SeMNPV and AcMNPV *ie1* genes may suggest that IE1 is an integral part of the DNA replication complex and interacts specifically with (an)other *trans*-acting DNA replication factor(s). It is also very likely that SeMNPV-IE1 is not able to transactivate (or shows limited transactivation of) the AcMNPV replication genes. Nevertheless it has been shown that SeMNPV IE1 could transactivate AcMNPV-39K-CAT constructs (D.R. Theilmann and E.A. van Strien, unpublished results). Characterization of other SeMNPV *trans*-acting DNA replication factors and detailed studies of their transactivation mechanism might shed some light on the functioning of SeMNPV and AcMNPV IE1 in this respect.

SeMNPV-p143 could not substitute its AcMNPV counterpart in the "complementation" assay. Since SeMNPV can replicate its own *cis*-acting elements in Sf-AE-21 cells (Heldens *et al.*, 1997; Heldens *et al.*, 1996b), it is likely that a functional SeMNPV p143 is generated. It can therefore be hypothesized that the interaction between p143 and the

other *trans*-acting DNA replication factors and/or the interaction between p143 and the functional domains in the *ori* is very specific. In contrast to our results with SeMNPV and AcMNPV p143 substitutions, AcMNPV p143 could substitute for its OpMNPV homologue

complete set	SeMNPV	AcMNPV
all Ac repli genes	-	+
- helicase	+	-
-DNA pol	+	-
- lef1	+	-
-lef2	+	-
-lef3	+	-
- ie1	+	-

Table 5.3.
Transient DNA replication assay using AcMNPV-*hr2* as reporter for DNA replication.
All individual AcMNPV *trans*-acting DNA replication factors (*lef1*, *lef2*, *lef2*, *lef3*, *DNA pol*, *helicase*, *ie1*, *ie2*, *pe38* and *p35*), provided as plasmids, were depleted one by one. The missing factors were complemented by SeMNPV infections. Positive (+) or negative (-) DNA replication signals are indicated.

with a 50% loss of replication activity in *L. dispar* cells, whereas OpMNPV p143 failed to substitute for AcMNPV p143 in *S. frugiperda* cells (Ahrens and Rohrmann, 1996). These observations suggest that the formation of an active baculovirus replisome depends on virus specific interactions between individual replication factors. Gel retardation and gel-supershift assays using purified protein and labeled origins of DNA replication might elucidate which parts of the individual proteins contribute to these specific interactions and what the nature of these interactions is.

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The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide sequence database under accession number AF021837.

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Chapter 6

A HIGHLY CONSERVED GENOMIC REGION IN BACULOVIRUSES: SEQUENCE AND TRANSCRIPTIONAL ANALYSIS OF A 11.3 KBP DNA FRAGMENT (MU 46.5-55.1) FROM THE *SPODOPTERA EXIGUA* MULTICAPSID NUCLEOPOLYHEDROVIRUS[5]

Summary

A DNA fragment of 11.3 kilobase pairs (kbp) in size of the baculovirus *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV) genome (mu 46.5 to 55.1) was completely sequenced. Analysis of the sequence revealed eleven potential open reading frames (ORF). Ten of these ORF showed significant amino acid identity to *Autographa californica* MNPV (AcMNPV) and *Orgyia pseudotsugata* MNPV (OpMNPV) p6.9, lef5, 38kDa, p19, p143, p25, p18, vp33, lef4, and vp39. One ORF (*XC12*) has no homolog in other baculoviruses and may be unique to SeMNPV. All but three of these putative genes are preceeded by the consensus baculovirus late promoter element (5'-ATAAG-3'). All ORFs were transcriptionally active, which is a novel finding for baculovirus *lef5*, *lef4*, *p18* and *vp33* genes. The genetic organization and transcriptional pattern of this fragment suggested that this region is highly similar to that of AcMNPV fragment *Eco*RI-D. Comparison of the genetic organization on the eleven kbp fragment in the genomes of AcMNPV, OpMNPV, *Bombyx mori* NPV and SeMNPV revealed that this region is highly conserved among baculovirus genomes. This is in contrast to the genetic organization of *polyhedrin-p10* region, which is much more diverged, but has been taken as point of reference to orient baculovirus physical maps. The latter region, however, would be an excellent candidate to determine baculovirus relatedness and phylogeny. The presence of conserved and diverged regions in baculovirus genomes with respect to gene order is reminiscent to the situation in other large DNA viruses, such as herpes- and poxviruses, where conserved central and diverged terminal

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[5] This chapter has been submitted as:
J.G.M. Heldens, Y. Liu, D. Zuidema, R.W. Goldbach and J.M. Vlak (1998). A highly conserved genomic region in baculoviruses: Sequence and transcriptional analysis of a 11.3 kbp DNA fragment (mu 46.5-55.1) from the *Spodoptera exigua* multicapsid nucleopolyhedrovirus. *Virus Research*

parts are common characteristics. The role of this feature in the genomic organization of large DNA viruses is discussed with particular emphasis on virus replication and evolution.

Introduction

The biological properties of *Spodoptera exigua* multicapsid nucleopolyhedrovirus (SeMNPV), a baculovirus of the beet army worm, are rather distinct from many other baculoviruses in their respective hosts. SeMNPV has a very narrow host range, it is only capable of infecting *S. exigua* larvae, and it is relatively virulent as compared to other baculoviruses infectious for this insect (Smits *et al.*, 1987). In addition, the genetic stability of the SeMNPV genome *in vitro* is, in contrast to the stability of the AcMNPV genome in *S. frugiperda* cell lines rather limited. During a few passages in *S. exigua* cell lines about 18% of the SeMNPV genome is deleted (Heldens *et al.*, 1996; E.M.M. Colbers and J.M. Vlak, unpublished results). The relatively high virulence and narrow host range render SeMNPV an attractive biological insecticide for *S. exigua* pests. However as is the case for all baculoviruses its speed of action needs to be improved to be comparable to most commonly used chemical insecticides. The improvement might be achieved via the genetic engineering of the SeMNPV genome in a similar way as has been achieved for *Autographa californica* MNPV (Bonning and Hammock, 1996). Therefore, to be able to engineer the viral genome succesfully and, more importantly, to elucidate the molecular basis of the distinct biological properties of SeMNPV with respect to other baculoviruses, detailed information about the genetic organization and gene regulation of the SeMNPV genome is essential.

A detailed physical map has recently been constructed for the US-isolate of SeMNPV, assumed to be the SeMNPV proto-type species (Heldens *et al.*, 1996). SeMNPV has a circular double stranded DNA genome of approximately 134 kb. A few genes, e.g. *polyhedrin* (van Strien *et al.*, 1992), *p10* (Zuidema *et al.*, 1993), *ubiquitin* (van Strien *et al.*, 1996), *ribonucleotide reductase* large subunit (van Strien *et al.*, 1997), *ecdysteroid-UDP-glucosyltransferase* (D. Zuidema, R.W. Mans, B.I.F Klassens, and J.M. Vlak, unpublished results) and *p143* (Heldens *et al.*, 1997) have been identified and characterized. The majority of these genes is, however, located in the *polyhedrin-p10* region. The location of these genes in other baculoviruses, such as AcMNPV (Ayres *et al.*, 1994), OpMNPV (Ahrens *et al.*, 1997), *Spodoptera litoralis* NPV (SpliNPV) (Faktor *et al.*, 1997) or *Bombyx mori* NPV (GenBank accession number L33180) e.g., varies considerably with SeMNPV, and suggests that the genetic organization of SeMNPV is significantly different.

Alignment and comparison of *polyhedrin* (van Strien *et al.*, 1992; van Strien unpublished results), *p10* (Zuidema *et al.*, 1993), *p143* (Heldens *et al.*, 1997) and *ie1* (E.A. van Strien, 1997) genes to homologues in other baculoviruses suggested that SeMNPV is on the molecular level only distantly related to AcMNPV and OpMNPV. Phylogenetic analysis using for instance parsimony on several of those genes revealed that SeMNPV is a member of a different clade than AcMNPV, OpMNPV and BmNPV (Zanotto *et al.*, 1993; Cowan *et al.*, 1994; Hu *et al.*, 1997; E.A. van Strien, 1997), confirming the distinct character of SeMNPV.

Here we report the complete nucleotide sequence and transcriptional analysis of an 11.3 kbp fragment in the SeMNPV genome located between map units 46.5 and 55.1 (*Xba*I-C). The availability of the complete nucleotide sequences of baculoviruses genomes, e.g. AcMNPV (Ayres *et al.*, 1994), OpMNPV (Ahrens *et al.*, 1997) and BmNPV, allows comparison of gene sequences and gene organization among these viruses and to determine their genetic relatedness and phylogeny. The order of genes located on fragment SeMNPV-*Xba*I-C was compared to OpMNPV, AcMNPV, BmNPV and *Cryptophlebia leucotreta* granulovirus (ClGV) (Jehle and Backhaus, 1994) genomes and found to be highly conserved. Based on this observation and the high variability in gene organization found in the *polyhdrin-p10* region, it is hypothesized that the presence of constant and variable regions is characteristic for baculovirus genomes. In this respect the genetic organization of baculoviruses is similar to that of other large DNA viruses such as herpes and pox viruses, and possibly a reflection of a common mode of replication (Davison and McGeoch, 1995; Gompels *et al.*, 1995; Senkevich *et al.*, 1996).

Materials and methods

Cells and Virus

S. frugiperda (Sf-AE-21) (Vaughn *et al.*, 1977) and *S. exigua* (Se-IZD-2109) cells (B. Möckel & H.G. Miltenburger, unpublished results) were cultured in TNM-FH medium (Hink, 1970), supplemented with 10% fetal calf serum (FCS). The US-isolate of SeMNPV (Gelernter and Federici, 1986) and the E2 strain of AcMNPV (Summers and Smith, 1987) were used as wild type (wt) viruses. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Summers and Smith, 1987; King and Possee, 1992). Budded virus (BV) used in time course infection experiments was obtained from the supernatant of Se-IZD-2109 cells infected with hemolymph from SeMNPV-infected fourth instar *S. exigua* larvae. AcMNPV BVs were obtained from the

supernatant of Sf-AE-21-infected cells. BV titers were determined by the end point dilution method (Vlak, 1979) and expressed as $TCID_{50}$ units per ml.

Plasmid constructions

SeMNPV subgenomic fragments were cloned into pUC19, pBluescript KS^+ (Stratagene) or $pGEM7zf^+$ (Promega) and transformed into *E. coli* DH5α using standard techniques (Sambrook *et al.*, 1989). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis and Southern blotting were carried out according to standard protocols (Sambrook *et al.*, 1989). Unidirectional deletion clones were generated from the cloned SeMNPV-*Xba*I-C fragment using the *Exo*III-based 'Erase-a-Base' kit according to the protocols of the manufacturer (Promega).

Isolation of total RNA and Northern blotting

Total RNA for Northern blot and primer extension analysis was isolated from SeMNPV-infected Se-IZD-2109 cells at several time points p.i., as described by Xie and Rothblum (1991). Total RNA was denatured, electrophoresed and blotted to Hybond N nylon membrane (van Strien *et al.*, 1992). To identify SeMNPV-*Xba*I-C derived transcripts, the blot was hybridized for 16 h at 65 °C with [$\alpha-^{32}$P]dATP-labeled probes. Probes were generated by elongation of random primers using Klenow DNA polymerase (Gibco-BRL) annealed to restriction fragments containing segments of the SeMNPV-*Xba*I-C fragment (Feinberg and Vogelstein, 1984). After hybridization at 65 °C (overnight) in Church buffer (0.25 M sodium phosphate pH 7.2, 7% SDS, 1% BSA, 1mM EDTA), the filters were washed for 5 min with 2*SSC, 0.5% SDS at room temperature, 30 min with 2*SSC, 0.1% SDS at 65 °C and 30 min with 0.1* SSC, 0.1% SDS at 65 °C. The filters were exposed to Kodak XAR film.

Sequencing

Both DNA strands of the *Xba*I-C fragment of SeMNPV and its flanking regions were sequenced from fragments generated with the 'Erase-a-Base' system (Promega) using an automated DNA sequencer (Applied Biosystems) and the dideoxy chain-termination protocol (Sanger *et al.*, 1977). Sequence analyses were carried out by the UWGCG computer programs (Devereux *et al.*, 1984). Deduced amino acid sequences were compared with the daily updated GenBank/EMBL, SWISSPROT and PIR data libraries using BLAST and FASTA programs.

Sequence analysis of the SeMNPV-XbaI-C region

The sequence of the ends of the SeMNPV *Xba*I-C fragment (mu 46.5-55.1) (Fig. 6. 1ab) showed high amino acid similarity to the vp39 and p6.9 polypeptides of AcMNPV and OpMNPV (Heldens *et al.*, 1997). An OpMNPV and AcMNPV-*p143* gene homolog was recently identified, characterized and also located on fragment *Xba*I-C (11.5 kbp) of SeMNPV (Heldens *et al.*, 1997). The location of these genes suggested conserved colinearity between the *p143* regions of SeMNPV with those of AcMNPV, OpMNPV and BmNPV. To verify this hypothesis complete sequence analysis of SeMNPV *Xba*I-C fragment was initiated. Firstly, a detailed physical map of *Xba*I-C was constructed using several restriction enzymes (Fig. 6.1b. Based on this map two subclones pCHK and pCSK were generated (Heldens *et al.*, 1997). Secondly, the complete sequence of the SeMNPV *Xba*I-C fragment and the 3' end of the *Xba*I-P fragment (Fig. 6.1b), encompassing 11297 basepairs in total, was determined using terminal sequence data from *Exo*III deletion clones of pCHK and pCSK. Sequence analysis revealed eleven open reading frames (encoding putative proteins of at least 50 amino acids in size), among which were the SeMNPV homologues of AcMNPV and OpMNPV *p6.9*, *lef5*, *38kd*, *p19*, *p143* (Heldens *et al.*, 1997), *p25*, *p18*, *vp33*, *lef4*, and *vp39* (Fig. 6.1c) (Table 6.1). Interestingly, these genes were also found clustered in a single locus in the genomes of AcMNPV and OpMNPV in an identical orientation. One additional ORF (ORF XC12) (Fig. 6.1c) (Table 6.1), not present in AcMNPV or OpMNPV, was located between SeMNPV-*p19* and *38kd*. The coding sequence of ORF *XC12* did not show homology on the nucleotide or amino acid level to any other gene or protein from GenBank and is unique to SeMNPV.

Mapping of transcripts of SeMNPV-XbaI-C

The highly conserved genetic organization and tight clustering of the genes on fragment SeMNPV-*Xba*I-C compared to the *Eco*RI-GD region in the genome of AcMNPV, suggested a similar gene expression profile in SeMNPV as compared to AcMNPV. Sequence analysis revealed consensus baculovirus late promoter elements (A/GTAAG) in the majority of the 5' untranslated leaders of these putative genes. This indicated that they are expressed after the onset of viral DNA replication (> 8 h p.i.) (Table 6.1) and hence considered to be late genes. The putative SeMNPV-*lef4*, *lef5* and *p143* genes did not contain a consensus baculovirus early (CAGT) or late (TAAG) promoter element as is the case in the promoters of the OpMNPV and AcMNPV-*lef4* and *lef5* genes (Ahrens *et al.*, 1997; Passarelli and Miller, 1993). Transcriptional analysis of SeMNPV-*p143* showed an early transcript

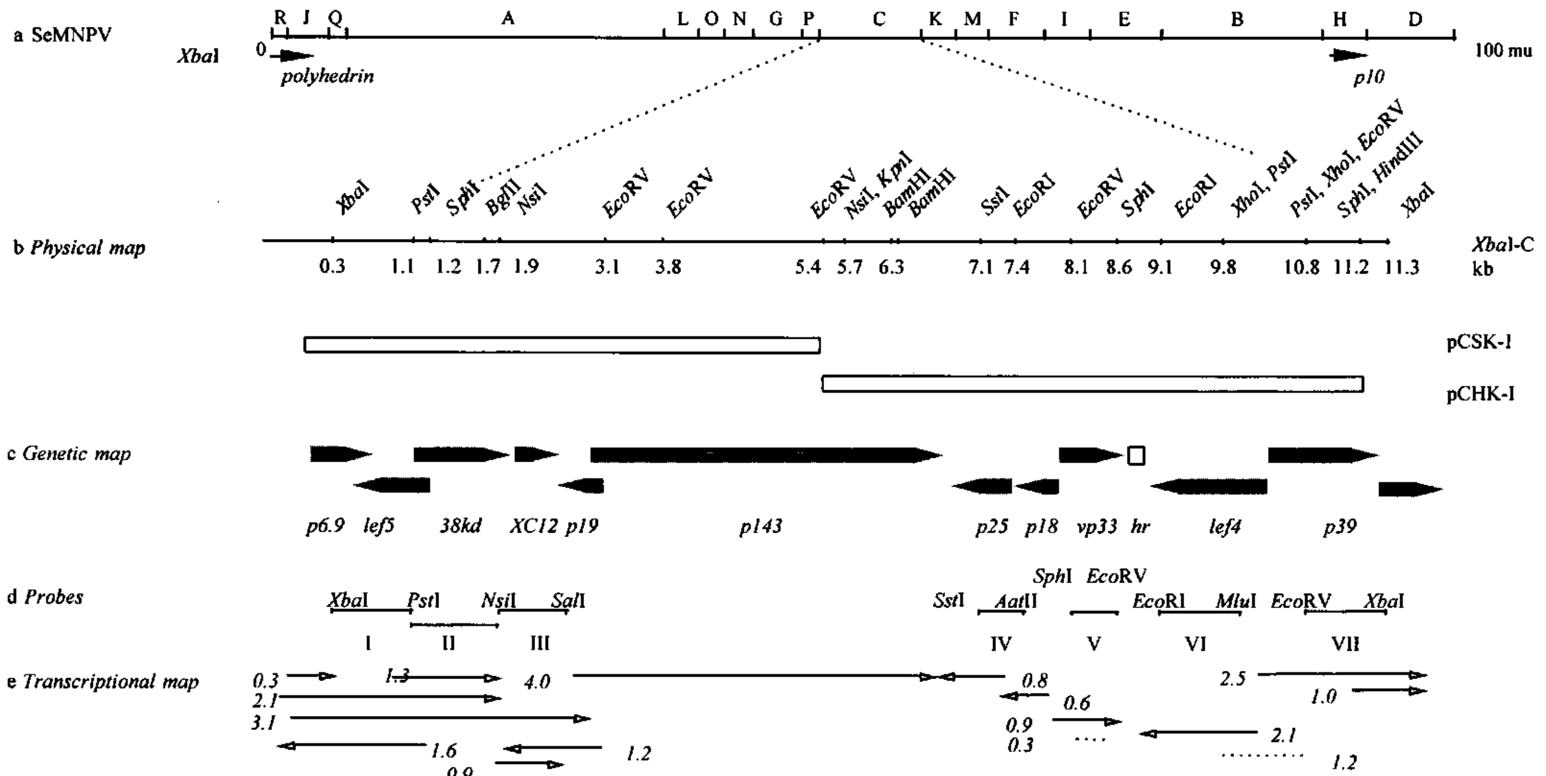

Figure 6.1.

a) *Xba*I map of the SeMNPV genome. The position and orientation of the SeMNPV *polyhedrin* and *p10*gene are indicated by arrowheads.

b) Detailed physical map of the sequenced fragment. The location of the restriction sites are indicated in kilobaspairs relative to the first nucleotide of the sequence.

c) Gene order in the *Xba*I-C fragment of SeMNPV.

d) Location of the restriction fragments that were labeled and used as probes to identify the transcriptional activities of the *Xba*I-C fragment.

e) Potential transcriptional map of fragment SeMNPV *Xba*I-C. Sizes of the transcripts are indicated in nucleotides and the arrowheads represent the possible orientation of the transcripts. Transcripts that could not be realted to any of the ORFs identified are represented by dashed lines.

(detectable at 4 h p.i.) originating from an unusual transcriptional initiation site (Heldens *et al.*, 1997). To investigate the temporal expression of the remaining putative genes on fragment *Xba*I-C of SeMNPV northern blots of total infected-cell RNA were hybridized with seven different ORF-specific probes (Fig. 6.2).

Probe I (Fig. 6.1d), specific for *p6.9* and *lef5*, detected three major transcripts of 0.3, 2.1 and 3.1 kb respectively and 1 minor transcript of approximately 1.6 kb (Fig. 6.2a). The transcript of 300 bp in size, present between 8 and 48 h p.i., originates probably from SeMNPV *p6.9* expression. It is in agreement with the size of the transcript predicted from sequence analysis (Table 6.1), but somewhat smaller than the predicted size of the AcMNPV-*p6.9* transcript (0.5 kb) (Lu and Carstens, 1992). The transcripts of 2.1 kb and 3.1 kb in size, present from 10 until 48 h p.i., might originate from readthrough transcription from the *p6.9* promoter, suggesting a similar expression pattern for this gene during SeMNPV and AcMNPV infections (Wilson *et al.*, 1987). The minor transcript of 1.6 kb is probably related to SeMNPV *lef5* expression. A transcript of similar size (1.4 kb) was identified during AcMNPV infections using a strand-specific probe complementary to *p6.9* (Wilson *et al.*, 1987). Lu and Carstens (1992) detected a 1.4 kb transcript as well in this region using a double-stranded DNA probe harboring the putative AcMNPV *lef5* and *p6.9* genes. Hybridizations using strand-specific riboprobes and primer extension analysis on AcMNPV, OpMNPV and SeMNPV mRNA may reveal a consensus transcriptional start of baculovirus *lef5* genes. A band of 1.35 kb is detected as well using this probe from 8 until 48 h p.i. (Fig. 6.2a). A transcript of similar size was also observed during AcMNPV infections originating from AcMNPV-*38kd* transcription (Lu and Carstens, 1992).

Probe II (Fig. 6.1d) harboring the *38kd* and part of the *lef5* genes revealed transcripts of 3.1 kb, 2.1 kb 1.6 kb and 1.3 kb respectively (Results not shown) suggesting that the 1.3 kb transcript originated indeed from *38kd* expression. The longer 2.1 and 3.1 kb transcripts are most likely the same as the 2.1 and 3.1 kb transcripts detected with probe I. From sequence analysis it can be deduced that the SeMNPV-*38kd* transcript must contain a poly A tail of approximately 300 bases to meet the size of the 1.3 kb detected on the northern blot (Table 6.1). It also suggests that *lef5* and *38kD* have a similar temporal expression pattern and transcript length during SeMNPV and AcMNPV infections.

Probe III (Fig. 6.1d) hybridized to a 3.1 kb mRNA from 10 to 48 h p.i., 1.2 kb mRNA from 8 to 24 h p.i. and 0.9 kb mRNAs from 8 to 48 h p.i. and with a transcript of approximately 150 nt (Fig. 6.2d). The 1.2 kb transcript most likely originates from SeMNPV-*p19* expression, which is in agreement with the size that can be predicted from sequence analysis (Fig. 6.2b; Table 6.1). The 900 bp transcript might originate from *XC12* expression assuming that the mRNA has a poly A tail of approximately 300 nt. The 3.1 kb transcript is probably the same as the 3.1 kb transcript detected by probe I and II (*p6.9*). The smaller

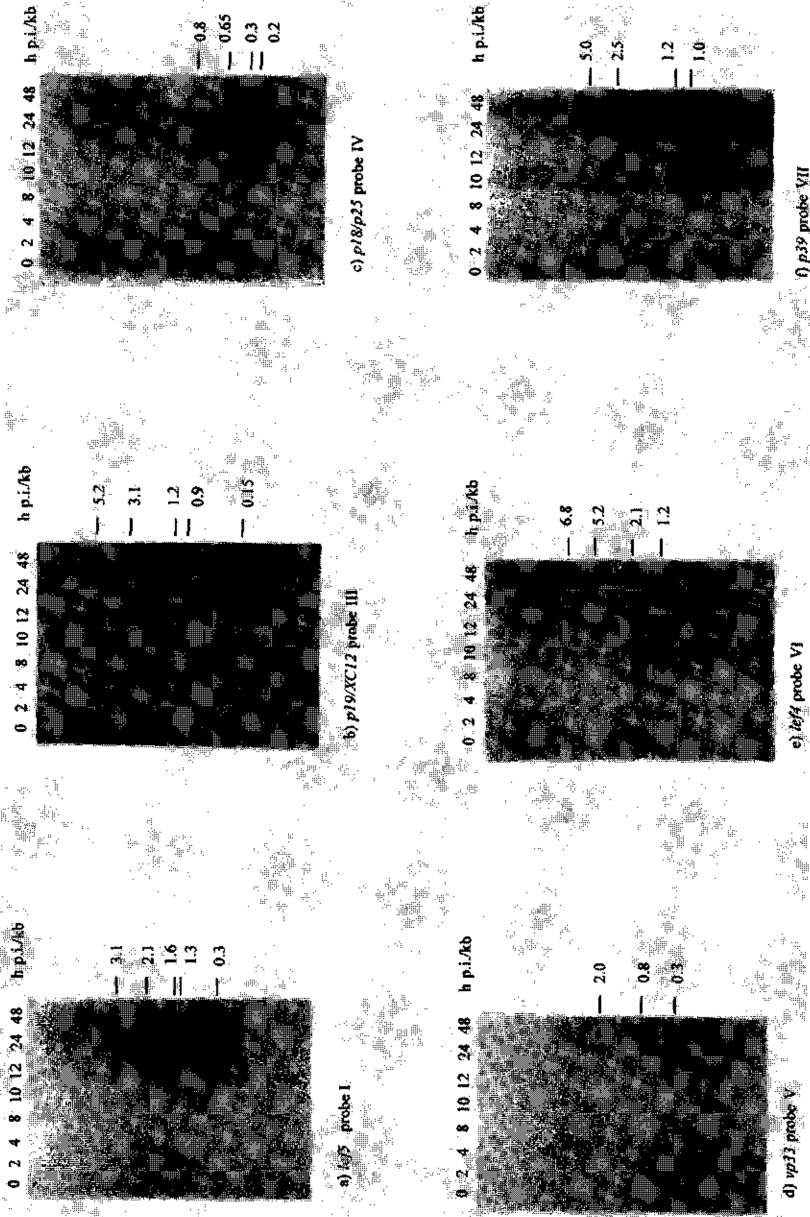

Figure 6.2.

Northern analysis of the transcripts in the SeMNPV *XbaI*-C fragment. Figure a through f. Total RNA from-SeMNPV infected insect cells harvested at 0, 2, 4, 8, 10, 12, 24 and 48 h p.i. was electrophoresed and blotted to nylon membranes. These blots were hybridized to six different ^{32}P-labeled DNA probes. The identity of these probes is indicated in figure 6.1d. a) Probe I, encompassing *p6.9* and *lef5*; b) Probe III, encompassing *XC12* and *p19*; c) Probe IV, ecompassing *p25* and *p18*; d) Probe V, harboring *vp33*; e) Probe VI, encompassing *lef4* and f) Probe VII, harboring *p39*.

transcripts might originate from starts located within *XC12* or *p19* genes present on either strand of the Se-*Xba*I-C fragment (Fig. 6.2b).

To detect transcripts of the putative *p18* and *p25* genes northern blots were hybridized with probe IV (Fig. 6.1.d). At least four major transcripts were observed from 10 to 24 h p.i. (Fig. 6.2c). The two larger transcripts, approximately 800 and 650 bases in size, are in agreement with the sizes predicted from sequence analysis for *p25* and *p18* transcripts, respectively (Fig 6.2c; Table 6.1). The smaller transcripts, 200 and 300 bases respectively, might originate from start sites located within the *p25* and *p18* coding regions. These transcripts could not be related to any open reading frame.

Probe V (Fig. 6.1d), hybridized to a transcript of approximately 900 bases present between 12 and 48 h p.i. (Fig. 6.2d), resembling the predicted size of the *vp33* transcript (Table 6.1). Overexposure of the blot revealed that this transcript was already present at 8 h p.i. A highly expressed transcript of approximately 300 bases was also detected between 12 and 48 h p.i. The origin of this transcript is unclear but *vp33* internal start sites might activate its transcription.

The coding sequence of the putative SeMNPV-*lef4* gene encompasses 1397 bases (Table 6.1). Probe VI (Fig. 6.1d), hybridized to 2.1 kb and 1.2 kb transcripts produced between 8 and 48 h p.i and to several smaller transcripts (approximately 500 bases in size) present from 10 until 48 h p.i. (Fig. 6.2e). The large transcripts of 5.2 and 6.8 kb were only present very late in the infection process at 24 and 48 h p.i. and are possible readthroughs. The size of the major 2.1 kb transcript is in good agreement with the predicted size of *lef4* transcripts (Table 6.1). Primer extension and northern blot analysis using single stranded riboprobes might reveal consensus transcriptional start sites not only for SeMNPV but also for AcMNPV and OpMNPV-*lef4* expression (Passarelli and Miller, 1993; Ahrens *et al.*, 1997).

Probe VII (Fig. 6.1d) hybridized to 1.0 kb, 1.2 kb, 2.5 kb and 5.0 kb transcripts (Fig. 6.2f). During AcMNPV infection *vp39* is expressed as a 2.2 kb transcript from 6 h p.i. onwards (Thiem and Miller, 1989). During OpMNPV infections, the *vp39* gene is expressed as a 2.6 kb transcript from 24 to 48 h p.i. (Blissard *et al.*, 1989). A 1.0 kb transcript is present from 8 h p.i. and could overlap with the SeMNPV-*vp39* transcript as calculated from sequence analysis, but it is relatively short as compared to the OpMNPV and AcMNPV-*vp39* transcripts. The transcript of 2.5 kb in length is in much better agreement with the length of the OpMNPV and AcMNPV-*vp39* transcripts and is in good agreement with the OpMNPV-*vp39* time course of expression (Blissard *et al.*, 1989).

Since during AcMNPV infection the transcript of the *cg30* gene starts within the coding sequence of *vp39* (Thiem and Miller, 1989b) it is possible that the 1.0 kb transcript,

detected by our DNA probe (Probe VII), originates from an internal start site within the SeMNPV-*cg30* gene assuming this is located downstream of SeMNPV-*vp39*. The AcMNPV-

ORF	Left	Right	Dir	aa	MW kDA	Trans	TATA	PolyA	% Id Ac	% Id Op
p6.9	112	337	>>	75	8.8	L55	15	381	59	62
lef5	338	1176	<<	279	33	nf	nf	-	54	47
38kDa	1071	1940	>>	290	34	L1003	906	1973	46	43
XC12	2004	2489	>>	161	19	L1985	nf	2519		
p19	2578	3090	<<	170	20	L3207 L3235	nf	2281	50	48
p143	3056	6722	>>	1221	143	E2994	3044	6733	43	40
p25	6808	7457	<<	216	24	L7531	7559	6766	51	45
p18	7455	7928	<<	157	18	L8070	8075	-	47	45
vp33	7940	8690	>>	250	31	L7758 L7775	nf	8694	55	55
lef4	8920	10317	<<	462	42	nf	10451	8863	50	41
vp39	10316	11294	>>	326	37	L10291 L10244	ns	10260	42	46

Table 6.1.
The selected ORFs are listed according to their relative position in the SeMNPV *Xba*I-C sequence. The columns left and right define the ends of the ORF irrespective of its orientation. The direction of the transcripts (Dir) that could express the ORF is indicated by arrows. The number of amino acids and the predicted molecular weight of the protein encoded by the ORF is indicated in columns aa and MW, respectively. The nucleotide position of an early (E) or late (L) transcriptional start site, a consensus TATA-box or a poly adenylation signal is indicated in the columns Trans, TATA and Poly A, respectively. The percentage of amino acid sequence identity of the protein encoded by the SeMNPV ORFs to their homologs in the genomes of AcMNPV and OpMNPV is listed in columns %Id Ac and %Id Op, respectively. Ns and nf represent not sequenced and not found respectively.

cg30 gene is highly-expressed from 2 h p.i. and reaches its maximum concentration around 12 h p.i. even in the presence of aphidicolin and cycloheximide (Thiem and Miller, 1989b). Sequence analysis of the left hand end of SeMNPV-*Xba*I-K (Fig. 6.1) revealed an ORF with strong homology to AcMNPV-*cg30*. A putative baculovirus early promoter element (CAGT) was found approximately 260 bp upstream of the putative translational start site of the SeMNPV-*cg30* gene. This CAGT motif is located at nt 11212 of the SeMNPV-*Xba*I-C sequence, suggesting that our probe had relatively short overlap of 90 nt with the *cg30* leader sequence. The short overlap might explain why during the initial stages of the infection process, when only low concentrations of the transcript are present, no SeMNPV-*cg30* messengers could be detected due to limited hybridization efficiency. These observations

suggest that the SeMNPV-*vp39* gene will be probably expressed as a 2.5 kb transcript between 12 and 48 h p.i. The 1.2 kb transcript might be the same as detected by probe VI.

Based on the results from these expression studies a transcriptional map of the SeMNPV *Xba*I-C region was compiled (Fig. 6.1e). It seems that all identified ORFs are transcriptionally active. Overall this map is highly similar to the map that can be compiled from the transcriptional analysis of the corresponding region in AcMNPV-*Eco*RI-D (Lu and Carstens, 1992) and the AcMNPV-*p6.9* gene (Wilson *et al.*, 1987). It should be noted, however, that primer extension analysis, northern blot analysis using strand-specific riboprobes should be used to confirm the origin of the proposed transcripts and to eliminate the occurrence of small transcripts that cannot be related to any open reading frame.

Discussion

The genetic organization and transcription profile of the SeMNPV-*Xba*I-C fragment showed a considerable degree of similarity to the AcMNPV-*p6.9-vp39* (Ayres *et al.*, 1994) and/or OpMNPV-*p6.9-vp39* (Ahrens *et al.*, 1997) region (Fig. 6.3). However, a number of unique features were noted. Firstly, the ORF encoding a hypothethical AcMNPV p24 protein was not identified in SeMNPV *Xba*I-C. ORF98 of OpMNPV (Ahrens *et al.*, 1997), located between *p19* and *p38* was not present in the SeMNPV *Xba*I-C sequence. In AcMNPV no OpMNPV-ORF98 homolog was present either (Ayres *et al.*, 1994). Secondly, the ORF encoding the putative AcMNPV-*p18* homolog (Lu and Carstens, 1991, 1992) does, in contrast to the situation in AcMNPV, not overlap with the SeMNPV-*p143* (Fig. 6.3). Thirdly, in the genome of SeMNPV a putative *hr*-like sequence element (R. Broer, J.G.M. Heldens, E.A. van Strien, D.Zuidema and J.M. Vlak, unpublished results) is located in the intergenic region between *vp33* and *lef4*, the position where in the genomes of AcMNPV and OpMNPV the putative *p24* gene is situated (Fig. 6.3). In the genomes of the latter viruses *hr*-like sequence could be identified about 5 kb upstream of this position (Ayres *et al.*, 1994; Ahrens *et al.*, 1997). Fourthly, all SeMNPV genes had an inverted genomic orientation with respect to the orientation of their homologues in the genomes of AcMNPV (Ayres *et al.*, 1994) and OpMNPV (Ahrens *et al.*, 1997). This suggested that the SeMNPV genome is colinear with the genomes of AcMNPV and OpMNPV in the *p143* locus but in an inverted orientation (Fig. 6.3).

Terminal sequence analysis of the *Xba*I-fragments flanking SeMNPV-*Xba*I-C (*Xba*I-P, *Xba*I-K,) revealed high similarities to *p40* and *p48* for *Xba*I-P, *cg30* and ORF82 for *Xba*I-K. Also the distance between these genes in the genome of SeMNPV is similar to the distance

between their homologues in the genomes of AcMNPV and OpMNPV. This further supports the notion that the genetic organization of the SeMNPV genome may be colinear with AcMNPV and OpMNPV over a much larger area than only the 11.3 kb of the SeMNPV-*Xba*I-C fragment (Fig. 6.3). Partial nucleotide sequence data from *Heliothis armigera* SNPV (X. Chen and J.M. Vlak, unpublished results), *Buzura suppressaria* SNPV (Z.H. Hu and J.M. Vlak, unpublished results), *Lymantria dispar* MNPV (LdMNPV) (G.F. Rohrmann, unpublished data) and *Cryptophlebia leucotreta* GV (Jehle and Backhaus, 1994), suggest that the genetic organization of the *vp39-lef5* gene cluster is not only conserved in AcMNPV, OpMNPV and SeMNPV but in many more if not all baculoviruses.

The presence in baculovirus genomes of a large genetically conserved part with respect to gene content and gene expression strategy is in sharp contrast to the hypervariable *polyhedrin-p10* region. The latter showed a pattern of limited similarity in genetic organization among SeMNPV, SpliMNPV, AcMNPV, LdMNPV and OpMNPV (E.A. van Strien, 1997; Faktor *et al.*, 1997; Ayres *et al.*, 1994; Rohrmann, unpublished results; Ahrens *et al.*, 1997). The orientation of this hypervariable region is however identical to the orientation in AcMNPV and OpMNPV. This would imply that for instance *p143* would be a better marker to set the orientation and zero point of baculovirus physical and genetic maps instead of the hypervariable *polyhedrin-p10* locus. It would also facilitate the localization of a number of genes in other less well-characterized baculoviruses as soon as one of the homologs of the AcMNPV genes of the *p6.9-vp39* cluster has been found. As a consequence SeMNPV should then show an inversed *polyhedrin-p10* orientation rather than an inverted *p6.9-p39* region consisting of at least *Xba*I-C, *Xba*I-K and *Xba*I-P.

The availablitity of the complete nucleotide sequences of the poxviruses *Molluscum contagiosum* (MCV) and *Vaccinia* (VV) allowed comparison of their genetic organization Gompels *et al.*, 1995; Davison and McGeoch, 1995; Senkevic *et al.*, 1996). It was observed that the central part of their genomes was conserved in gene order, whereas the termini displayed a more diverged genetic organization (Senkevich *et al.*, 1996). Comparison of the genetic organization in alpha, beta and gamma herpesviruses also showed that conserved blocks are flanked by more diversed sequences. In general, herpesviruses contain central conserved areas and more diverged and rearranged termini (Gompels *et al.*, 1995).

The presence of a genetically conserved region and other more diverged regions in the baculovirus genome, is so far similar with the situation in herpes and poxvirus genomes. In herpesvirus genomes it seems that extensive nucleotide substitutions, gene rearrangements, gene duplications and gene captures occur throughout herpesviruses genomes most frequently at their termini. During baculovirus evolution such rearrangements, duplications and captures might have occurred as well. The arrangement of genes in the *p6.9-lef5* region may not have

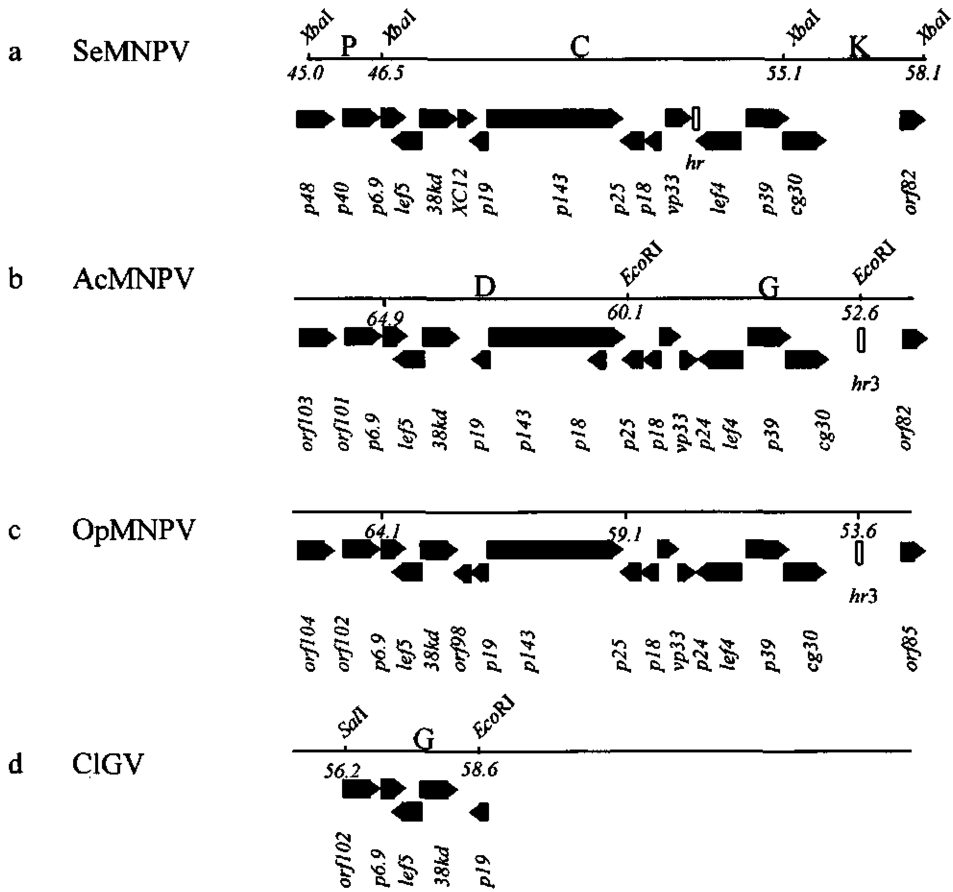

Figure 6.3.
Comparison of the gene organization in the *p6.9-p39* gene region of SeMNPV, AcMNPV, OpMNPV and ClGV. The exact position of the region in the different genomes is indicated in map units.

occured recently in baculovirus evolution, since the genetic organization and gene expression profile is so well conserved. The mechanisms underlaying herpes virus rearrangements (reviewed by Davison and McGeoch, 1995) may be similar in baculovirus genomes. Whether the numerous *hr*s interspersed in the baculovirus genomes play, besides their role as transcriptional enhancers and as putative origins of DNA replication, a role in this process remains to be investigated. A theta mode of DNA replication mechanism during the first rounds genome amplification (bidirectional using multiple origins at the same time) as postulated by Kool *et al.* (1995) would allow a rearrangement mechanism in which genes or

gene clusters are readily inversed easily upon the coiling of the produced progeny DNA. The further this theta replication form has proceeded the more chance there is that a rearrangement event occurs.

The gene organization of baculovirus genomes may provide supplementary phylogenetic information. Gene order has been used to determine herpesvirus phylogeny (Hannenhalli *et al.*, 1995). The method relies on the presence of homologous genes throughout the virus species. In contrast to herpesviridae, most baculoviruses contain a similar set of genes with relatively high amino acid sequence indentity. If all baculoviruses contain a conserved part of the genome as hypothesized here, the divergence in the genetic organization of the *polyhedrin-p10* locus would be a measure for the evolutionary relatedness of baculoviruses. Complete sequence analysis of the genome of not only SeMNPV but many other baculoviruses and comparison of their gene organization, in especially the *polyhedrin-p10* and *p143 loci*, would verify this hypothesis.

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The nucleotide sequence data reported in this paper will appear in the EMBL nucleotide sequence database under accession number XXXXXXX

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Chapter 7

GENERAL DISCUSSION[6]

Introduction

Baculoviruses are widely used for the high level expression of foreign proteins in insect cells (Luckow and Summers, 1988). The *polyhedrin* and *p10* genes are abundantly expressed very late in the infection process and their promoters are used to drive the expression of the foreign genes. The expressed proteins are normally processed as in higher eukaryotic cells (only glycosylation occurs slightly different) and they are often biologically active (Jarvis *et al.*, 1997). Furthermore, recombinant baculoviruses with increased insecticidal properties have been produced for use in biological pest management programs (Bonning and Hammock, 1996, for review). Recombinant baculoviruses are not only engineered for biotechnological purposes but also for fundamental research to determine the role of specific viral gene products in the infection or replication process (null-mutants). Insight into the molecular genetic characteristics of SeMNPV that underlie the biology of a SeMNPV infection, is a prerequisite towards the successful improvement of its bio-insecticidal properties by genetic engineering. The understanding of key steps in this infection process such as virus entry, gene expression, genome replication or virion assembly, is required prior to the development and application of recombination strategies. Previous (Smits, 1987) and concomitant (van Strien, 1997) research on SeMNPV focused on its application as safe bio-insecticide and on the characterization of selected parts of its genome, respectively. The research described in this thesis aimed at the unravelling of the SeMNPV DNA replication process *in vitro* and hence at the identification of *cis*- and *trans*-acting elements involved in DNA replication, and at their capability to act in the replisomes of heterologous baculoviruses. In this chapter the genetic organization of the SeMNPV genome,

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SeMNPV DNA replication and future perspectives regarding genetic modification of the SeMNPV genome, are discussed.

Genetic organization of the SeMNPV genome

To date approximately 71 kb or 52% of the 134 kb SeMNPV genome have been sequenced and analyzed (Fig. 7.1). Within these 71 kb of nucleotide sequence, 68 open reading frames with considerable homology to genes from other baculoviruses have been identified (Fig. 7.1). Assuming that baculovirus genomes encode approximately 150 functional genes (Ayres *et al.*, 1994; Ahrens *et al.*, 1997), about 45% of the genes is identified. The amino acid identity among the different gene products varies considerably between SeMNPV, AcMNPV and OpMNPV. Relative to AcMNPV and OpMNPV the most conserved SeMNPV gene products include v-ubiquitin (80%) (van Strien *et al.*, 1996) and polyhedrin (85%) (van Strien *et al.*, 1992). Conserved to only a (very) limited extent are, on the other hand, SeMNPV-xj462 (van Strien, 1997), p10 (van Strien, 1997) and p143 (Heldens *et al.*, 1997). Phylogenetic analysis using parsimony on polyhedrin or EGT clustered SeMNPV apart from *Lymantria dispar* MNPV (LdMNPV), *Spodoptera litoralis* NPV (SpliNPV) and from the AcMNPV/OpMNPV clade (Zanotto *et al.*, 1993; Cowan *et al.*, 1994; Hu *et al.*, 1997), indicating that SeMNPV is distantly related to AcMNPV and OpMNPV.

This distant genetic relationship between SeMNPV, AcMNPV and OpMNPV is also reflected by the deviate gene order and by the genetic distance between its *polyhedrin* (van Strien *et al.*, 1992) and *p10* (Zuidema *et al.*, 1993) genes (van Strien *et al.*, 1997). The physical distance between these genes is in the SeMNPV genome relatively small (11 kb), compared to the distance between these genes in the genomes of AcMNPV (19kb) and OpMNPV (22 kb). The gene content of a 20 kb region containing SeMNPV-*p10-polyhedrin* gene cluster is different not only among SeMNPV, AcMNPV and OpMNPV, but also in many other baculoviruses such as LdMNPV (Rohrmann, personal communication) or SpliNPV (Faktor *et al.*, 1997). Remarkably, the homologs of the immediate early genes *ie2* (Carson *et al.*, 1991; Theilmann and Stewart, 1992) and *ie3* (Krappa and Knebel-Mörsdorf, 1991; Ahrens *et al.*, 1997) (AcMNPV ORF151, ORF153; OpMNPV ORF151, ORF152) are absent in the 20 kb region of the SeMNPV genome whereas the *ribonucleotide reductase* large subunit gene (not in AcMNPV; in OpMNPV ORF32) is present (van Strien *et al.*, 1997). It has been shown that *ie2* and *ie3* have auxiliary functions in baculovirus DNA replication (Kool *et al.*, 1995 for review; Lu *et al.*, 1997). If IE2 and IE3 represent essential

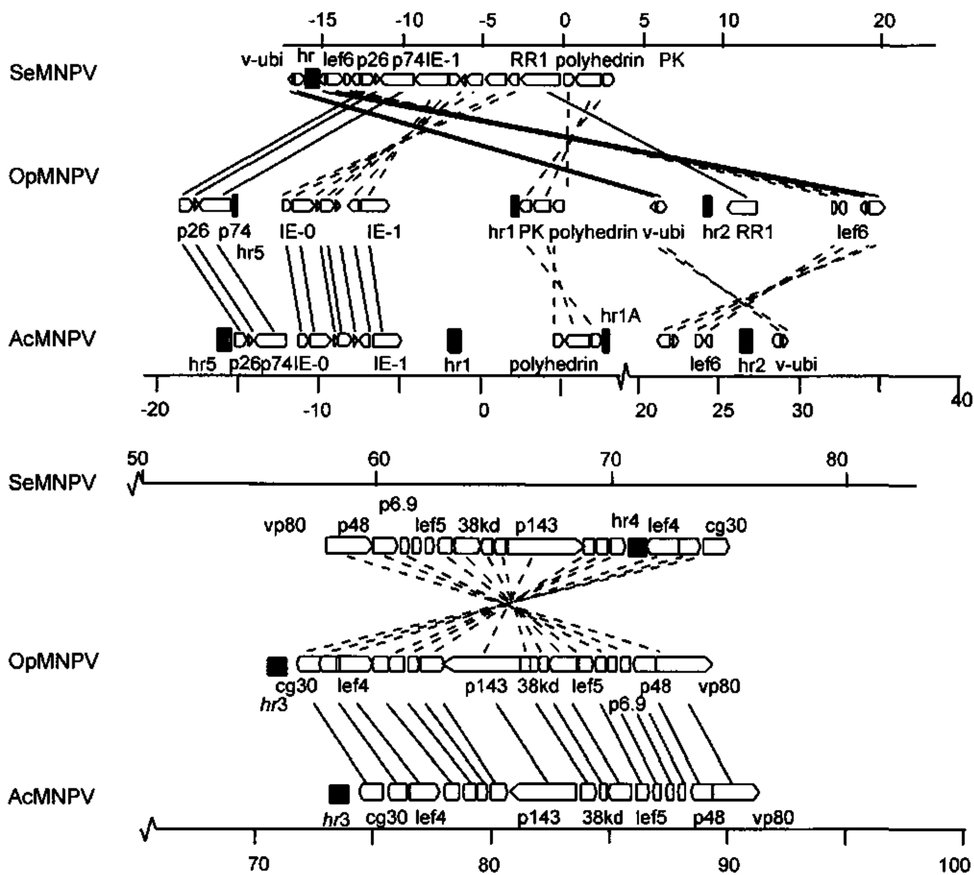


Figure 7.1.

Physical map and partial genetic organization of the SeMNPV genome. Ac-xx numbers refer to the ORF numbers in the AcMNPV genome.

transactivators in the baculovirus gene expression cascade or in the DNA replication process, their SeMNPV homologs must be located elsewhere in the genome. Among other genes in the *polyhedrin-p10* locus that are absent from AcMNPV, but present in OpMNPV, are ribonucleotide reductase and dUTPase (Ahrens *et al.*, 1997) (Fig. 7.2).

The rather diverged gene order in the baculovirus *p10-polyhedrin* locus is in sharp contrast to the conserved gene order in the *p143* locus of many baculoviruses including SeMNPV, AcMNPV, OpMNPV and *Cryptophlebia leucotreta* GV (ClGV) (Jehle and Backhaus, 1994; Heldens *et al.*, 1997) (Fig. 7.2). Further sequence analysis from the *p143*

region suggests that the SeMNPV genome is colinear to the genomes of AcMNPV and OpMNPV over a much longer stretch (Fig. 7.1). The orientation of this colinear stretch is in SeMNPV however antigenomic. Only the *iap*2-Ac-76 region containing the genes encoding DNA polymerase and LEF3 apparently has undergone a double inversion, suggesting that the Ac-63 and Ac-64 homologs are located close to the non-*hr ori* (Fig. 7.1). Further analysis is required to determine the exact position of the individual genes in this region and to locate the inversion sites.

It has been suggested that the alternation of conserved and diverged gene blocks in baculovirus genomes is somewhat comparable to the situation in the genomes of other large eukaryotic DNA viruses such as herpesviruses (Chapter 6 and Heldens *et al.*, 1998; van Strien *et al.*, 1997). Complete sequence analysis of a number of baculovirus genomes including the SeMNPV genome to determine the full gene content and gene order of this virus is essential to consolidate this view. In line with the herpes and poxviruses, the alternation and actual content of these conserved and diverged gene blocks might serve as (phylogenetic) markers in the classification of baculoviruses. Gene rearrangements, however, in blocks or as individual genes occur more frequently throughout herpesvirus genomes than in baculovirus genomes (Gompels *et al.*, 1995; Davison and McGeoch, 1995; Senkevic *et al.*, 1996; Ayres *et al.*, 1994; Ahrens *et al.*, 1997). Comparison of the gene order in SeMNPV to AcMNPV and OpMNPV, and a number of other baculoviruses such as LdMNPV, SpliNPV, SNPVs and GVs could provide more solid genetic evidence for the distant relatedness of this virus to other baculoviruses, than has been provided by single gene phylogenetic analyses.

Host range determination

Insight into the molecular principles of host range determination of baculoviruses is desired for environmentally safe application of these viruses as insecticides in the field. To date little is known about the molecular mechanisms that determine baculovirus host range. Single amino acid changes in AcMNPV-p143 resulted in an expanded host range of AcMNPV towards BmN cells (Kondo and Maeda, 1991; Croizier *et al.*, 1994). Only a few unique baculovirus host-range determinants have been identified. Mixed infection of Ld-652Y cells with AcMNPV and LdMNPV led to generation of a recombinant LdMNPV-*hrf*1. The *hrf*1 is a gene capable of expanding the host range of AcMNPV towards Ld-652Y cells (Thiem *et al.*, 1996; Du and Thiem, 1997). Interestingly OpMNPV, which is routinely propagated in Ld652Y cells, does not encode an *hrf*1 homolog with substantial nucleotide sequence homology to LdMNPV-*hrf*1 (Thiem *et al.*, 1996). The factor promotes AcMNPV

replication in the non-permissive cell lines as Ld652Y cells (Du *et al.*, 1997). A host cell specific factor (*hcf1*) from AcMNPV appeared to have a function in the DNA replication process of the virus in TN-368 cells and *Trichoplusia ni* larvae. The factor was not required for AcMNPV replication in *S. frugiperda* larvae or Sf-AE-21 cells (Lu and Miller, 1995; Lu and Miller, 1996). It is very likely that SeMNPV also encodes factors that direct its specificity for only *S. exigua*. Whether the host range specificity of SeMNPV for only *S. exigua* cells or insects can be correlated to functional homologs of one or a few of these genes remains to be investigated.

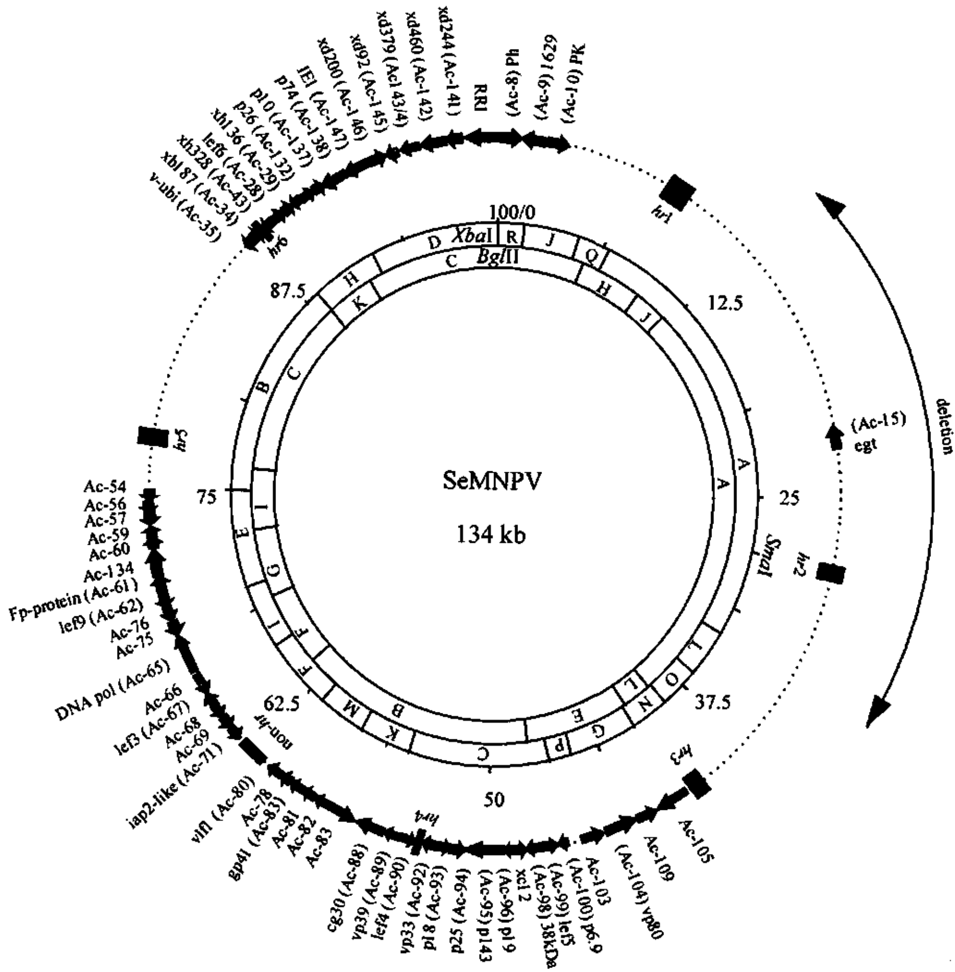


Figure 7.2.
Comparison between the genetic organization of the SeMNPV, OpMNPV and AcMNPV genomes in the *polyhedrin-p10* and *helicase* loci.

DNA replication

The baculovirus genome replication mechanism, a "rolling circle", a "theta-like" bipartite mechanism or a combination of both as has been suggested by Kool *et al.* (1995) is another area of speculation. The AcMNPV and OpMNPV virus encoded factors involved in DNA replication have all been identified. Among these virus encoded factors are *cis*- and *trans*-acting elements (Lu *et al.*, 1997 for review). The involvement of cellular encoded factors in the replication process cannot be excluded. In AcMNPV late gene transcription clearly cellular encoded proteins are involved (Jain and Hasnain, 1996). It has been suggested in this perspective that DNA replication and gene transcription are closely related processes in eukaryotic cells (DePamphilis, 1993).

Two types of *cis*-acting elements can be distinguished so far in baculovirus DNA replication, i.e. homologous region-like (*hr*) and non-*hr*. The *hr*s have a double function, as transcriptional enhancers and as origins of DNA replication. In SeMNPV (Chapter 3), but also in AcMNPV and OpMNPV *hr*s (Kool *et al.*, 1995; Lu *et al.*, 1997 for review) and in many more baculoviruses, *hr*s are characterized by an array of palindromic repeats interspersed with short direct repeats. An important feature of baculovirus *hr* elements is the presence of mismatches at conserved positions in these palindromes. These mismatches may mediate most likely the formation of subtle tertiary structures in the *hr*-motif which might be essential for optimal functioning as origin of DNA replication or as enhancer of transcription. The funtioning of *hr*s probably involves the acquisition of *trans*-acting elements, transactivators or other factors involved in gene transcription. For SeMNPV and AcMNPV *hr*s one palindromic repeat was sufficient for the support of transcriptional enhancement and/or transient DNA replication *in vitro* (Leisy *et al.*, 1993; Broer *et al.*, 1997). Although little or no sequence homology between *hr*s exists, some can be replicated by heterologous viruses whereas others cannot (Broer *et al.*, 1997). Apparently the actual sequence or tertiary structure of the *hr* is to a certain extent involved in the direction of the transcriptional machinery.

Crucial in the functioning of *hr*s as transcriptional enhancer is their capability to bind IE1. SeMNPV-*hr*1 is able to enhance CAT expression from AcMNPV-39K-CAT constructs in the presence of SeMNPV and AcMNPV-IE1 (Theilmann and van Strien, unpublished data). In AcMNPV-*hr*1a and *hr*5 the IE1 binding motif has been mapped in the central 42 bp region of a palindromic repeat (Rodems and Friesen, 1995; Leisy *et al.*, 1995; Guarino and Dong, 1994). A putative IE1 binding motif, 5'-ACBYGTAA-3', located within these 42 bp, could be deduced from sequence alignment of the AcMNPV *hr*s (Rasmussen *et al.*, 1996). Although this consensus could not be found in SeMNPV-*hr*s, the motif 5'-AAACGAAA-3'

was present in all identified SeMNPV palindromes. DNase protection and gel mobility shift assays using various radioactive labelled segments from a SeMNPV-*hr* related palindrome might reveal whether this motif is the SeMNPV-IE1 binding site and furthermore whether AcMNPV-IE1 is able to bind the same motif.

Although the non-*hr ori* of SeMNPV (Chapter 4), AcMNPV (Kool *et al.*, 1994) and OpMNPV (Pearson *et al.*, 1993) resembles consensus eukaryotic origins of DNA replication (DePamphilis, 1993) its role in baculovirus DNA replication is still enigmatic. Besides direct and indirect repeats these elements contain a number of other sequence motifs, such as putative eukaryotic transcription factor binding sites. Whether the baculovirus *trans*-acting elements are able to bind to the non-*hr* elements or whether other factors able to bind the non-*hr* element exist, in infected insect cells has not been established.

The non-*hr* sequence of SeMNPV is, unlike AcMNPV and OpMNPV, located in a region which does not contain any ORF. Multiple passaging of AcMNPV in bioreactors rendered eventually defective interfering viruses (DIs) with large genomic deletions (Kool *et al.*, 1991; Lee and Krell, 1992). Inside the nucleus of the infected insect cells even smaller particles were found that consist mainly of reiterated non-*hr* like sequences (Lee and Krell, 1994). Serial passaging of SeMNPV-wt and non-*hr* deletion mutants in insect cell lines would reveal whether Dis arise from SeMNPV. Analysis of the *de novo* synthesized genomes would elucidate if non-*hr* related sequences accumulate in their genomes and whether this sequence itself plays a critical role in the occurrence of DIs. A SeMNPV genome which can be stably maintained during replication in both *S. exigua* cell lines, *S. exigua* larvae and in bioreactors is required for a successful, large scale production of this virus as bio-insecticide or expression vector.

In the genome of SeMNPV two *trans*-acting DNA replication factors have been identified and characterized, *ie1* (van Strien *et al.*, 1997) and *helicase* (Chapter 5). The sequence analysis of the complete SeMNPV genome should reveal all other *trans*-acting DNA replication factors, which may include the late expression factors (LEF) 1, 2 and 3, DNA polymerase, IE2, IE3 and possibly an inhibitor of apoptosis. The inhibitor of apoptosis of SeMNPV might be either a functional P35 homolog as is the case for AcMNPV or a gene belonging to the *iap* gene family as is the case in OpMNPV and *Cydia pomonella* GV (Crook *et al.*, 1993).

The position of the SeMNPV *DNA polymerase* and *lef3* genes has already been proposed (Fig. 7.1). Based on sequence alignments it has been proposed that *lef1*, *lef2* and *lef3* encode the baculovirus homologs of Herpes Simplex Virus 1 (HSV) UL8 a primase-associated protein, HSV UL42 a putative DNA polymerase processivity factor, and HSV UL52 a putative primase, respectively (Kool, 1994). Biochemical assays would elucidate the exact role of the *trans*-acting DNA replication factors in the baculovirus DNA

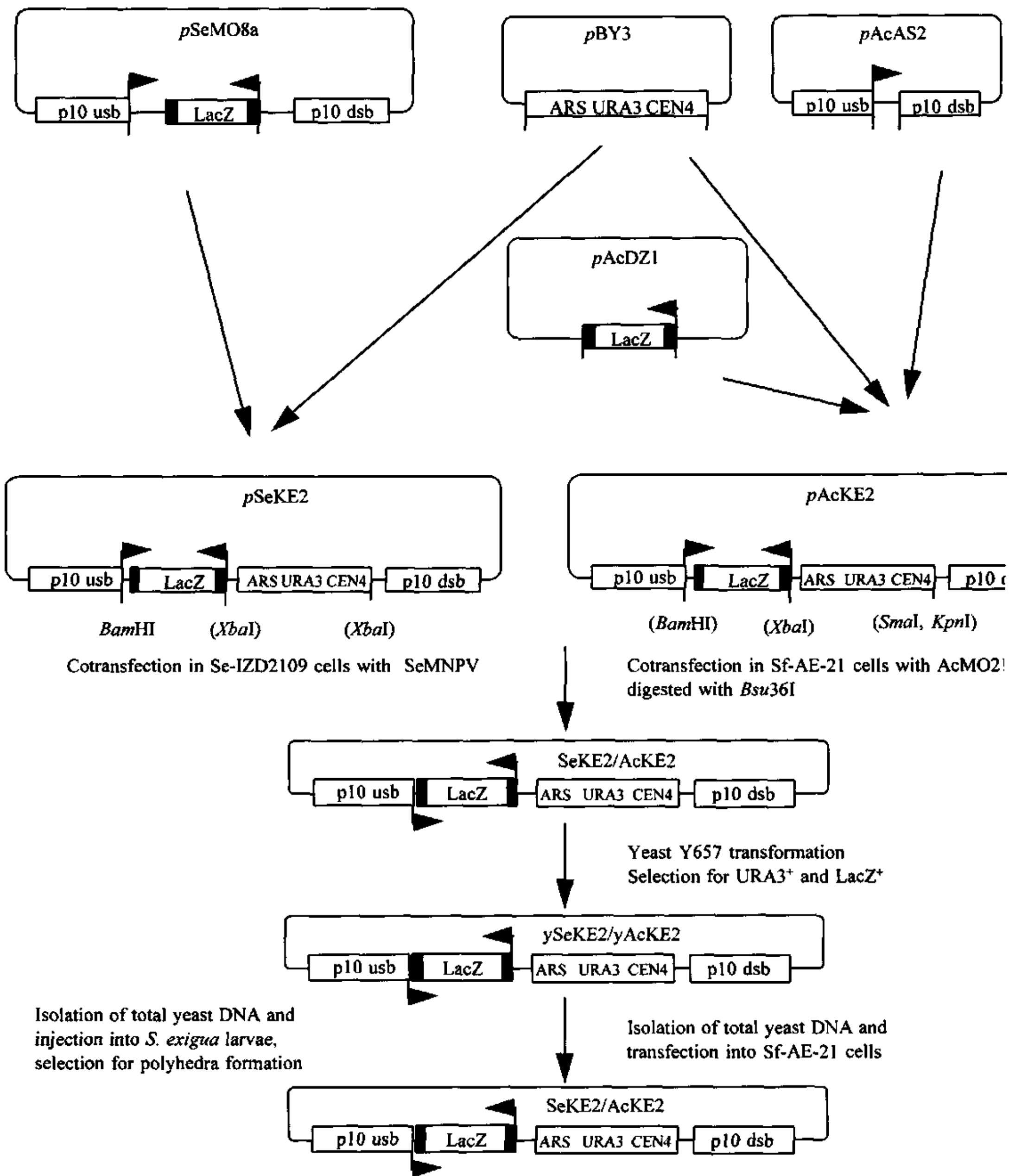


Figure 7.3.
Generation of baculovirus recombinant yAcKE2/ySeKE2 containing an ARS-CEN-URA3-LacZ cassette. usb = upstream border, dsb = downstream border

replication process. Immunoprecipitation of AcMNPV helicase from infected insect cells suggested already that this protein is active as a dimer or hexamer and strongly interacts with a multimeric protein (Carstens *et al.*, 1997). Circumstantial evidence for its activity as helicase, i.e. its capacity to bind *hr*-like sequences (*ori*s), and the necessity of intact helicase motifs for efficient DNA repliation in transient DNA replication assays, has been provided (Laufs *et al.*, 1997; Liu and Carstens, 1996). The construction of mutant viruses with specific alterations in the coding sequence of the *trans*-acting DNA replication genes would further address the functionality of specific domains of these proteins and their relative contribution in baculovirus DNA replication. Recent studies suggest that AcMNPV LEF1 interacts withLEF2 and that LEF3 is present *in vivo* as a homotrimer (Evans *et al.*, 1997; Evans and Rohrmann, 1997), which interacts with helicase (Evans *et al.*, 1997). Based on gel-mobility shift assays, Hang *et al.* (1995) showed that *lef3* encodes a single stranded DNA binding protein, rather than a UL52-like primase as sugested by Kool *et al.* (1995). Since many eukaryotic DNA helicases interact directly with a ssDNA binding protein (Lohman and Bjornson, 1996 for review) it can be hypothesized that the protein co-precipitating with AcMNPV helicase is LEF3.

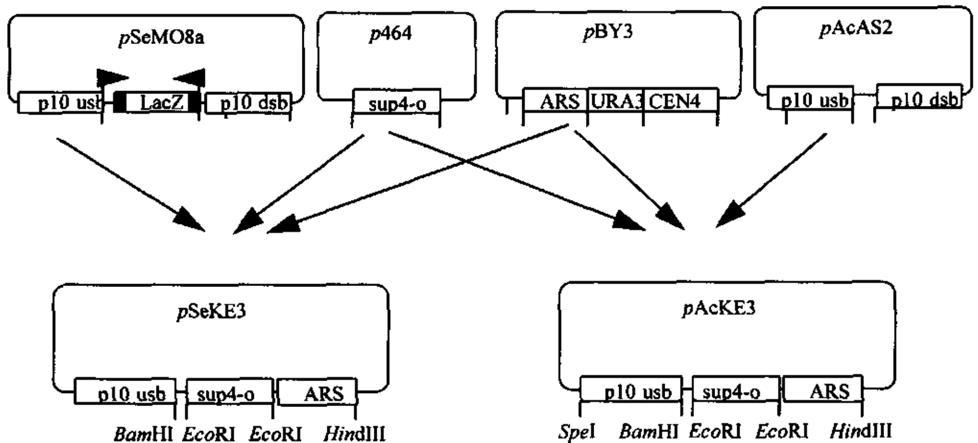
Interactions between replication origins on one hand and *trans*-acting elements on the other is essential to form replisomes. In contrast to substitutions among AcMNPV/SeMNPV *trans*-acting elements, AcMNPV and OpMNPV could functionally substitute each others DNA polymerase in transient DNA replication assays. AcMNPV helicases could substitute OpMNPV helicase, but OpMNPV could not substitute AcMNPV helicase (Ahrens and Rohrmann, 1996). A similar incompatiblity was observed when OpMNPV-*hrs* were assayed for DNA replication in AcMNPV infected cells and when AcMNPV-*hrs* were assayed in OpMNPV infected cells (Chapter 3). Apparently *trans*-acting DNA replication factors from closely related baculoviruses cannot necessarily substitute each other. These observations suggest once more that DNA replication is a highly virus-specific process that requires delicate interaction between its different components. The yeast two-hybrid system would allow the study whether the (in)ability of heterologous *trans*-acting elements to act in DNA replications assays is due to their (in)ability to interact with their respective heterologous elements to form a functional replisome or is due to other unkown factor(s).

Genetic modification of SeMNPV

Commonly, foreign genes or specific site directed mutations are introduced into the baculovirus genome by homologous recombination in insect cells between parental

baculovirus DNA and a transfer plasmid containing the foreign gene or a mutated gene under control of a baculovirus promoter flanked by baculovirus sequences. Homologous recombination in insect cells can not always be applied. Firstly, only genes that are non-essential for the infection process can be deleted or mutated, and secondly for many baculoviruses no permissive cell lines are available. Since both Se-UCR1 (Gelernter and Federici, 1986) and Se-IZD2109 (Colbers and Vlak, unpublished results) cell lines generate SeMNPV-mutants that lack virulence *in vivo* (Heldens *et al.*, 1996), recombinant viruses cannot be constructed using this conventional approach. Therefore two alternative recombination strategies are proposed in this chapter.

The formation of polyhedra is pivotal for the infection and production of recombinant proteins in insect larvae, in particular for the production of genetically improved baculoviruses in insect pest control. For biological containment *p10*-minus baculoviruses



- Transformation of pSeKE3 or pAcKE3 into ySeKE2 or yACKE2.
- Selection for URA3$^+$, ADE$^+$ and LacZ$^-$ yeast clones.
- Isolation of total yeast DNA and injection in S.exigua larvae or transfection in Sf-AE-21 cells, repectively.
- Selection for polyhedra and diseased larvae

Figure 7.4.
Cloning strategy of yeast p10 transfer vectors pAcKE3 and pSeKE3

could be generated, since the release of polyhedra from the nuclei is impaired which may result in limited spread of the virus in the environment (Van Oers *et al.*, 1993). The maintenance and replication of the baculovirus genome in the yeast *Saccharomyces cerevisiae* or in an extrachromosomal state in bacteria would allow the genetic modification of baculovirus genomes without the use of the insect cell lines. A general strategy would be the construction of a SeMNPV variant that can be stably maintained and genetically modified via Yeast Artificial Chromosome (YAC) or Yeast Centromere plasmid (YCp) technology (Guthrie and Fink, 1991). To test the feasibility of this approach the technology was first applied on the AcMNPV genome (Patel *et al.*, 1992; Heldens *et al.*, 1997b) (Fig. 7.3 and 7.4). In brief, a yeast autonomous replicating sequence (ARS), a centromere (CEN) and a selectable marker (URA3) can be inserted in the *polyhedrin* or *p10* loci to maintain the baculovirus genome in the yeast. Recombinants can be generated via homologous recombination in *S. cerevisiae* between transfer vectors containing the gene of interest under control of the polyhedrin or p10 promoter, respectively, the yeast ARS sequences, and the baculovirus DNA thereby (counter)selecting for the presence of the sup4-o gene (Patel *et al.*, 1992) and/or ß-galactosidase expression (Heldens *et al.*, 1997) (Fig. 7.4).

An alternative strategy to maintain SeMNPV in yeast aims at the exploitation of the single *Sma*I restriction site present in the SeMNPV genome (Fig. 7.1). Both YAC, to maintain the SeMNPV genome in a linear state in the yeast cells and YCp approaches can be considered (Fig. 7.5). The SeMNPV 5.6 kb *Bam*HI-*Pst*I fragment (mu 27.7-32.2) containing both this single *Sma*I site, has been cloned and sequenced. The *Sma*I site appeared to be located in an open reading frame with considerable homology to AcMNPV-ORF119 (Ayres *et al.*, 1994). If this ORF is non-essential for replication *in vivo* and *in vitro*, the system can be used to introduce foreign genes to improve the insecticidal properties of the virus (Fig 7.3; Fig. 7.4; Fig. 7.5). Environmentally safe baculovirus based insecticides require the absence of heterologous eukaryotic origins of DNA replication to prevent potential spread of the virus into other insect species or organisms via their ability to replicate in field persistent yeasts. To delete the ARS-CEN-URA cassette from and to recircularize the SeMNPV-YAC or YCp a second, or an *in vivo*, recombination strategy was developed, using marker genes to monitor this recombination event (Fig. 7.4; Fig. 7.5).

Epilogue

S. exigua has a devastating effect on the yields of agriculturally important crops in green houses and in the tropics. The use of this virus as a potent biological insecticide is a safe alternative for environmentally hazardous chemicals. The application of SeMNPV for

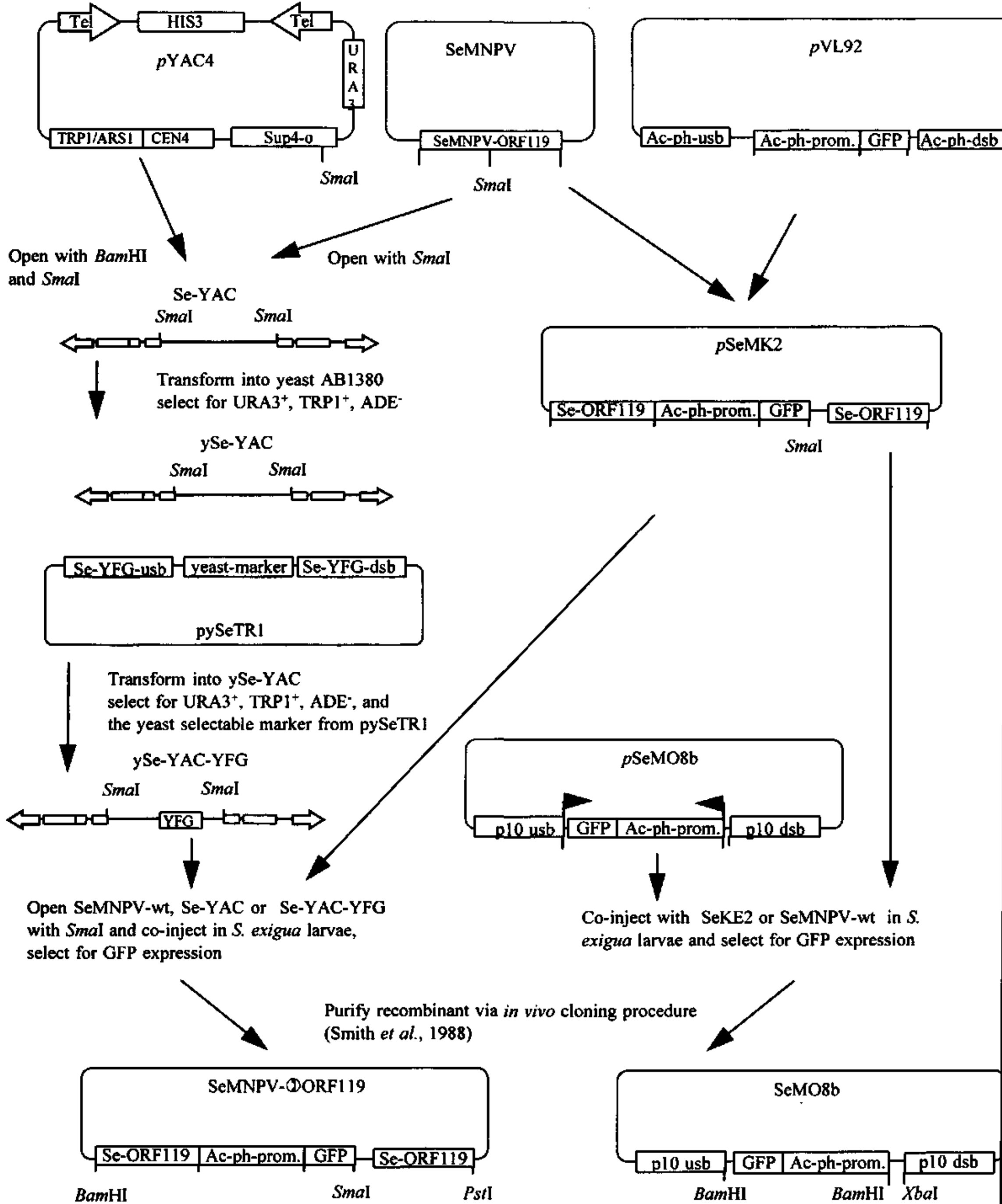


Figure 7.5.
Generation of ySe-YAC, the cloning strategy of pSeMO9 and pSeMK2, and the *in vivo* recombination and selection procedure.

this purpose is attractive since the virus has a limited host range and is relatively virulent for *S. exigua* larvae as compared to other baculoviruses pathogenic for this insect. SeMNPV has been registered as a bio-insecticide in several countries (Smits and Vlak, 1994). However, as is the case for all baculoviruses, the time between a virus infection and the cessation of feeding is long compared to the speed of action of chemical insecticides. Improvement of the insecticidal properties of baculoviruses has been achieved via genetic engineering (Bonning and Hammock, 1996). With the information about the genetic organization and genome replication of SeMNPV, now available and the developed strategies to engineer the viral genome, the virus is amenable to further improvement of its insecticidal properties.

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Chapter 8

SUMMARY

Spodoptera exigua multicapsid nucleopolyhedrovirus (SeMNPV) is an attractive biological control agent for the beet army worm *S. exigua*. This baculovirus has a narrow host range and is relatively, compared to other baculoviruses, virulent for beet army worm larvae. The molecular principles that specify the host range and virulence of SeMNPV are unknown. This thesis describes studies aimed at the unravelling of the molecular genetics of this baculovirus and the key steps in the infection and genome replication process.

As a first step SeMNPV was multiplied in an established cell line of *S. exigua* to obtain a better understanding of the replication process. Polyhedra derived from cell culture were unable to infect *S. exigua* larvae, although the hemolymph isolated from these larvae did contain budded virus able to infect insect cell lines (Chapter 2). This suggested the generation of a genetic defect in the SeMNPV genome during replication and/or maintenance in cell culture. A partial plasmid library and complete cosmid library were made to construct a physical map of the SeMNPV genome and locate the genetic defect(s). The position of the *polyhedrin* gene and the transcriptional direction of the *p10* gene allowed the pin-pointing of the zero point and orientation of the circular SeMNPV genome. A large deletion (20-25 kilobase pairs), turned out to be the genetic defect, arising upon limited passaging of the virus in cell culture. This deletion was located between map unit 12.9 and 31.3 in the SeMNPV genome. The occurrence of this deletion implied that the construction of SeMNPV recombinant viruses still virulent *in vivo* cannot be achieved via conventional techniques, i.e. homologous recombination in cell culture (Chapter 2). Alternative recombinationstrategies involving yeast artificial chromosomes and *in vivo* cloning were considered and partially tested (Chapter 7).

In Chapters 3 and 4 the identification and characterization of *cis*-acting elements in SeMNPV DNA replication are described. Transient DNA replication assays, sequence analyses and hybridization experiments identified one non-*hr* (*hr* homologous region) and six *hr* origins of DNA replication. SeMNPV *hr*s contained one (*hr*4) up to nine (*hr*1) repeated, near-identical 68-bp long palindromes. The SeMNPV *hr*s, located in non-coding regions, were found dispersed in the viral genome as observed in the genomes of two other baculoviruses, *Autographa californica* MNPV and *Orgyia pseudotsugata* MNPV. Transient

DNA replication assays in AcMNPV-infected insect cells revealed no replication of SeMNPV-*hr*s and, in SeMNPV-infected insect cells no replication of AcMNPV-*hr*s could be observed, suggesting that these elements display specificity (Chapter 3). In the SeMNPV-*Xba*I library one additional genomic fragment unrelated to *hr*s and reminiscent of AcMNPV and OpMNPV non-*hr* origins of DNA replication that underwent SeMNPV dependent DNA replication was identified. By deletion analysis the core of this non-*hr* origin was mapped within a 800 bp region of non-coding sequence. This sequence contained also several motifs such as multiple palindromes, direct repeats, putative transcriptional factor binding sites and multiple polyadenylation signals, characteristic for baculovirus non-*hr* and other eukaryotic origins of DNA replication. In contrast to the *hr*s, the SeMNPV non-*hr* origin could be replicated by the replication machinery of the heterologous AcMNPV (Chapter 4).

The putative helicase is the most intriguing *trans*-acting DNA replication factor of baculoviruses, since it may be involved in both DNA replication and host range determination. An open reading frame (ORF) potentially encoding a polypeptide of 143 kDa (p143) with considerable amino acid identity to the putative helicases of AcMNPV, BmNPV and OpMNPV was identified in SeMNPV. Sequence alignment of the SeMNPV p143 indicated that it is somewhat diverged from its AcMNPV, BmNPV and OpMNPV homologs. Whether the protein is also involved in the host range of SeMNPV determination remained unsolved. The ORF is expressed as a 4 kb transcript between 4 and 24 h p.i., starting from an unusual transcriptional initiation site present eleven nucleotides upstream of the translational start. Transient plasmid dependent DNA replication assays showed that not only helicase plays a crucial role in SeMNPV and AcMNPV replication specificity, but also one or more of the other previously mentioned essential *trans*-acting DNA replication factors. Apparently the interaction between the origins of DNA replication and/or the assembly of the replisome is a highly virus specific process (Chapter 5).

Complete sequence and transcriptional analysis of the 11.3 kb SeMNPV-*XbaI-C* fragment containing the *p143* gene revealed twelve ORFs that all showed high amino acid identity to AcMNPV and OpMNPV homologs. The genetic organization of the SeMNPV-*Xba*I-C fragment was identical to the AcMNPV and OpMNPV helicase region, although in an antigenomic orientation. In line with recent observations in herpes- and poxvirus genomes, which contain conserved central parts of their genomes and more diverged termini, it is hypothesized that baculoviruses genomes could also contain a highly conserved gene block centered around the *p143* gene and a more diverged region at the *polyhedrin-p10* loci. This hypothesis is further supported by partial sequence and hybridization data from other baculovirus genomes. The organization of the less conserved *polyhedrin-p10* region could be a marker for the genetic relatedness of baculoviruses (Chapter 6). The state of the art on the sequence analysis of the complete SeMNPV genome is described in Chapter 7.

The availability of a physical map, the insght in the genetic organization of the SeMNPV genome and the occurence of the spontaneous deletion mutants in cell culture prompted the development of alternative recombination strategies to bypass the use of the insect cell lines (Chapter 7). To this end a recombination strategy using yeast genetics was employed. Deletion of the yeast autonomous replicating sequences prior to the application of the recombinant baculoviruses in the field is recommended and can be achieved for SeMNPV using a direct *in vivo* recombination and selection protocol. The strategy proposed is based on the occurrence of homologous recombination of baculoviruses in the insect (Chapter 7).

The research on SeMNPV described in this thesis, has created a good starting point to study the molecular basis of virulence and host range. The development of the recombination system described in chapter 7 could offer a tool for the insertion and deletion of specific (viral) genes for this purpose. Moreover exploiting the proposed recombination system, the improvement of the insecticidal properties of SeMNPV can be pursued.

Chapter 9

SAMENVATTING

Spodoptera exigua multicapsid nucleopolyhedrovirus (SeMNPV) wordt in de praktijk reeds toegepast als biologisch bestrijdingsmiddel tegen de floridamot (*S. exigua*). Het virus is alleen infectieus voor de larvale stadia van de mot en in vergelijking tot andere baculovirussen is het relatief virulent. De moleculair genetische achtergrond van de gastheerspecificiteit en de virulentie van SeMNPV is niet bekend. Het onderzoek beschreven in dit proefschrift werd gestart om meer inzicht te verkrijgen in de moleculaire genetica van dit baculovirus en in de cruciale stappen van het infectieproces en het genoomreplicatie mechanisme van dit virus.

Om het SeMNPV infectieproces beter te kunnen bestuderen werd als eerste stap getracht SeMNPV te vermeerderen in een cellijn van *S. exigua*. Polyeders, geïsoleerd uit deze geïnfecteerde cellen, bleken niet meer infectieus te zijn voor *S. exigua* larven. De hemolymf van larven die geïnfecteerd waren met deze polyeders, bevatte daarentegen wel "budded virus", dat infectieus was voor de cellijnen. Dit suggereerde dat er een genetisch defect in het SeMNPV genoom ontstaat tijdens de vermenigvuldiging van het virus in cel-kweek (Hoofdstuk 2). Om de structuur van het virale genoom in kaart te brengen en het genetische defect te lokaliseren, werden plasmiden- en cosmiden bibliotheken van het SeMNPV-genoom geconstrueerd. De reeds bekende posities van de *polyhedrine p10* genen op het circulaire SeMNPV genoom, bepaalden het nulpunt en de oriëntatie van de kaart. Het in celkweek opgelopen genetsche defect bleek een grote deletie (20-25 kbp) te zijn, gelegen tussen map unit 12.9 en 31.3 van de kaart van het SeMNPV genoom (Hoofdstuk 2). Deze resultaten impliceren dat de constructie van recombinant SeMNPV, bijvoorbeeld om genfuncties te bestuderen of om de inzetbaarheid van het virus bij biologische bestrijding te vergroten, niet kan worden bereikt met behulp van conventionele methoden, waarbij gebruik gemaakt wordt van homologe recombinatie in cellijnen. Derhalve werden alternatieve recombinatie strategieën voor het SeMNPV genoom door middel van gist- en *in vivo* cloning overwogen en getest aan de hand van het prototypische *Autographa californica* MNPV (AcMNPV) genoom (Hoofdstuk 7).

DNA replicatie is een essentiële stap in het vermenigvuldigingsproces van baculovirussen. In de hoofdstukken 3 en 4 is de isolatie en karakterisering van de "*cis*-acting"

elementen die bij SeMNPV DNA replicatie zijn betrokken, beschreven. Met behulp van transiënte DNA replicatie-assays, sequentie-analyse en hybridisatie experimenten werden zes startpunten van DNA replicatie van het *hr*-type (*hr* homologous region) gevonden en één van het non-*hr* type. De SeMNPV *hr*s bevatten één (*hr*4) tot negen (*hr*1) gerepeteerde, bijna identieke, 68 basenparen-lange palindromen. Deze *hr*s bleken verspreid over het virale genoom te liggen in gebieden welke niet voor eiwitten coderen en zijn verspreid over het genoom gevonden. Dit is ook het geval voor AcMNPV en *Orgyia pseudotsugata* MNPV (OpMNPV). Met AcMNPV geïnfecteerde insectencellen bleken niet in staat te zijn de SeMNPV *hrs* te repliceren. Omgekeerd bleken AcMNPV-*hr*s niet te repliceren in SeMNPV geïnfecteerde cellen. *Hrs* blijken dus slechts herkend te worden door de viruseigen replicatiefactoren (Hoofdstuk 3). De non-*hr* replicatiestartplaats werd uit de *Xba*I-plasmide bibliotheek geïsoleerd met behulp van SeMNPV-afhankelijke DNA replicatie assays (Hoofdstuk 4). Door middel van deletie analyse werd de "core" van dit replicatiestartpunt binnen een segment van 800 baseparen, dat niet voor eiwitten codeerde, gelokaliseerd. De nucleotidenvolgorde van dit element vertoonde noch overeenkomst met die van de *hr*s van SeMNPV, noch met de non-*hr* elementen van andere baculovirussen. De sequentie bevatte echter meerdere palindromen, "direct repeats", potentiële bindingsplaatsen voor transcriptiefactoren en poly-adenyleringsignalen, die allen karakteristiek lijken te zijn voor zowel baculovirus non-*hr* sequenties als eukaryotische startpunten van DNA replicatie. De non-*hr* werd wel door de replicatiemachinerie van AcMNPV herkend en is in tegenstelling tot de *hr*s niet virus specifiek (Hoofdstuk 4). Ondanks deze informatie kan over de rol van de *hrs* en non-*hr* tijdens virale DNA replicatie *in vivo* echter nog geen uitspraak worden gedaan.

Van alle "*trans*-acting" DNA replicatie factoren is het vermeende helicase het meest interessant. Dit eiwit heeft mogelijk een dubbele functie, in zowel DNA replicatie als in gastheerspecificiteit. In het genoom van SeMNPV werd een open leesraam aangetroffen dat voor een polypeptide van 143 kDa codeert met een aanzienlijke homologie met de helicases van AcMNPV, OpMNPV en *Bombyx mori* NPV (Hoofdstuk 5). Op belangrijke punten week de aminozuurvolgorde van het SeMNPV helicase echter af van die van andere baculovirus helicases. Gezien de geringe verwantschap van SeMNPV met AcMNPV en BmNPV valt nog te bezien of het SeMNPV helicase eveneens het gastheer bereik van dit virus bepaalt. Het SeMNPV-*helicase* gen wordt tussen 4 en 8 uur na infectie tot expressie gebracht als een 4 kilobasen mRNA vanaf een ongewone transcriptie startpositie, elf nucleotiden stroomopwaarts van de translatie start. Transiënte DNA replicatie testen toonden aan dat niet alleen het helicase, maar ook de andere "*trans*-acting" factoren, de gevonden replicatiespecificiteit van SeMNPV en AcMNPV bepalen. Klaarblijkelijk is de interactie tussen de startpunten van DNA replicatie en/of de assemblage van het replisoom een zeer virusspecifiek proces (Hoofdstuk 5). De isolatie van alle andere SeMNPV gecodeerde "*trans*-

acting" elementen is vereist voor de verdere ontrafeling van het replicatiemechanisme en de moleculaire principes, die ten grondslag liggen aan de replicatiespecificiteit van dit virus (Hoofdstuk 7).

Een gedetailleerde analyse van het 11,3 kilobasen-lange *Xba*I-C fragment, dat het helicase gen van SeMNPV bevat, toonde twaalf open leesramen aan, waarvan de translatieproducten een hoge aminozuur identiteit bleken te hebben met bekende polypeptiden van AcMNPV en OpMNPV (Hoofdstuk 6). De genetische organisatie van het SeMNPV-*Xba*I-C fragment bleek gelijk te zijn aan die van het AcMNPV en OpMNPV helicase gebied. In vergelijking met AcMNPV en OpMNPV is deze regio in SeMNPV op ongeveer dezelfde plaats gelegen, maar in een anti-genomische oriëntatie. Overeenkomstig de genetische organisatie van herpes- en pokkenvirussen, wijzen voorlopige analyses erop dat baculovirusgenomen gekenmerkt worden door een zeer sterk geconserveerde helicase regio en een meer gedivergeerde *polyhedrine-p10* regio (Hoofdstuk 6). De genetische organisatie van het *polyhedrine-p10* gebied zou derhlave een goede fylogenetische merker kunnen zijn voor de bepaling van intervirale verwantschappen bij baculovirussen.

Het beschikbaar komen van de fysische kaart, het voorlopig inzicht in de genetische organisatie van SeMNPV en de onmogelijkheid om het virus genetisch stabiel in cellijnen te vermeerderen, leidde tot de ontwikkeling van alternatieve recombinatiestrategieën. Vooralsnog komt het baculovirus-gist recombinatiesysteem als eerste in aanmerking (Hoofdstuk 7). Deletie van de zogenaamde autonoom replicerende gist sequenties (ARS) uit artificiële chromosomen en plasmiden is daarbij aan te bevelen alvorens het recombinante virus toe te passen als bio-insecticide in het veld. Dit kan worden bereikt door middel van *in vivo* recombinatie, clonering en selectiemethoden.

Het verbeterde inzicht in de genetische organisatie en het DNA replicatiemechanisme van SeMNPV vormt de benodigde aanzet om door midel van verder onderzoek de moleculaire achtergrond van zowel virulentie als gastheerbereik van SeMNPV te doorgronden. De verdere ontwikkeling van het voorgestelde recombinatiesysteem (Hoofstuk 7) zou vervolgens kunnen leiden tot SeMNPV recombinanten, die enerzijds gerichte studie naar deze biologische eigenschappen mogelijk maken en anderzijds als verbeterd bio-insecticide toegepast kunnen worden.

CURRICULUM VITAE

Jacobus Gerardus Maria Heldens werd geboren op 11 oktober 1968 in Leunen. Hij behaalde in 1987 het gymnasium-ß diploma aan het Bisschoppelijk College in Sittard. In datzelfde jaar werd aangevangen met de studie Moleculaire Wetenschappen, oriëntatie biotechnologie, aan de Landbouwuniversiteit van Wageningen. In maart 1993 werd het ingenieurs diploma behaald. Tijdens de doctoraalfase heeft hij afstudeeronderzoek verricht in de Moleculaire Biologie aan de Landbouwuniversiteit (Prof.dr. A. van Kammen), in de Biochemie aan de faculteit Diergeneeskunde van de Rijks Universiteit Utrecht (Prof.dr. S.G. van de Bergh en Dr. A.G.M. Tielens), in Plant Pathology aan de University of California at Riverside in de Verenigde Staten van Amerika (Prof.dr. N.T. Keen en Dr. J. Visser) en in de Moleculaire Genetica van Industriële Microorganismen aan de Landbouwuniversiteit (Dr. J. Visser en Dr. J. van den Hombergh). Van september 1993 tot september 1997 was hij werkzaam als Assistent in Opleiding bij de vakgroep Virologie van de Landbouwuniversiteit Wageningen onder begeleiding van Prof.dr. J.M. Vlak en Prof.dr. R.W. Goldbach. Van het daar uitgevoerde onderzoek, dat onder andere gefinancierd werd door de Lanbouwuniversiteit, het Dutch Israeli Agricultural Research Project en de Nederlandse Organisatie voor Wetenschappelijk Onderzoek, staan de resultaten beschreven in dit proefschrift. In periode maart-april van 1994 is een werkbezoek gebracht aan het laboratorium van Dr. D.R. O'Reilly, Imperial College of Science and Technology van London University in het Verenigd Koninkrijk. Vanaf november 1997 is hij werkzaam als vakspecialist vaccins bij Fort Dodge Animal Health Holland in Weesp.

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