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**Molecular Dissection of the *Spodoptera littoralis* Nucleopolyhedrovirus:
Virus-host Cell Interaction and Virus DNA Replication**

by

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Abstract

Baculoviruses are viruses of arthropods with large rod-shaped virions that contain super-coiled double-stranded DNA genomes. These viruses have been used as gene expression vectors and insect biological control agents, and have been studied as a virus model for the investigation of molecular mechanisms, such as apoptosis, gene expression, DNA replication, and virus-host interaction. Our current knowledge about baculovirus is largely based on the studies of the *Autographa californica* nucleopolyhedrovirus and the closely related species. In spite of the increasing interest of recombinant baculoviruses as gene expression and delivery vectors and bioinsecticides, the mechanisms of baculovirus DNA replication and virus-host interaction are still poorly understood. To take advantage of baculovirus diversity and their specific host-ranges, I studied the *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV). Previous investigations indicated that SpliNPV possesses a unique host-range and genetic organization. In this dissertation, I studied the SpliNPV infection of an orthopteran cell line derived from the grasshopper, *Melanopus sanguinipes*, and provided evidence of viral DNA replication and production of viable virus progeny. I next investigated SpliNPV infection in five cell lines derived from three lepidopteran families: Sf9, CLS79 and Se1 cell lines from *Spodoptera* (*Noctuidae*), Ld652Y cells from *Lymantria dispar* (*Lymantriidae*), and Md210 cells from *Malacosoma disstria* (*Lasiocampidae*), which represented permissive (Sf9, CLS79, and Se1), semi-permissive (Ld652Y), and non-permissive (Md210) cell lines. SpliNPV infection in permissive cell lines resulted in viral gene expression, DNA replication, and production of viable progeny. While the semi-permissive cell line displayed reduced and delayed transcription of viral genes and supported limited viral DNA replication, the non-permissive

cell line displayed dramatically reduced viral transcription and abolished viral progeny. Transient expression assays using SpliNPV early- or very late-promoter reporters suggested that non-productive infection of SpliNPV in semi- or non-permissive cell lines was a consequence of limited viral specific transcription at the early phase of viral infection.

Having documented the infection events in these cell lines, I investigated the mechanism of SpliNPV DNA replication. Using transient replication assays I have identified a non-*hr* origin of SpliNPV DNA replication. With limited sequence similarity to other NPV non-*hr* origins, the putative SpliNPV origin consists of sequence motifs found in other origins of virus DNA replication, such as imperfect palindromes, direct repeats, and transcription factor binding sites. Transient expression assays indicated that the putative non-*hr* origin represses the SpliNPV early gene, *lef-3*. Gel mobility shift analyses confirmed that nuclear proteins from both infected and uninfected cells bound with specificity to the putative origin.

After identification and characterization of the *cis*-acting factor involved in viral DNA replication, I then identified a *trans*-acting factor involved in viral DNA replication. I have sequenced a 6.4 kb DNA from SpliNPV genome that contains an ORF encoding a predicated polypeptide of 998 amino acid sequences. Comparative sequence analyses demonstrated that the ORF encoded a DNA polymerase (*dnapol*) that consists of conserved exonuclease domains and DNA polymerase motifs found in other eukaryotic DNA viruses and in cellular DNA polymerases. The transcription initiation site of the 3 kb SpliNPV *dnapol* transcript was mapped to an NPV early promoter element, ACGT. The transcript terminated at the polyadenylation signal AATAAA. Using *E. coli* and baculovirus expression systems, I over-expressed a 110 kDa full-length SpliNPV DNA polymerase

(DNAPOL) and a truncated 96 kDa protein, in which the amino terminal 80 amino acids were deleted. Enzymatic analyses demonstrated that the DNA polymerase and 3'-5' exonuclease activities are intrinsic to the SpliNPV DNAPOL. Deletion of the 80 amino acid residues at the N-terminal of the DNAPOL did not affect DNA polymerase and exonuclease activities. Replication products from single-stranded M13 DNA revealed that SpliNPV DNAPOL possesses a processive activity.

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Table of Contents

Abstract.....	ii
Table of Contents.....	v
List of Abbreviations.....	ix
List of Tables.....	xi
List of Figures.....	xii
Acknowledgments.....	xiv
Chapter 1. General Introduction.....	1
1.1. Baculovirus.....	1
1.2. Baculovirus life cycle.....	2
1.3. Baculovirus host range.....	3
1.4. Baculovirus gene expression.....	5
1.5. Baculovirus DNA replication.....	7
1.5.1. Baculovirus replication origins.....	8
1.5.1.1. Homologous regions as replication origins.....	8
1.5.1.2. Non-homologous origins of replication.....	9
1.5.2. Genes involved in baculovirus DNA replication.....	10
1.5.3. Protein-protein interactions.....	17
1.6. Baculovirus for insect control.....	18
1.7. Baculovirus as gene expression and delivery vectors.....	19
1.8. The <i>Spodoptera littoralis</i> nucleopolyhedrovirus and research objectives.....	21
Chapter 2. The <i>Spodoptera littoralis</i> Nucleopolyhedrovirus Infection of an Orthopteran Cell Line.....	24
2.1. Abstract.....	24
2.2. Introduction.....	25
2.3. Materials and Methods.....	25
2.3.1. Cells and virus.....	25
2.3.2. RAPD analysis of cell lines.....	26
2.3.3. Infection of cells with SpliNPV.....	26
2.3.4. Dot blot analysis of viral DNA replication.....	27
2.3.5. Polymerase chain reaction.....	27

2.3.6. Electron microscopy.....	28
2.4. Results.....	28
2.4.1. Cytopathic effect.....	28
2.4.2. Viral DNA replication.....	29
2.4.3. Viral specific gene amplification.....	35
2.4.4. Virion detection by electron microscopy.....	35
2.5. Discussion.....	38

Chapter 3. Molecular Characterization of the *Spodoptera littoralis*

Nucleopolyhedrovirus Infection in Permissive, Semi-permissive and Non-permissive Cell Lines.....	40
3.1. Abstract.....	40
3.2. Introduction.....	41
3.3. Materials and Methods.....	42
3.3.1. Cells and viruses.....	42
3.3.2. Infection of cells with SpliNPV.....	42
3.3.3. Plasmids.....	43
3.3.4. Dot blot analysis.....	46
3.3.5. RNA isolation and Northern blot analysis.....	46
3.3.6. Transient expression assays.....	47
3.4. Results.....	47
3.4.1. SpliNPV DNA replication.....	47
3.4.2. Production of viable progeny.....	48
3.4.3. Northern blot analysis of global SpliNPV transcription in infected lepidopteran cell lines.....	52
3.4.4. Levels of SpliNPV-specific RNAs in three infected lepidopteran cell lines.....	52
3.4.5. Transcriptional activation of SpliNPV early and very late promoters.....	56
3.4.6. Comparison of the SpliNPV and AcMNPV promoter activity in the presence of heterologous virus.....	60
3.5. Discussion.....	64

Chapter 4. Identification and Functional Analysis of a Putative Non-*hr* origin of DNA replication from the *Spodoptera littoralis*

Nucleopolyhedrovirus.....	69
4.1. Abstract.....	69
4.2. Introduction.....	70
4.3. Materials and Methods.....	71
4.3.1. Cells and virus.....	71
4.3.2. Recombinant plasmids.....	72
4.3.3. Transient replication assay.....	75
4.3.4. DNA sequence analysis.....	75
4.3.5. Luciferase assays.....	76
4.3.6. Preparation of nuclear extracts and gel mobility shift assays.....	76
4.4. Results.....	77
4.4.1. Identification of an origin of SpliNPV DNA replication.....	77
4.4.2. Location, DNA sequence analysis, and structural features of the origin.....	78
4.4.3. Functional analysis of <i>cis</i> -acting sequences of the origin.....	84
4.4.4. Cellular proteins bind to the putative SpliNPV origin sequence.....	87
4.5. Discussion.....	91
Chapter 5. Identification, Transcription and Sequence Analysis of the <i>Spodoptera littoralis</i> Nucleopolyhedrovirus DNA Polymerase Gene.....	97
5.1. Abstract.....	97
5.2. Introduction.....	98
5.3. Materials and Methods.....	99
5.3.1. Cells and virus.....	99
5.3.2. Molecular cloning, PCR and sequencing.....	100
5.3.3. Southern blot analysis.....	100
5.3.4. RNA isolation and Northern blot analysis.....	101
5.3.5. Mapping ends of the SpliNPV <i>dnapol</i> transcript.....	101
5.3.6. GenBank accession number.....	102
5.4. Results.....	102
5.4.1. Identification, location and sequence analysis of the SpliNPV <i>dnapol</i> gene.....	102

5.4.2. Transcriptional analysis of the SpliNPV <i>dnapol</i> gene.....	106
5.4.3. Mapping ends of the SpliNPV <i>dnapol</i> transcript.....	106
5.4.4. Amino acid sequence conservation among baculovirus DNA polymerases.....	108
5.5. Discussion.....	118
Chapter 6. Expression and Characterization of the <i>Spodoptera littoralis</i>	
 Nucleopolyhedrovirus DNA Polymerase.....	127
6.1. Abstract.....	127
6.2. Introduction.....	128
6.3. Materials and Methods.....	129
6.3.1. Cells and virus.....	129
6.3.2. PCR and cloning of the SpliNPV <i>dnapol</i> gene.....	129
6.3.3. Expression and purification of SpliNPV DNAPOL in <i>E. coli</i>	131
6.3.4. Expression and purification of SpliNPV DNAPOL in Sf9 cells.....	131
6.3.5. SDS-PAGE and Western blot analysis.....	132
6.3.6. DNA templates.....	132
6.3.7. DNA polymerase assay.....	133
6.3.8. Exonuclease assay.....	133
6.4. Results.....	134
6.4.1. Cloning of the full-length SpliNPV <i>dnapol</i> gene and the 80 mutant.....	134
6.4.2. Overexpression and purification of SpliNPV DNAPOL and the 80 mutant proteins.....	135
6.4.3. DNA polymerase activity.....	141
6.4.4. 3'-5' exonuclease activity.....	145
6.4.5. Replication of singly-primed single-stranded M13 DNA.....	149
6.5. Discussion.....	149
Conclusions.....	153
Bibliography.....	154
Appendix. The <i>Spodoptera littoralis</i> nucleopolyhedrovirus <i>dnapol</i> sequence.....	177

List of Abbreviations

aa amino acid	DNA deoxyribonucleic acid
AAV adeno-associated virus	DNase deoxyribonuclease
A₆₀₀ Absorbance at 600 nm	dNTP deoxynucleoside triphosphate
A adenine or adenosine	DTT dithiothreitol
ADP adenosine 5'-diphosphate	dTTP deoxythymidine triphosphate
AMP adenosine 5'-monophosphate	dUTP deoxyuridine triphosphate
AMV avian myeloblastosis virus	DBP DNA-binding protein
ATP adenosine 5'-triphosphate	EDTA ethylenediaminetetraacetic acid
bp base pair(s)	egt ecdysteroid UDP-glucosyl transferases
BSA bovine serum albumin	exo exonuclease
BrdU 5-bromodeoxyuridine	G guanine or guanosine
BV budded viurs	GST glutathionine S-transferase
C cytosine or cytidine	GTP guanosine 5'-triphosphate
cDNA complementary deoxyribonucleic acid	GV granulovirus
CDS cDNA synthesis	HB hunchback factor
CMV cytomegalovirus	HBV hepatitis B virus
cpm counts per minute	HCV hepatitis C virus
CTP cytidine 5'-triphosphate	HEPES <i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid
d(A-T) dexyadenylate-deoxythymidylate	hpi hours post infection
Da Dalton	hr homologous region
dATP deoxyadenosine triphosphate	HSV herpes simplex virus
dCTP deoxycytidine triphosphate	IE immediately early protein
ddATP dideoxyadenosine triphosphate	Ig immunoglobulin
DCTA 1,2 diaminocyclohexane- <i>N,N,N',N'</i> -tetraactic acid	IPTG isopropyl-1-thio- β -D-galactoside
ddCTP dideoxycytidine triphosphate	ITR inverted terminal repeats
ddGTP dideoxyguanosine triphosphate	lef late expression factor
ddNTP dideoxythymidine triphosphate	MOI multiplicity of infection
DEPC diethylpyrocarbonate	MOPS morpholinepropanesulfonic acid
DFD deformed factor	mRNA messenger ribonucleic acid
dGTP deoxyguanosine triphosphate	m.u. map units
	NFI nuclear factor I

NFIII nuclear factor III	RING really interesting new gene
non-hr ori non-homologous origin	RNA ribonucleic acid
NPV nucleopolyhedrovirus	RNase ribonuclease
NTP nucleoside triphosphate	RPA replication protein A
OD₂₆₀ optical density at 260 nm	RPC replication protein C
oligo(dT) oligodeoxythymidylic acid	RT reverse transcriptase
oligo oligonucleotide	SDS sodium dodecyl sulfate
ORF open reading frame	SP1 stimulating protein 1
PAGE polyacrylamide gel electrophoresis	SSB single-stranded DNA binding protein
PBS phosphate buffered saline	ssDNA single-stranded DNA
PCR polymerase chain reaction	SV40 simian virus 40
PIB polyhedral inclusion bodies	T thymine or thymidine
PMSF phenylmethylsulfonyl fluoride	TBP TATA binding protein
Pol α DNA polymerase α	TCA trichloroacetic acid
Pol β DNA polymerase β	TCID₅₀ 50% tissue-culture infectious dose
Pol δ DNA polymerase δ	TEM transmission electron microscope
Pol ϵ DNA polymerase ϵ	TE Tris-HCl EDTA (buffer)
Polh polyhedrin gene	Tris tris(hydroxymethyl)aminomethane
Poly(dA-dT) polydeoxyadenylic acid- polydeoxythymidylic acid or polydeoxyadenylate- polydeoxythymidylate	Tris-HCl Tris hydrochloride
RACE rapid amplification of cDNA end	TTP thymidine 5'-triphosphate
RAPD random amplified polymorphic DNA	U unit
REN restriction endonuclease	UAR upstream activation regions
RF replication form	USF upstream stimulatory factor
	UV ultraviolet
	VLP virus-like particles
	X-gal 5-Bromo-4-chloro-3-indolyl- β -D-galactoside

List of Tables

Table 1.1. Baculovirus DNA replication proteins.....	12
Table 2.1. Budded virus production from SpliNPV-infected cell lines.....	32
Table 3.1. SpliNPV budded virus production in selected cell lines.....	51
Table 4.1. Structural motifs within the putative SpliNPV non- <i>hr ori</i> sequence.....	83
Table 5.1. Percentage of aa sequence identity of baculovirus DNAPOLs.....	117
Table 6.1. Primers used for PCR amplification.....	130
Table 6.2. Specificity of SpliNPV DNAPOL.....	138

List of Figures

Figure 2.1. RAPD analysis of Sf9, MSE4, and Md210 cell lines.....	30
Figure 2.2. SpliNPV infection in Sf9 and MSE4 cells.....	31
Figure 2.3. Dot blot replication assays of SpliNPV-infected cell lines.....	33
Figure 2.4. PCR analysis of cell pellets and supernatants from infected cells.....	36
Figure 2.5. Transmission electron micrographs of SpliNPV-infected Sf9 and MSE4 cells.....	37
Figure 3.1. Plasmid construction.....	45
Figure 3.2. Dot blot analysis of the extent of SpliNPV DNA replication.	49
Figure 3.3. Global SpliNPV transcription in infected cell lines.....	54
Figure 3.4. Levels of SpliNPV-specific RNAs in infected cell lines.....	55
Figure 3.5. Analysis of SpliNPV promoter activity in permissive and non-permissive cells by transient expression assays.....	58
Figure 3.6. Analysis of AcMNPV promoter activity in permissive and non-permissive cell lines by transient expression assays.....	61
Figure 4.1. Plasmid constructs.....	73
Figure 4.2. Transient replication assays.....	80
Figure 4.3. Location and DNA sequence analysis of the origin.....	81
Figure 4.4. Functional analysis of <i>cis</i> -acting sequences in the origin.....	85
Figure 4.5. Gel mobility shift assays with the SpliNPV origin.....	88
Figure 4.6. Comparison of the structural organization of selected <i>oris</i>	94
Figure 5.1. Localization of the SpliNPV <i>dnapol</i>	103

Figure 5.2. Sequencing strategy of the SpliNPV <i>dnapol</i> gene.....	104
Figure 5.3. Transcriptional analysis of the SpliNPV <i>dnapol</i> gene.....	107
Figure 5.4. Mapping ends of the SpliNPV <i>dnapol</i> transcript.....	109
Figure 5.5. Amino acid sequence conservation among baculovirus DNA Polymerases.....	112
Figure 5.6. Comparison of 5'flanking region of the NPV <i>dnapol</i> genes.....	125
Figure 6.1. Cloning the full-length SpliNPV <i>dnapol</i> and the 80 mutant.....	137
Figure 6.2. Expression of SpliNPV DNAPOL and the 80 mutant.....	139
Figure 6.3. Recombinant SpliNPV DNAPOL DNA polymerase activity.....	142
Figure 6.4. Recombinant SpliNPV DNAPOL DNA exonuclease activity	146
Figure 6.5. Replication of singly-primed M13 DNA by SpliNPV DNAPOL.....	150

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Chapter 1. General Introduction

1.1. Baculovirus

Viruses are intracellular obligate parasites that can infect prokaryotic and eukaryotic cells in nature. Viruses contain either DNA or RNA as their genomic material. Viruses of the family *Baculoviridae* infect arthropods and consist of rod-shaped virions that contain double-stranded supercoiled DNA genomes ranging in size from 88 to 173 kb (Ayres *et al.*, 1994). All members of the *Baculoviridae* replicate in the host cell nucleus, and have similar virion structure and genome organization. The baculoviruses are classified into two genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Volkman *et al.*, 1995). The NPVs contain virions that can be singly or multiple embedded in a crystalline matrix of the polyhedrin protein. The occluded viruses are referred to as polyhedra. The GVs contain virions that are enveloped singly, and only one or rarely two, virions are embedded in a crystalline matrix of granulin (Funk *et al.*, 1997).

For more than two decades, studies on molecular biology of baculoviruses, particularly NPVs, have been of great interest. First, they have been extensively used as gene expression vectors for expressing heterologous proteins in cultured insect cells (Griffiths & Page, 1997; Jarvis, 1997). Proteins expressed in this system are similar to their authentic counterparts; they are appropriately modified, processed, secreted, and correctly folded to give high yields of biologically active proteins. Infection of insect cells with recombinant NPV also provides a useful system for studying viral particle assembly processes and the development of vaccine candidates (Shi *et al.*, 1999). Second, NPVs have been used, and continue to be investigated, for use as rapid-action biological insecticides (Cory *et al.*, 1994; Black *et al.*, 1997). Third, modified NPV vectors have been used for efficient transient and stable transduction of diverse mammalian cell types. The application of modified NPV vectors for gene expression in mammalian cells continues to expand (Condreay *et al.*, 1999). Fourth, investigations of baculovirus genetics have shed light on fundamental questions in biology, such as the function and nature of apoptosis, gene expression, regulation, and DNA replication. Therefore, as concluded by the late baculovirologist, Lois Miller, “of all the viruses known to

mankind, baculoviruses are the most beneficial from an anthropocentric viewpoint” (Miller, 1997).

1.2. Baculovirus life cycle

Being specially designed to survive outside their host due to their protective polyhedra, baculoviruses can reside for years in soil or in the crevices of plants or other refugia (Miller, 1997). Infection is initiated when susceptible larvae ingest the occlusion bodies. The alkaline-soluble polyhedral inclusion bodies (PIBs) are dissolved by the high pH of the insect midgut, which releases the virions. The virions, released from the matrix of the polyhedron, establish sites of primary infection in the cells comprising the midgut. After passing through the peritrophic membrane, the virions come in contact with the microvillar membrane of midgut epithelial cells by receptor-mediated fusion. With the aid of microtubules, the virions are transported to the nucleus where the viral genomes are released from the capsid to initiate viral transcription and replication (Blissard, 1996).

Following infection of an insect cell, baculovirus gene expression occurs in a temporally regulated cascade. Unlike gene transcription in the three other temporal phases, transcription of the immediate early (IE) genes does not depend on production of other viral proteins. Their products up-regulate the delayed early genes. Late gene expression occurs concurrently with the onset of viral DNA replication, and these gene products involved in the final stages of infection and polyhedron morphogenesis, including the p10 and polyhedrin proteins (Bonning & Hammock, 1996). Initial rounds of viral replication within the nucleus of the infected cell produce a second viral phenotype, the budded virus, which spreads the infection to other cells and tissues. The budded viruses move through the cell membrane and become coated with a viral protein-modified basal plasma membrane (Blissard, 1996). Infection of different larval tissues occurs in a sequential manner, and the virus is hypothesized to use the tracheal system of the insect as a conduit. Budded viruses appear to enter cells by endocytosis. Interactions between virions and a host receptor lead to the invagination of the plasma membrane and formation of an endocytic vesicle containing the enveloped virion. The endosome is then acidified, which activates fusion of the viral and endosomal membrane, thereby releasing

the nucleocapsid into the cytoplasm (Blissard, 1996). At later stages of virus infection, progeny viruses become occluded within the nuclei of the infected cells (Federici, 1997). These occluded virions are released upon disintegration of dead insects and contaminated foliage, which is subsequently ingested by other susceptible insects (Bonning & Hammock, 1996).

1.3. Baculovirus host-range

Baculoviruses are usually very host-specific, in most cases infecting a single species or several closely related species. The host-range is determined by the ability of virus to enter host cells and tissues, to replicate, and to release new infectious virus particles. While other animal virus host-ranges are frequently determined by the presence of suitable receptors that facilitate virus attachment and entry into a host cell, baculoviruses are able to enter nonpermissive insect cells and mammalian cells, suggesting that if specific receptors are required by baculoviruses, they are common to both insect and mammalian cells (Miller & Lu, 1997). Studies of the *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV) replication in cultured insect cells indicated that while some cell lines can fully support viral DNA replication and production of viable progeny (permissive cells), other cell lines support only limited replication of the viral genome without (or with very limited) production of viable progeny (semi-permissive cells). Finally, there are cell lines that support neither viral replication nor production of viable progeny (non-permissive cells). Studies with recombinant AcMNPV bearing reporter genes have demonstrated that although these viruses do not replicate in non-permissive insect cells, they are able to enter and express some viral encoded genes (Carbonell *et al.*, 1985; Morris & Miller, 1992). Thus, the block to productive infections in semi-permissive and non-permissive insect cells occurs subsequent to viral entry and uncoating (Guzo *et al.*, 1992; Thiem *et al.*, 1996).

The mechanisms of NPV-host-cell interactions are not well understood. Many studies have attempted to establish the causes of abortive virus infection in non-permissive cell lines. Analyses of gene expression in heterologous systems, as well as the use of transient expression and viral replication assays, have permitted the identification

of several viral genes (*lef-7*, *hcf-1*, *p35*, *p143* and *hrf-1*) that play roles in determining host range. In AcMNPV, eighteen late expression factors (*lefs*) have been demonstrated to be required for optimal late promoter-mediated reporter gene expression (Lu & Miller, 1995a; Todd *et al.*, 1995). Among these, the *ie-1*, *ie-2*, *lef-1*, *lef-2*, *lef-3*, *p143*, *pe-38*, *dnapol*, and *p35* genes are involved in viral DNA replication (Kool *et al.*, 1994a; Lu & Carstens, 1993).

However, different *lef* genes may be required for virus replication in different cell lines. AcMNPV *ie-2*, *lef-7* and *p35* are not essential for late gene expression in transient expression assays in *Trichoplusia ni* TN368 cells (Lu & Miller, 1995b). Virus-encoded host cell-specific factor 1 (*hcf-1*) is required to support optimal reporter gene expression in TN368 cells, but is not required for expression in Sf21 cells (Lu & Miller, 1995b). Deletion of the AcMNPV P35, an inhibitor of apoptosis, results in premature cell death and aborted viral DNA replication in infected *Spodoptera frugiperda* (Sf21) and *Bombyx mori* (Bm5) cells but does not impair virus replication in TN368 cells or *T. ni* larvae (Clem & Miller, 1993). The product of the *p143* gene, a putative DNA helicase, affects host range. Recombinant AcMNPVs, in which the region between amino acids 536 and 584 of the *p143* gene are exchanged with the homologous region from the NPV of *B. mori* (BmNPV), enable the mutant AcMNPV to replicate in Bm5 cells and kill *B. mori* larvae (Argaud *et al.*, 1998; Croizier *et al.*, 1994; Maeda *et al.*, 1993). While early viral genes were expressed at normal levels in Sf9 cells infected with one such expanded-host range mutant (*eh2*-AcMNPV) at a low multiplicity of infection (MOI) inoculum, viral DNA replication, and late gene expression were dramatically reduced (Kamita & Maeda, 1996).

Virus-host interactions at the translational level may directly or indirectly affect the ability of a baculovirus to replicate in a given cell line. Infection of *Lymantria dispar* (Ld652Y) cells with AcMNPV resulted in shutoff of both viral and cellular protein synthesis between 16 and 20 hours post-infection (hpi), and production of infectious virions was abolished (Guzo *et al.*, 1992). However, viral DNA was replicated and viral mRNA from all temporal classes was isolated from AcMNPV-infected Ld652Y cells (Guzo *et al.*, 1992; Morris & Miller, 1992; Morris & Miller, 1993). Thiem *et al.* (1996) identified a unique gene from the NPV of *L. dispar* (LdMNPV) genome, host-range

factor 1 (*hrf-1*), that alleviates the block in protein synthesis observed in Ld652Y cells infected with wild-type AcMNPV and permits replication of a recombinant AcMNPV that expresses the *hrf-1* gene product.

The outcome of viral infection in a host cell is determined by the nature of the interactions between the virus and host cell constituents. During replication within permissive cells, viruses exploit cellular processes at the expense of the host cells, resulting in coordinated expression of virus encoded genes, viral DNA replication, and packaging of viral progeny. At later stages of virus infection, virus progenies become occluded within the host nuclei.

1.4. Baculovirus gene expression

Our understanding of baculovirus gene expression and DNA replication is largely based on studies of AcMNPV, which has a genome of 134 kb and encodes approximately 150 genes (Ayres *et al.*, 1994). The viral genome is made up of three types of genes: a) genes encoding enzymes required for the replication of the viral genome; b) genes encoding proteins involved in regulatory processes; and c) genes that encode the viral structural proteins such as capsid and envelop proteins. Transcription of the NPV genome is tightly regulated and involves the sequential expression of the immediately early gene, delayed early gene, late gene and very late gene. Viral early gene products function to interact with host transcription machinery to prepare for viral replication, and to transactivate other viral genes whose products are essential for the replication. It is generally believed that the transcription of the viral immediate early genes depends on the host transcription system. Mounting evidence indicates that host transcription machinery is responsible for baculovirus early gene expression: a) viral DNA without virion components is infectious in *in vitro* transfection; b) viral early genes are transcribed *in vitro* using uninfected cell nuclear extracts (Pullen & Friesen, 1995a, Blissard *et al.*, 1992); c) viral early promoter-reporter constructs are expressed in the absence of virus in transient expression assays; d) viral early transcription is inhibited by the host RNA polymerase inhibitor α -amanitin, a fungal toxin that specifically inhibits eukaryotic RNA polymerase II (Huh & Weaver, 1990); and e) viral early promoter

sequences resemble insect RNA polymerase II-responsive gene promoters (Friesen, 1997).

Early NPV promoters can be categorized as: I) conventional TATA-containing; II) initiator-containing; III) composite promoter; and IV) unconventional promoter element (Friesen, 1997). In conventional TATA-containing promoters, a TATA box is usually found approximately 30 base pairs (bp) upstream of the transcription start site. The TATA box in eukaryotic RNA polymerase II promoters is well defined as a binding site of TATA binding protein (TBP). Binding of TBP to TATA sequences recruits the transcription initiation complex to a site about 30 bp downstream. Mutagenesis has been used to functionally define TATA boxes from early NPV promoters (Blizzard *et al.*, 1992; Guarino & Smith 1992; Kogan *et al.*, 1995; Theilmann & Stewart, 1991).

Some early NPV gene promoters contain a conserved CAGT sequence at the transcription initiation site. The CAGT sequence functions as an initiator-containing promoter and is similar to host insect promoters that utilize RNA polymerase II (Friesen, 1997). The CAGT promoter function was first demonstrated in the immediately early gene, *ie-1*, whose gene product functions as a transcriptional activator (Pullen & Friesen, 1995b). Transient expression assays demonstrated that the initiator-containing promoter sequence, CAGT, contributes to the overall promoter activity (Kogan *et al.*, 1995; Blissard *et al.*, 1992; Carson *et al.*, 1991).

The composite promoters consist of TATA box and CAGT sequence as promoter elements. In the majority of cases where NPV early transcription initiation has been mapped within the CAGT sequence, a TATA box is also located upstream, such as in the *ie-2*, *gp64*, and *39k* (39K) genes (Kogan *et al.*, 1995; Blissard *et al.*, 1992; Guarino & Smith, 1992; Carson *et al.*, 1991). The organization of composite promoters may recruit host transcription factors to the initiation site, where the transcription factors stabilize or enhance viral specific transcription.

Several important early promoters lack the conventional TATA box and CAGT elements (Friesen, 1997). The *cis*-acting elements mediating transcription are not well understood. One such important viral early genes, *dnapol*, contains unconventional promoter elements. During AcMNPV infection, the *dnapol* transcription initiates from multiple start sites (Ohresser *et al.*, 1994; Tomalski *et al.*, 1988). It is believed that such

unconventional promoters are dependent on virus-encoded transactivator proteins for their expression (Friesen, 1997).

The transition from the early to the late phases of the NPV infection cycle is characterized by replication of viral DNA and activation of an α -amanitin resistant DNA-dependent RNA polymerase activity (Blissard, 1996). Concomitant with viral DNA replication, host mRNA transcription levels decline substantially (Ooi *et al.*, 1989). While late gene expression occurs concurrently with the onset of viral DNA replication, these genes encode the structural proteins of the virus particles. The very late genes encode proteins involved in the final stages of infection and polyhedron morphogenesis, including the p10 and polyhedrin protein (Lu & Miller, 1997). Several viral gene products required for late gene expression have been identified, such as *late expression factor (lef)* genes (Todd *et al.*, 1995).

Most NPV late transcription initiates within a conserved TAAG sequence that comprises the core of the NPV late promoter (Eldridge *et al.*, 1992; Lu & Carstens, 1992; Lu & Miller, 1997). The conserved TAAG sequence is frequently preceded by an A nucleotide. Mutational analyses suggested that sequences within 6-8 nt adjacent to the TAAG motif significantly affect late transcription (Morris & Miller, 1994; Ooi *et al.*, 1989). Because of their extremely high levels of transcription and hyper-expression, the *polyhedrin* and *p10* genes have been used extensively for heterologous gene expression.

In the cascade of NPV regulatory events, successive stages of virus replication are dependent on proper expression of genes within the preceding stages. The appropriate expression and regulation of viral early genes is critical to baculovirus reproductive success. The products of immediate early genes function both to accelerate replicative events and to prepare the host cell for virus multiplication, which represents an enormous tax on cellular biosynthetic capacity.

1.5. Baculovirus DNA replication

Genetically defined *cis*-acting elements which function as viral origins of DNA replication frequently comprise both a core element, which is absolutely required for replication, and one or more auxiliary components that are composed of promoter and

enhancer elements that are dispensable for replication (DePamphilis, 1996). Replication initiation typically begins within the core element that contains A/T-rich motifs capable of being easily unwound, while the auxiliary elements may determine the replication efficiency or interact with host transcription factors. In order for viral DNA synthesis to begin, usually a sequence-specific recognition event by an initiator protein that is encoded by virus is required (DePamphilis, 1996). Following the initiation event, the replication machinery continues as the origin binding proteins recruit other replication proteins to unwind DNA, to synthesize new DNA primers, and to elongate the synthesized DNA from both strands.

1.5.1. Baculovirus replication origins

Insight into the identification of possible replication origins was facilitated by the analysis of defective genomes of AcMNPV that arise from undiluted serial passage of the virus in cell culture (Lee & Krell, 1994). These defective viruses are propagated along with helper wild-type virus and gradually evolve into heterogeneous populations composed of virions that lack major segments of their genomes, and instead contain tandemly repeated viral sequences that behave as replication origins. These defective genomes possess DNA sequence elements that allow amplification and packaging. Subsequent evidence for the existence of distinct origins came from the infection-dependent plasmid DNA replication assays, in which the genome of baculovirus was explored for the presence of origins (Lu *et al.*, 1997).

1.5.1.1. Homologous regions as replication origins

All well-characterized baculovirus genomes contain a set of closely related sequences known as homologous regions (*hrs*), which are interspersed throughout the genome. These *hrs* share a number of common sequence features: (1) a core sequence consisting of an imperfect palindrome flanked by direct repeats; and (2) multiple copies of this core sequence separated by variable lengths of DNA. In AcMNPV, the *hrs* consist of one to eight copies of a repeated sequence composed of 30 bp palindromes flanked by

20 bp direct repeats and separated by approximately 80 to 120 bp of non-repetitive DNA (Kool *et al.*, 1994b).

Functional analyses demonstrated that a single palindrome from an AcMNPV *hr* could support limited plasmid DNA replication (Leisy *et al.*, 1995), although the relative efficiency of replication of a particular *hr*-containing plasmid increases as the number of palindromes present in that *hr* increases. Plasmids containing half of the palindrome or modified palindromes are severely compromised in their ability to replicate in infected cells (Wu & Carstens, 1996). Elements flanking *hr* sequences have been shown to be necessary for optimal infection-dependent plasmid replication (Leisy *et al.*, 1995).

Identification of similar DNA elements in the genomes of other NPVs such as the NPVs of *Orgyia pseudotsugata* (OpMNPV; Ahrens *et al.*, 1995b), *Choristoneura fumiferana* (CfMNPV; Xie *et al.*, 1995), *S. exigua* (SeMNPV; Broer *et al.*, 1998), and LdMNPV (Pearson & Rohrmann, 1995) suggests that *hrs* perform an essential function during the replication cycle of these viruses. Currently, however, there is no direct evidence that *hrs* function as origins of replication in the context of virus infection. Some *hrs* from AcMNPV and OpMNPV have also been demonstrated to function as *cis*-acting enhancers of IE-1-mediated early gene expression (Rodems & Friesen, 1993; Kool *et al.*, 1995; Leisy *et al.*, 1995).

1.5.1.2. Non-homologous origins of replication

A second type of putative NPV origin of replication, referred to as non-*hr* origins (non-*hr oris*), has been described in AcMNPV (Kool *et al.*, 1994b), OpMNPV (Pearson *et al.*, 1993), and SeMNPV (Heldens *et al.*, 1997). Non-*hr oris* contain unique palindromic and repetitive sequences that are not found in baculovirus *hr* sequences and are relatively complex in organization. Comparison of the non-*hr oris* from AcMNPV, OpMNPV, and SeMNPV demonstrates some striking similarities with the consensus *oris* of eukaryotes as proposed by DePamphilis (1996).

Only one copy of a non-*hr* sequence was identified in the genome of AcMNPV (Kool *et al.*, 1994b). Sequences in the AcMNPV *Hind*III-K region (84.9 to 87.3 map units, m.u.) support replication of plasmids in transient replication assays (Kool *et al.*,

1994b) and become enriched in defective AcMNPV genomes (Lee & Krell, 1994). Deletion analysis of the *Hind*III-K fragment indicated that the sequences required for optimal replication are contained within a relatively large region between 84.9 and 85.9 m.u., within the *p94* gene. The function of *ori*-K *in vivo* is unknown, but its conservation in defective AcMNPV genomes (Lee & Krell, 1994) and in the genome of BmNPV, which is closely related but lacks the *p94* gene (Kool *et al.*, 1994b), suggests that non-*hr* elements may play an important role in the replication of NPVs. Using a method of origin mapping by competitive PCR, Habib and Hasnain (2000) demonstrated that AcMNPV DNA replication is initiated at the *Hind*III-K origin region throughout the viral replication phase, with maximal utilization of the *Hind*III-K origin in the late replication phase.

Deletion analysis of the OpMNPV non-*hr* sequence, located within the *Hind*III-N fragment, revealed a complex organization, since deletion of any portion of the *Hind*III-N fragment resulted in reduced replication efficiency, suggesting that sequences affecting *ori* activity were distributed throughout the fragment. Sequence analysis identified a variety of direct and inverted repeat sequences, and palindromic sequences (Pearson *et al.*, 1993). The non-*hr* sequence of SeMNPV (Heldens *et al.*, 1997) was mapped to a 800 bp within *Xba*I-F fragment. Sequence analysis revealed a unique distribution of six different imperfect palindromes, several polyadenylation motifs, multiple direct repeats, and several putative transcription factor binding sites.

1.5.2. Genes involved in baculovirus DNA replication

Large viruses, such as herpes, vaccinia viruses and baculoviruses (80-300 kb genome size) and bacterial phages, such as T4 and T7, contain several genes encoding enzymes that direct the synthesis of precursor proteins as well as a relatively complete and independent replication apparatus. NPVs are believed to encode most of their own DNA replication machinery as well as other enzymes required for nucleotide metabolism including a ribonucleotide reductase (van Strien *et al.*, 1997). The development of a transient DNA replication assay in which origin-containing plasmids are replicated by transfected NPV sequences that supply *trans*-acting factors, led to major advances in the

identification of the essential NPV DNA replication genes. Using this procedure, six genes (*dnapol*, *p143*, *ie-1*, *lef-1*, *lef-2*, and *lef-3*) in AcMNPV have been shown to be both necessary and sufficient for origin dependent DNA replication in tissue culture cells. In addition, three genes (*p35*, *ie-2*, and *pe-38*) that stimulate transient replication were identified in AcMNPV (Lu *et al.*, 1997; Kool *et al.*, 1994a) and OpMNPV (Ahrens *et al.*, 1995a). The functions of these proteins are summarized in Table 1.1.

Table 1.1. Baculovirus DNA replication proteins

Essential DNA replication proteins			
Protein	Gene	Size (kDa)	Activities
DNA polymerase	<i>dnapol</i>	114	DNA polymerase, 3'-5' exonuclease
SSB	<i>lef-3</i>	44	single-stranded DNA-binding protein
IE-1	<i>ie-1</i>	67	DNA-binding protein, transactivator
Primase	<i>lef-1</i>	23	transactivator, DNA primase
Primase accessory factor	<i>lef-2</i>	30	NTPase, primase accessory factor
DNA helicase	<i>p143</i>	143	5'-3' DNA helicase, NTP binding
Nonessential DNA replication proteins			
IE-2	<i>ie-2</i>	47	transactivator
P35	<i>p35</i>	35	antiapoptosis
PE-38	<i>pe-38</i>	38	transactivator

DNA polymerase

Early studies of NPV-infected cells demonstrated the presence of a novel DNA polymerase activity that was distinct from host cell DNA polymerases (Miller *et al.*, 1981; Wang & Kelly, 1983). A 3'-5' exonuclease activity, specific for single-stranded DNA, was shown to be associated with the DNA polymerase of BmNPV (Mikhailov *et al.*, 1986). Sequence analyses from baculovirus DNA polymerases have revealed a virus-encoded DNA polymerase that shares significant amino acid sequence structural and functional similarity with family B DNA polymerases (Tomalski *et al.*, 1988; Bulach *et al.*, 1999; Huang & Levin, 2000). In AcMNPV, DNA polymerase was purified either from virus-infected cells or from cells infected with recombinant virus. Functional analyses of these native or recombinant DNA polymerases demonstrated conventional DNA polymerase and exonuclease activities (Hang & Guarino, 1999; McDougal & Guarino, 1999). Furthermore, the recombinant protein was shown to process processivity and moderate strand-displacement activity. The strand-displacement ability of the DNA polymerase was stimulated by a single-stranded DNA-binding protein (SSB) encoded by the viral gene, *lef-3* (McDougal & Guarino, 1999).

Helicase

DNA helicases are essential for the replication of double-stranded DNA. Helicases aid in progressively catalyzing strand displacement ahead of a growing polynucleotide chain and, thus, are critical enzymes for semi-conservative DNA replication (Matson & Kaiser-Rogers, 1990). While ATP is the preferred energy source, helicases bind and hydrolyze the γ -phosphates of NTPs. The ATPase activity of helicase is DNA dependent or DNA stimulated. The energy released by ATPase activity is coupled to the breaking of hydrogen bonds in duplex DNA or to translocation of helicase along DNA (McDougal & Guarino, 2000). A baculovirus gene with limited sequence similarity to helicases was identified by sequencing of an open reading frame (ORF) containing a temperature-sensitive mutation that resulted in virus defective for DNA synthesis (Lu *et al.*, 1991). This gene encodes a predicted protein of 143 kDa that contains a number of motifs characteristic of helicases including NTP binding and DNA

/RNA unwinding domains. P143 has been shown to possess a nonspecific double-stranded DNA binding activity (Laufs *et al.*, 1997). A more recent report indicated that AcMNPV P143 bound single-stranded DNA (ssDNA), supporting its function as a helicase (McDougal & Guarino, 2000). In addition, sequences in the putative helicase that are involved in specifying host-range have been identified. Recombinant AcMNPVs, in which the region between amino acids 536 and 584 of the *p143* gene are exchanged with the homologous region from BmNPV, enable the mutant AcMNPV to replicate in Bm5 cells and kill *B. mori* larvae (Kamita & Meada, 1996).

IE-1

Immediate early gene 1 (*ie-1*) is the only baculovirus gene for which splicing has been reported (Chisholm & Henner, 1988). In AcMNPV, the unspliced form encodes a protein with a predicted molecular mass of 67 kDa, whereas splicing results in 54 additional amino acids at the N-terminus (Lu *et al.*, 1997). Plasmids expressing unspliced IE-1 are essential for transient baculovirus DNA replication (Ahrens & Rohrmann, 1995b). IE-1 activates a variety of baculovirus early gene promoter-reporter constructs when they are cotransfected into uninfected insect cells (Lu & Carstens, 1993). This activation is greatly enhanced when the constructs are linked to *hr* sequences. The requirement for *ie-1* in baculovirus DNA replication may result from its function in activating the expression of early genes, some of which are required for viral DNA replication; however, the direct role in origin binding and initiation of the early steps leading to the assembly of a replication complex is still unclear (Lu *et al.*, 1997).

LEF-1 and LEF-2

The *lef-1* gene has been identified in AcMNPV (Passarelli & Miller, 1993), OpMNPV (Ahrens & Rohrmann, 1995a), and CfMNPV (Barrett *et al.*, 1996). The *lef-1* gene was initially recognized as an early gene important for late and very late gene expression (Passarelli & Miller, 1993). It was later shown that *lef-1* was essential for transient DNA replication for both AcMNPV and OpMNPV (Passarelli & Miller, 1993; Ahrens & Rohrmann, 1995a). Alignment of LEF-1 from these NPVs revealed four conserved domains homologous to the DNA primase genes of several organisms,

suggesting that LEF-1 may be an NPV primase. LEF-2 is essential for baculovirus late gene expression in transient expression assays (Passarelli & Miller, 1993) and was also found to be essential for DNA replication (Ahrens & Rohrmann, 1995a). Yeast two-hybrid and glutathione S transferase interaction assays indicated that LEF-2 interacted with LEF-1, suggesting that these proteins may form a hetero-oligomeric complex involved in replication (Evans *et al.*, 1997). Characterization of the interaction between LEF-1 and LEF-2 indicated that LEF-1 contains a primase motif and LEF-2 may be a primase accessory factor (Evans *et al.*, 1997).

LEF-3

The binding of SSB proteins favors single-stranded regions resulting from DNA breathing in regions of double-stranded DNA. This destabilizes the double-helix structure and reduces the temperature required for its melting. For this reason, SSB proteins are called "helix-destabilizing proteins" (Chase & Williams, 1986; Meyer & Laine, 1990). AcMNPV *lef-3*, an essential gene for DNA replication in transient assays, encodes a polypeptide of 385 amino acids (44 kDa). Biochemical evidence suggests that the AcMNPV LEF-3 is a single-stranded DNA binding protein (Hang *et al.*, 1995). The purified SSB protein had a preference for single-stranded DNA and demonstrated nonspecificity and cooperativity of binding on DNA. Further investigation revealed that LEF-3 interacts with itself to form a homotrimer and that this interaction is essential for the proper function of LEF-3 (Evans & Rohrmann, 1997). In addition to its single-stranded DNA binding activity, AcMNPV LEF-3 was shown to interact with P143 and mediated nuclear translocation of P143 (Wu & Carstens, 1998).

It has been demonstrated that the *dbp* gene from the BmNPV encodes a 38 kDa DNA-binding protein (DBP) that can destabilize duplex DNA (Mikhailov *et al.*, 1998). While BmNPV DBP could destabilize duplexed DNA, LEF-3 could not, suggesting that LEF-3 may not play a role as a "helix-destablizing protein" in the baculovirus replication initiation complex. However, LEF-3 may function as an SSB in other roles during viral DNA replication. LEF-3 has been co-purified with the viral helicase gene product (Evans *et al.*, 1997; Laufs *et al.*, 1997), suggesting that it may associate with the helicase during replication. In contrast to *lef-3*, BmNPV *dbp* was not found to be essential for transient

replication assays with AcMNPV and OpMNPV (Kool *et al.*, 1995; Ahrens *et al.*, 1995a). It is possible that a host-encoded nuclear SSB protein, such as RPA, may substitute for DBP and that this protein (and/or other factors) may permit initiation of viral DNA replication in transient replication assays (Mikhailov *et al.*, 1998).

P35, IE-2 and PE-38

Baculoviruses possess two types of genes with antiapoptotic activity, *iap* and *p35*, which can suppress apoptosis induced by virus infection or by diverse stimuli in vertebrates or invertebrates (Clem, 1997). The AcMNPV *p35* gene greatly stimulated DNA replication (Kool *et al.*, 1994a) and is an inhibitor of AcMNPV-induced apoptosis in Sf9 cells. Its major role in the replication could be to inhibit apoptosis by preventing infected cells from dying during the course of infection. The role of P35 in transient expression assays is to prevent apoptosis in transfected cells triggered by either plasmid DNA replication or a product of one or more replication genes, such as the *ie-1* gene. Apoptosis induced by transient expression of *ie-1* may be related to potentially high levels of IE-1 expression in transfected cells (Prihodko & Miller, 1996). Therefore, P35 may be stimulatory in the replication assay because it suppresses death of transfected cells caused by their response to IE-1 or a combination of IE-1 expression and plasmid DNA replication.

Two other genes, *ie-2* and *pe-38*, which stimulate DNA replication, encode transactivators of early gene transcription (Lu & Carstens, 1993). AcMNPV IE-2 is a 47 kDa nuclear-associated protein that stimulates plasmid DNA replication through the indirect transactivation of genes essential for replication (Kool *et al.*, 1995). The immediate early gene, *pe-38*, encodes a 38 kDa nuclear protein that contains an N-terminal RING finger and a C-terminal leucine zipper motif, typical of transcriptional regulator (Wu *et al.*, 1993; Krappa & Knobel-Morsdorf, 1991). In particular, PE-38 contributes to the activation of the baculovirus helicase expression, whereas IE-2 stimulates *pe-38* and *ie-1* expression. The stimulatory role of IE-2 and PE-38, therefore, may involve their activation of essential replication genes (Lu *et al.*, 1997).

1.5.3. Protein-protein interactions

The important events during DNA replication are mediated by specific protein-protein interactions. These protein-protein interactions serve to assemble multiple protein complexes that recruit other essential proteins to the origin of DNA replication and stabilize the replication forks to promote efficient DNA replication. Researchers have begun to address both the functional and physical interactions among the NPV DNA replication proteins. These investigations are stimulated by the identification of essential genes and by characterization of these gene products required for origin-specific DNA replication in other viruses, such as herpes simplex virus (HSV) (Boehmer & Lehman, 1997). Based on sequence alignment analyses, it has been suggested that the NPV replisome includes a DNA polymerase, a helicase, a primase, a primase-associated protein, and SSB involved in origin recognition and stabilization of single-stranded regions of the replication fork (Lu *et al.*, 1997). The presence of conserved amino acid motifs found in other replicative proteins strongly suggest that DNAPOL, P143, and LEF-1 of NPVs function within the replisome complex as a DNA polymerase, a helicase, and a primase, respectively. Since LEF-3 cooperatively binds to single-stranded DNA, its role in DNA replication may be to bind to single-stranded DNA formed at the replication fork by the unwinding of parental duplex DNA by P143. In HSV, the helicase is a component of a multisubunit complex that contains helicase/primase and DNA-binding activities. If a similar complex is found in NPV-infected cells, then P143 may interact with LEF-1, possibly through its leucine zipper motif (Lu & Carstens, 1991). The interaction of LEF-1 and LEF-2 in the yeast two-hybrid system (Evans *et al.*, 1997) suggests that LEF-2 might function as a primase-associated protein.

The origin binding protein in HSV is encoded by the *UL9* gene. In addition to its origin DNA binding activity, the UL9 protein possesses DNA-stimulated nucleoside triphosphatase and DNA helicase activities (Boehmer & Lehman, 1997). In contrast, a protein equivalent to UL9 protein has not been identified in NPVs. However, there are some candidates that show properties of UL9 activity. IE-1 is a promising candidate, given its requirement in transient replication assays and its ability to bind to *hrs*. If IE-1 conducts origin-binding activity, then the DNA unwinding activity normally associated with origin-binding proteins such as UL9 may be supplied by P143, since it is the only

protein in the baculovirus proteins containing a conserved ATP-binding motif (Lu *et al.*, 1997). Thus, the initiation of DNA replication would be mediated by the binding of IE-1 to the *hrs*, leading to localized melting of duplex DNA that would allow the assembly of a complex of P143, LEF-1, and LEF-2 at the origin. This complex would subsequently also prime lagging-strand DNA synthesis carried out by DNA polymerase, while simultaneously unwinding DNA at the replication fork. The single-stranded regions resulting from unwinding of the DNA would be stabilized by LEF-3. The contribution of accessory factors such as P35, IE-2, and PE-38, would then be to maximize DNA replication in a specific host, or the presence of host-factors that may otherwise interfere with the viral replication process (Wu & Carstens, 1998; Lu *et al.*, 1997). Following initiation at an origin(s), it is believed that DNA replication proceeds by a rolling-circle mechanism that generates long head-to-tail concatemers that are concomitantly cleaved into unit-length genomes and packaged into preformed capsids (Leisy & Rohrmann, 1993).

1.6. Baculovirus for insect control

Two properties of baculoviruses have made their use as bioinsecticides particularly attractive. First, they are highly pathogenic to permissive invertebrate hosts and established infection results in death of the host, although sublethal infections may result in slower developmental rates, lower pupae and adult weights, shorter adult longevity, and reduced reproductive capacity (Rothman & Myers, 1996). Second, they have a remarkable degree of host-specificity. NPVs have been isolated only from arthropods, with most isolates infecting only a narrow range of closely related insect species (Groner, 1987). Wild-type baculoviruses are an integral component of the natural biological control of many species, and application of wild-type viruses has been very effective for pest management in several cases (Bonning & Hammock, 1996). However, these wild type viruses have limited success for various reasons. The main drawback is the relatively long time taken to suppress pest populations below economic thresholds. Another deterrent to commercialization of baculoviruses as insect control agents is their

limited market size because of the high degree of host-specificity and narrow host-range displayed by most NPVs (Bonning & Hammock, 1996).

The opportunity to enhance the insecticidal potential of baculoviruses arose with the advent of recombinant DNA technology. Genetic engineering of NPVs to reduce the time taken by the virus to kill the host insect, and to extend the host range, will yield viruses that are more economically competitive with classical insecticides. The aim of genetic engineering of NPVs for use as insecticides is to combine the pathogenicity of the virus with the insecticidal action of an insect-specific bioactive molecular. This has been accomplished by deleting certain viral genes that delay host mortality and/or with insertion of genes encoding insecticidal proteins and other insecticidal products, such as insect-specific neurotoxins, modified enzymes (juvenile hormone esterase), and growth regulators (Maeda, 1995; Wood & Granados, 1991; Bonning & Hammock, 1996; Black *et al.*, 1997).

1.7. Baculovirus as gene expression and gene delivery vectors

An important consideration of expression of cloned genes in recombinant expression systems is the ability of the foreign host to produce the protein in a form that is similar to or identical to its authentic form (Makrides, 1999). Three important features of NPVs account for the success of these viruses as expression vectors. First, the viral genome contains a number of nonessential genes that can be replaced by an exogenous gene. Second, many of these non-essential genes, particularly the very late genes, are under the control of powerful promoters that allow abundant expression of the exogenous gene. Third, the protein expressed in this system is often very similar to its authentic counterpart; recombinant proteins are appropriately modified, processed, secreted, and correctly folded to give high yields of biologically active proteins. These include cytosolic, nuclear, mitochondrial, membrane bound, and secreted proteins, such as human interferon, Human Factor VIII, tissue-plasminogen activator, β -galactosidase, c-myc, interleukin-2, and influenza hemagglutinin (Miller, 1993; Smith *et al.*, 1992; Jones & Morikawa, 1996; Jarvis, 1997; Possee, 1997; Jarvis *et al.*, 1998; Pfeifer, 1998). This system is also being used for studying the viral particle assembly processes and for the

development of vaccine candidates based on the production of virus-like particles (VLPs) and conventional recombinant antigens (Newcomb *et al.*, 1996). Co-expression of proteins using multiple recombinant baculoviruses can be achieved not only to enhance the production of functional proteins, but also to study the protein-protein interactions.

Gene therapy is a rapidly emerging field that aims to treat a variety of genetic or acquired diseases through the transfer of functional genetic material into cells both *in vitro* and *in vivo* (Anderson, 1992; Miller, 1992). Critical to the success of gene therapy is the development of safe and efficient gene transfer vehicles. Various strategies have been developed for the transfer of therapeutic genes, which include viral and nonviral vectors. Among the viral vectors utilized for gene transfer protocols, adenovirus (Ad) vectors deliver genes to a wide variety of cell types and tissues independently of their proliferative state (Bramson *et al.*, 1995). The major disadvantage of this type of vector is the instability of the genes transferred into the target cell and the substantial pathology that develops at the site of gene transfer. Retroviruses, the viral vectors currently most widely used, offer the desirable feature of being able to insert a gene of interest into the host genome, thus contributing to the stability of the transduced gene (Smith, 1995). However, retroviruses have a limited host range, and successful infection occurs only in mitotic cells, with the exception of the human immunodeficiency virus (Lewis *et al.*, 1992). Also, retroviruses integrate randomly into the host cell chromosome, which has raised concern about the potential activation of transcriptionally silent oncogenes, as well as the possible inactivation of tumor suppressor genes mediated by insertional mutagenesis (Smith, 1995). The adeno-associated virus (AAV) is used for gene delivery protocols as well, because of the lack of obvious pathogenic effects associated with AAV infection and the stability of the viral particle.

Recently, a hybrid baculovirus was used as a gene delivery system in mammalian cells. The recombinant NPV contained expression cassettes, controlled by mammalian promoters, flanked by the inverted terminal repeats (ITR) of AAV, to take advantage of the ability of AAV to integrate its genome into that of its host cells. The recombinant virus gave rise to a low frequency of stable colonies of transformed kidney 293 cells. It was suggested that the frequency could be increased if the virus is able to direct expression of the AAV *rep* gene. The ITR-flanked cassettes directed integration of the

recombinant virus into the host genome at the specific site on chromosome 19q13.3 that is characteristic of AAV integration (Palombo *et al.*, 1998). However, there is evidence indicating that blood components interact with baculovirus, suggesting further work should be done for enabling to use recombinant NPVs as *in vivo* gene therapy vectors (Sandig *et al.*, 1996).

The use of NPV chimeras to launch the infection of another virus in mammalian cells has been another area of interesting research. This could be a valuable approach to the study of viruses that lack a suitable *in vitro* infection model (Kost & Condreay, 1999). The first efforts in this research have been in hepatotropic viruses, as studies of baculovirus-mediated gene delivery to mammalian cells suggested that hepatic cells were most susceptible (Condreay *et al.*, 1999). A hepatitis B virus (HBV) genome was placed into a recombinant NPV in the antisense orientation to the polyhedrin promoter. This construct contains sufficient contiguous HBV sequences to synthesize all of the HBV mRNAs from its endogenous promoters in liver-derived cell lines (Delaney & Isom, 1998). Upon transduction of NPV-HBV hybrid into human hepatoma HepG2 cells, high levels of HBV gene products are detected and extracellular HBV virions are produced. Another report has exploited NPV transduction to study the replication of hepatitis C virus (HCV) by placing the entire HCV cDNA under the control of the cytomegalovirus (CMV) promoter in a recombinant NPV. Transduction of Human hepatoma HuH7 cells with the NPV-HCV elicited long-term expression of the HCV polyprotein and its correct processing into HCV structural and non-structural gene products (Fipaidini *et al.*, 1999). The use of recombinant NPV containing mammalian gene regulatory elements will prove to be a useful tool for gene delivery and expression in mammalian cells.

1.8. The *Spodoptera littoralis* nucleopolyhedrovirus and research objectives

The *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV) is a member of the *Baculoviridae* (Volkman *et al.*, 1995). SpliNPV was isolated from lepidopteran insect, *S. littoralis*, which is a polyphagous pest of economically important field- and greenhouse-grown crops worldwide (Jones *et al.*, 1994). The ability of SpliNPV to successfully infect several *Spodoptera* species, including *S. exigua*, *S. exempta*, *S. frugiperda*, and *S. litura*,

makes it a suitable candidate for use as a microbial pest control agent. SpliNPV replicates well in cell lines established from *S. frugiperda* (Sf9 and Sf21) and *S. littoralis* (CLS79), but does not grow in cell lines derived from *Trichoplusia ni* (TN368) or *B. mori* (BmN) (Maeda *et al.*, 1990). Two reports have suggested that SpliNPV can infect species outside the Order *Lepidoptera*: one suggested that SpliNPV infected two species of locust (*Orthoptera*); the African migratory locust, *Locusta migratoria migratorioides*; and the desert locust, *Schistocerca gregaria* (Bensimon *et al.*, 1987). The other study indicated that an uncharacterized SpliNPV isolate from Egypt could infect the wood-dwelling termite (*Isoptera*), *Kaloterms flavicollis* (Fazairy & Hassan, 1988).

SpliNPV appears distantly related to AcMNPV and other more extensively studied baculoviruses. Recent molecular studies have classified SpliNPV as a Group II NPV of the *Baculoviridae* (Volkman *et al.*, 1995; Zanotto *et al.*, 1993). Phylogenetic analyses (Hu *et al.*, 1997; Levin *et al.*, 1997; Smith & Goodale, 1998) have suggested that SpliNPV represents a more ancient lineage of NPVs that is distantly related to more commonly studied NPVs that cluster together in a clade referred to as the Group I NPVs (Zanotto *et al.*, 1993). Nucleotide sequence analyses of several SpliNPV genes [*polh* (Croizier & Croizier, 1994; Faktor *et al.*, 1997a), *egt* (Faktor *et al.*, 1995), *p10* (Faktor *et al.*, 1997b), *rr1* (van Strien *et al.*, 1997), *lef-3* (Wolff *et al.*, 1998), *lef-8* (Faktor & Kamenski, 1997), and *p49* (Du *et al.*, 1999)] have revealed a number of unique features about this virus that are not found in other NPVs studied to date.

With the progress of biotechnology and development of recombinant baculovirus as gene expression and delivery vectors, interest in molecular baculovirology continues to increase. To date, five NPV genomes have been sequenced completely: AcMNPV (Ayres *et al.*, 1994), BmNPV (Gomi *et al.*, 1999), OpMNPV (Ahrens *et al.*, 1997), LdMNPV (Kuzio *et al.*, 1999), and SeMNPV (Ijkel *et al.*, 1999). Most of the molecular information of NPVs is, therefore, based on the studies of the Group I NPVs, such as AcMNPV, BmNPV, and OpMNPV, which are closely related. Moreover, the mechanisms of NPV DNA replication are not well understood and the mechanisms of NPV host-specificity are still unresolved. To further study the mechanisms of DNA replication and gene expression by which viruses rearrange the cellular environment in the process of virus replication, I have studied SpliNPV, a virus that is genetically distinct from the better known Group I NPVs.

First, I studied the SpliNPV infection of an orthopteran cell line derived from the grasshopper, *Melanopus sanguinipes*, and investigated viral DNA replication, production of viable virus progeny, and presence of virus particles in infected cells (Chapter 2). Second, I asked questions regarding the SpliNPV infection process in permissive, semi-permissive, and non-permissive cell lines. By studying the viral DNA replication, viral early gene and late gene transcription, and viral promoter transactivation in the presence of either homologous virus or heterologous virus, I was able to document the events that hampered the SpliNPV infection in semi- and non-permissive cell lines (Chapter 3). Third, having characterized the SpliNPV infection in different cell lines, I further investigated the *cis*-acting factor that determines the viral DNA replication initiation. Gel mobility shift analyses demonstrated that both host and viral proteins bind to the non-*hr* origin (Chapter 4). Fourth, central to understanding virus replication is the need to understand the functions of both *cis*-acting factors and *trans*-acting factors during viral replication initiation. I further identified and characterized the transcription of a *trans*-acting factor gene, the SpliNPV DNA polymerase gene, which showed substantial sequence similarity to other eukaryotic DNA virus and cellular DNA polymerases (Chapter 5). Fifth, it was of great interest to characterize the DNA polymerase protein. Using prokaryotic and baculovirus expression systems, I over-expressed the SpliNPV DNA polymerase protein (DNAPOL) and a mutant in which the first 80 amino acids were deleted, and demonstrated that the polymerase and exonuclease activities are intrinsic to the SpliNPV DNAPOL (Chapter 6). These studies are highly relevant to the future development of this virus as an efficient pest control agent in particular, and to molecular baculovirology in general.

Chapter 2. The *Spodoptera littoralis* Nucleopolyhedrovirus Infection of an Orthopteran Cell Line

2.1. Abstract

I have determined that the *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV) can infect the cell line, MSE4, derived from a grasshopper, *Melanopus sanguinipes*. I compared the infectivity of SpliNPV in two lepidopteran cell lines (Sf9 and Md210) and in the grasshopper cell line. Both Sf9 and MSE4 cells were permissive for SpliNPV replication and supported production of viable progeny. Md210 cells were nonpermissive for SpliNPV, and although the virus entered into these cells, they supported neither viral replication nor production of viable progeny. Infection of MSE4 cells with SpliNPV resulted in cytopathic effects within 48 hours post-infection and complete destruction of the cells within 5 days. Both virions and polyhedra were detected within virus-infected MSE4 cells by transmission electron microscopy. Extracellular virions were detected in the culture medium and were infectious to Sf9 cells, indicating that the MSE4 cells supported production of viable virus progeny.

2.2. Introduction

Two reports have suggested that SpliNPV can infect species outside the Order *Lepidoptera*: one suggested that SpliNPV infected two species of locust (*Orthoptera*), the African migratory locust, *Locusta migratoria migratorioides* and the desert locust, *Schistocerca gregaria* (Bensimon *et al.*, 1987); the other report indicated that an uncharacterized SpliNPV isolate from Egypt could infect wood-dwelling termites (*Isoptera*), *Kaloterms flavicollis* (Fazairy & Hassan, 1988). Faktor and Raviv (1996), however, were unable to detect the presence of virus in SpliNPV-infected *L. migratoria* by polymerase chain reaction (PCR).

The observation that SpliNPV can infect species outside the *Lepidoptera* is very unusual for NPVs, as most isolates infect only a narrow range of closely related insect species (Groner, 1987). Studies of SpliNPV infection of cultured insect cells indicated that SpliNPV replicates well in cell lines established from *S. frugiperda* (Sf9 and Sf21) and *S. littoralis* (CLS79) but does not grow in cell lines derived from *T. ni* (TN368) or *B. mori* (BmN) (Maeda *et al.*, 1990). In this report, I demonstrate that a cell line, MSE4, derived from the western migratory grasshopper, *Melanopus sanguinipes* (*Orthoptera*, *Acrididae*), was permissive for SpliNPV infection, lending support to the previous observations that SpliNPV can infect cells derived from an orthopteran insect, at least *in vitro*.

2.3. Materials and Methods

2.3.1. Cells and virus

Sf9 cells were obtained commercially (PharMingen). A *Malancosoma distria* cell line (*Lepidoptera*, *Lasiocampidae*), Md210, was obtained from Dr. A. Keddie, University of Alberta, Canada. The *M. sanguinipes* cell line, MSE4, was a gift from Dr. T. Kurtti, University of Minnesota, U.S.A. Cell lines were cultured according to described procedures (Sohi, 1971; Munderloh & Kurtti, 1989, Munderloh *et al.*, 1994). SpliNPV isolate M-2 was obtained from Dr. G. Croizier, Station de Recherches de

Pathologie Comparée INRA-CNRS, F-30380 Saint-Christol-Les-Ales, France. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (O'Reilly *et al.*, 1992).

2.3.2. RAPD analysis of cell lines

Reports of baculovirus replication in cell lines derived from non-host species have been tainted by cross-contamination with cells from permissive hosts (Markovic & Markovic, 1998). To ensure that the MSE4 cells used were indeed uncontaminated by the permissive lepidopteran cells used in my studies, I first performed random amplified polymorphic DNA (RAPD) analysis. DNA extractions from uninfected Sf9, MSE4 and Md210 cells were subjected to PCR, following the procedure of Kawai and Mitsuhashi (1997), with two modifications. First, Ready-To-Go RAPD analysis beads were used (Amersham Pharmacia) to provide optimized PCR buffer, dNTPs and thermal stable enzymes. Second, a primer (5'-GTAGACCCCGT-3', Amersham Pharmacia) was complimentary to the optimal primer described by Kawai and Mitsuhashi (1997) but contained three nucleotide mismatches (mismatches are underlined). The Kawai and Mitsuhashi (1997) primer was 5'-CCGCATCTAC-3' (compliment 5'-GTAGATGCGG-3'). PCR products were separated on a 1.0 % agarose gel in TAE buffer following standard procedures (Sambrook *et al.*, 1989), and photographed using the EagleEye system (Stratagene).

2.3.3. Infection of cells with SpliNPV

Cells were plated in 6-well plates at a density of 10^6 per well and infected with SpliNPV at an MOI of 10. After incubation for 1 h at 28°C, the infectious medium was removed and the cells were washed twice with phosphate-buffered saline (PBS) and then overlaid with fresh medium. Cells were incubated for various time points post infection, and harvested along with mock-infected cells. The supernatants were saved and stored at 4°C until use. To evaluate the ability of each cell line to support production of viable SpliNPV progeny, supernatants removed from virus-infected cells at 48 hours post

infection (hpi) were used to determine the titer of virus in the media by end-point dilution assays in Sf9 cells and for PCR experiments. The cell pellets were suspended in PBS, counted, pelleted and stored at -80°C until required. Cell pellets were used in dot blot hybridization assays for DNA replication, PCR, and electron microscopy.

2.3.4. Dot blot analysis of viral DNA replication

Cell pellets were resuspended in PBS and 2.0×10^5 cells were added to dot blot lysis buffer (0.4 M NaOH, 10 mM EDTA), heat denatured at 100°C for 10 min, and placed on ice. Samples were applied to a Hybond-N membrane (Amersham) using a dot blot manifold (Gibco/BRL) under vacuum. The membranes were washed twice with 0.4 M NaOH, rinsed twice with 2 X SSC buffer, dried and exposed to UV light for 2 min. The membranes were hybridized with total SpliNPV genomic DNA that was gel purified. Probe DNA was labeled by random primed PCR, according to the protocol provided in the Tag-It Kit (Bios). The extent of hybridization was visualized by exposing and quantified by using EagleEye photo-documentation system. Data derived from this analysis was plotted with Microsoft Excel 7.0.

2.3.5. Polymerase chain reaction

To confirm the presence of SpliNPV DNA within infected cells and to determine whether viral progeny were extruded into the culture medium, cell pellets and supernatants were subjected to PCR analysis using SpliNPV *polh*-specific primers. The primers (forward 5'-TCATGAACATGTTCCACAATATGC-3'; reverse 5'-TTAAAGACGCAGAGCG-3') were generated using the SpliNPV *polh* sequence (Croizier & Croizier, 1994). Supernatants from mock-infected or virus-infected cells were centrifuged at 15,000g for 30 min to pellet extracellular virions. The pellets were washed twice with PBS and centrifuged at 15,000g for 15 min. Cell pellets or pellets from supernatants were used as templates for PCR. PCR reactions contain 10 mM Tris-HCl, pH 9.0, 60 mM KCl, 2.5 mM MgCl_2 , 200 μM each dNTP, 144 nM each primer, 0.45% Triton X-100, 1.45 % Tween 20, and 10 mg/ml proteinase K. PCR reactions were

incubated in a thermocycle machine (Perkin-Elmer 9600) at 60°C for 1 h and then at 95°C for 10 min to inactivate the proteinase K. Taq DNA polymerase (10 units/reaction) was added and the reactions were subjected to 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, following by extension at 72°C for 60 s. The PCR products were separated on a 0.8 % agarose gel (Sambrook *et al.*, 1989).

2.3.6. Electron microscopy

Supernatants from mock-infected or virus-infected cells were centrifuged at 15,000g for 30 min to pellet extracellular virions. Pellets were resuspended and washed twice with sterile distilled, deionized water (ddH₂O) by centrifugation at 15,000g for 15 min each and resuspended in a final volume of 20 ul of ddH₂O. One-tenth of the volume was spotted onto copper grids and incubated in 0.5 M phosphorystic acid for 30 s. The excess solution was absorbed and the grids were allowed to stand at room temperature for 2 min to dry and subjected to transmission electron microscope (TEM) examination. Mock-infected or SpliNPV-infected cell pellets were washed twice in PBS, fixed, and embedded, following standard procedures. All samples were examined with a Hitachi H-7000 transmission electron microscope.

2.4. Results

2.4.1. Cytopathic effect

The banding patterns derived from RAPD analysis of Sf9, MSE4, and Md210 cell lines were clearly different, demonstrating unequivocally that the MSE4 cell line was distinct from other cell lines used in this study and that the MES4 cell line was not contaminated with permissive Sf9 cells (Figure 2.1). Cytopathic effects were observed in SpliNPV-infected Sf9 cells within 48 hpi and polyhedral inclusion bodies were clearly visible by 5 days post infection (Figure 2.2). The average titer of budded virions in the supernatants of SpliNPV-infected Sf9 cells was 1.4×10^8 TCID₅₀ units/ml at 48 hpi (Table 2.1). Cytopathic effect was visible in MSE4 cells within 48 hpi and many cells were clearly moribund by 5 days post infection. While uninfected MSE4 cells were

predominantly squamous in shape, SpliNPV-infected cells were small and round, and some cells appeared to contain small, highly refractive bodies within them that resembled PIBs. SpliNPV-infected Md210 cells did not exhibit cytopathic effects and appeared to grow at the same rate as uninfected MD210 cells.

Incubation of Sf9 cells with supernatants from SpliNPV-infected MSE4 cells resulted in productive infection, and titration by end-point dilution indicated a virus titer of 2.8×10^5 TCID₅₀ units/ml (Table 2.1) at 48 hpi. Incubation of Sf9 cells with supernatants from SpliNPV-infected Md210 cells did not result in infection, indicating that these cells did not support production of viable progeny (Table 2.1).

2.4.2. Viral DNA replication

Dot blot analyses revealed that SpliNPV replication occurred in Sf9 and MSE4 cell lines, but not in Md210 cells, which did not exhibit a time-dependent increase in intracellular viral DNA (Figure 2.3). SpliNPV DNA was detected in all cells within 4 hpi (Figure 2.3a). The intensity of hybridization with SpliNPV DNA from virus-infected Sf9 cells decreased between 4 and 12 hpi and then increased rapidly from 16 to 48 hpi (Figure 2.3b). The intensity of hybridization with SpliNPV DNA from virus-infected MSE4 cells decreased between 4 and 8 hpi and then slowly increased from 12 to 48 hpi. While the level of SpliNPV replication was approximately fourfold lower in MSE4 cells than in Sf9 cells, replication was clearly detected. SpliNPV entered into, and was detectable within Md210 cells, within 4 hpi. However, the amounts of SpliNPV DNA detected in Md210 cells steadily decreased in intensity from 4 to 48 hpi, suggesting that SpliNPV did not replicate in these cells.

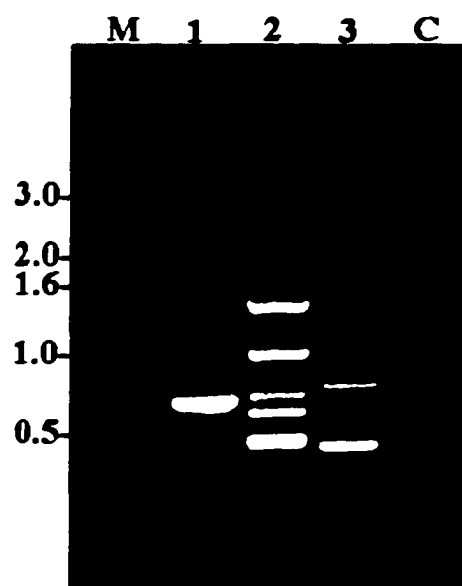


Figure 2.1. RAPD analysis of Sf9, MSE4, and Md210 cell lines. Agarose gel electrophoresis of PCR products after RAPD analysis of Sf9 cells (lane 1), MSE4 cells (line 2), and Md210 cells (line 3). M, 1-kb ladder used as molecular size marker; C, control PCR lacking DNA template.

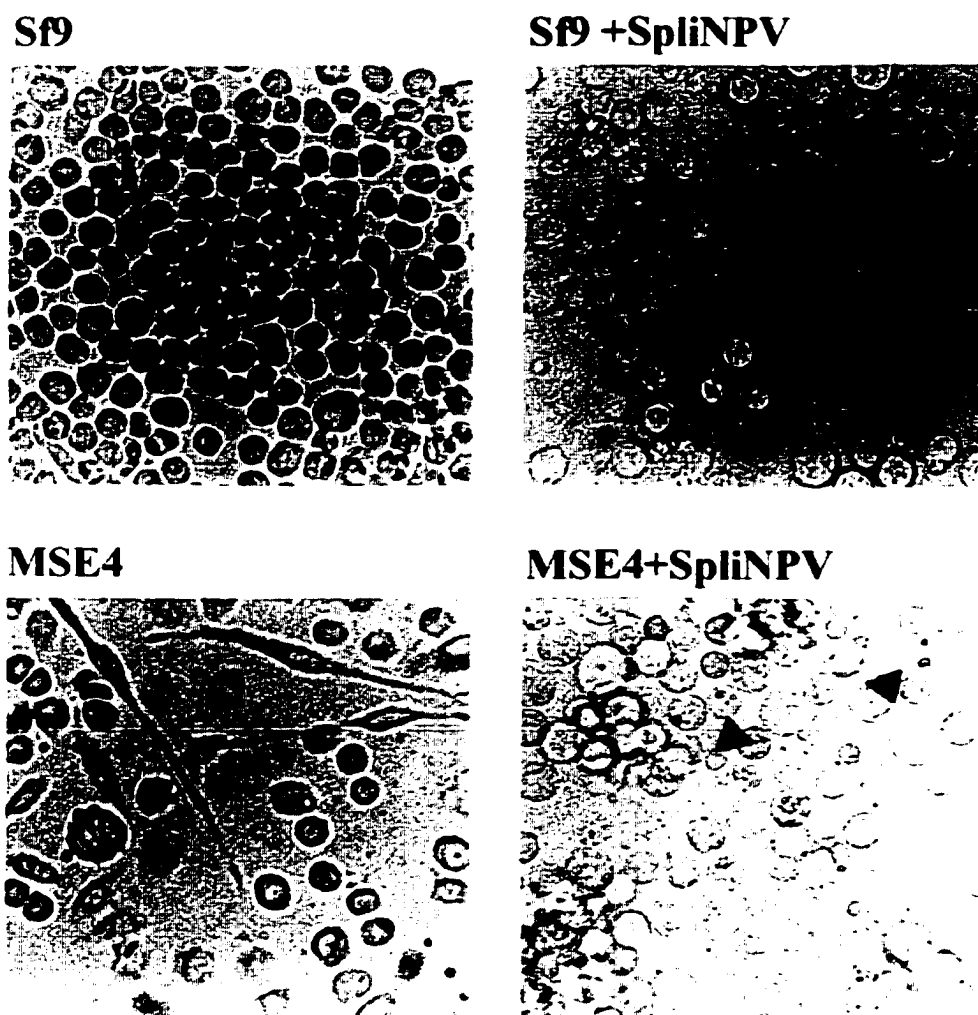


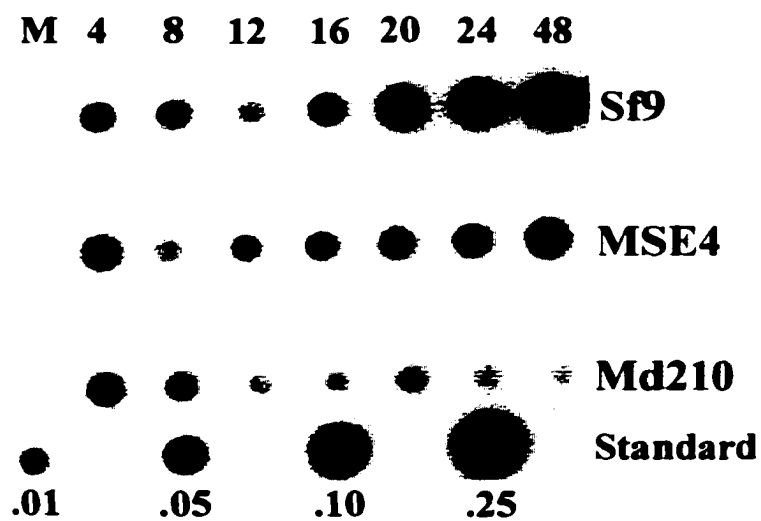
Figure 2.2. SpliNPV infection in Sf9 and MSE4 cells. Mock-infected and SpliNPV-infected Sf9 and MSE4 cells, photographed at five days post infection.

Table 2.1. Budded virus production from SpliNPV-infected cell lines

Cell line	TCID ₅₀ at 48 hpi*
Sf9	1.4 X 10 ⁸
MSE4	2.8 X 10 ⁵
Md210	No productive infection

* Media were removed from SpliNPV-infected cells (of the type indicated) at 48 hpi and titrated using fresh Sf9 cells. Indicated TCID₅₀s were obtained from the average of three separate titration experiments for each cell line.

(a)



(b)

SpliNPV DNA replication in selected cell lines

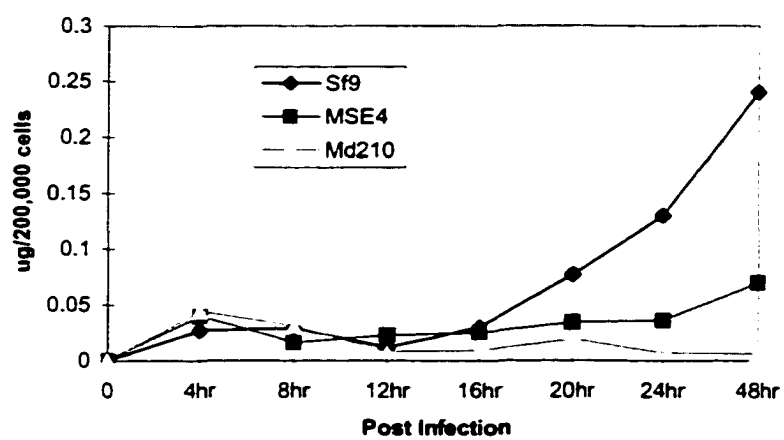


Figure 2.3. Dot blot replication assays of SpliNPV-infected cell lines. a) SpliNPV DNA synthesis in two lepidopteran cell lines and a grasshopper cell line. Dot blot of total cellular DNA from 2.0×10^5 mock-infected (M) or SpliNPV-infected Sf9, MSE4, and Md210 cells with SpliNPV at an MOI of 10, harvested at indicated time points, probed with labeled SpliNPV DNA. Known amounts of SpliNPV DNA, as indicated, were used as standards. **b)** Graphic representation of the data after integration of the density of each spot on the Dot blot autoradiogram. Quantities of viral DNA (ug/200,000 cells) were calculated using known amounts of SpliNPV DNA as standards in the Dot blot.

2.4.3. Viral specific gene amplification

PCR amplification of the SpliNPV *polh* gene confirmed the presence of SpliNPV DNA within all three virus-infected cell lines (Figure 2.4a). The SpliNPV *polh* PCR product was also detected in the supernatants of infected Sf9 and MSE4 cells (Figure 2.4b, lanes 2 and 3), suggesting that both of these cell lines extruded extracellular virions. The *polh* PCR product was not detected, however, in the medium of SpliNPV-infected Md210 cells (Figure 2.4b, lane 3).

2.4.4. Virion detection by electron microscopy

Transmission electron microscopy confirmed the presence of SpliNPV budded virions in the supernatants from infected Sf9 and MSE4 cells (Figures 2.5A and 2.5B, respectively). Furthermore, electron microscopic studies provided the greatest source of information on SpliNPV infection in Sf9 and MSE4 cells (Figures 2.5C and 2.5D, respectively). Virogenic stroma were observed and virus particles were visibly interspersed in the stromal network. Polyhedra formation was detected by 48 hpi and enveloped virions were occluded within the polyhedra of SpliNPV-infected Sf9 and MSE4 cells (Figures 2.5E and 2.5F, respectively). The polyhedra were surrounded by an electron dense polyhedron membrane, which is believed to form by the condensation of a fibrous substance associated with developing occlusion bodies (Federici, 1997).

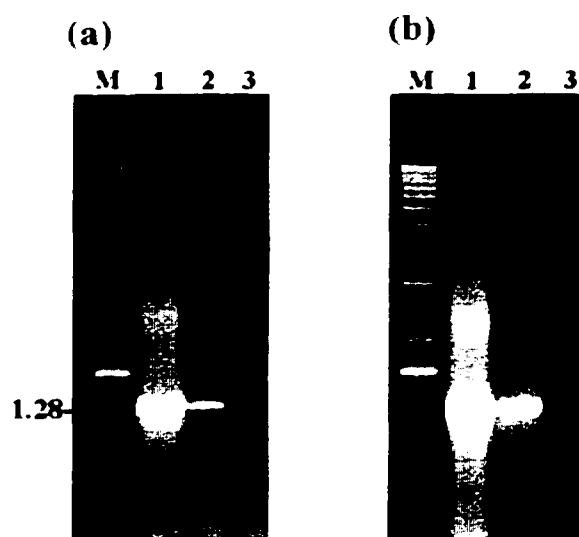


Figure 2.4. PCR analysis of cell pellets and supernatants from infected cells. PCR analysis using SpliNPV polyhedrin gene-specific primers to amplify a 1.28-kb fragment from cell pellets or supernatants of SpliNPV-infected cell lines. **a)** PCR of intracellular virus from cell pellets of SpliNPV-infected Sf9 (lane 1), MSE4 (lane 2), and Md210 (lane 3) cells harvested 48 hpi. **b)** PCR of extracellular virions from supernatants of SpliNPV-infected Sf9 (lane 1), MSE4 (lane 2), and Md210 (lane 3) cells harvested at 48 hpi. M, 1-kb ladder used as molecular size marker.

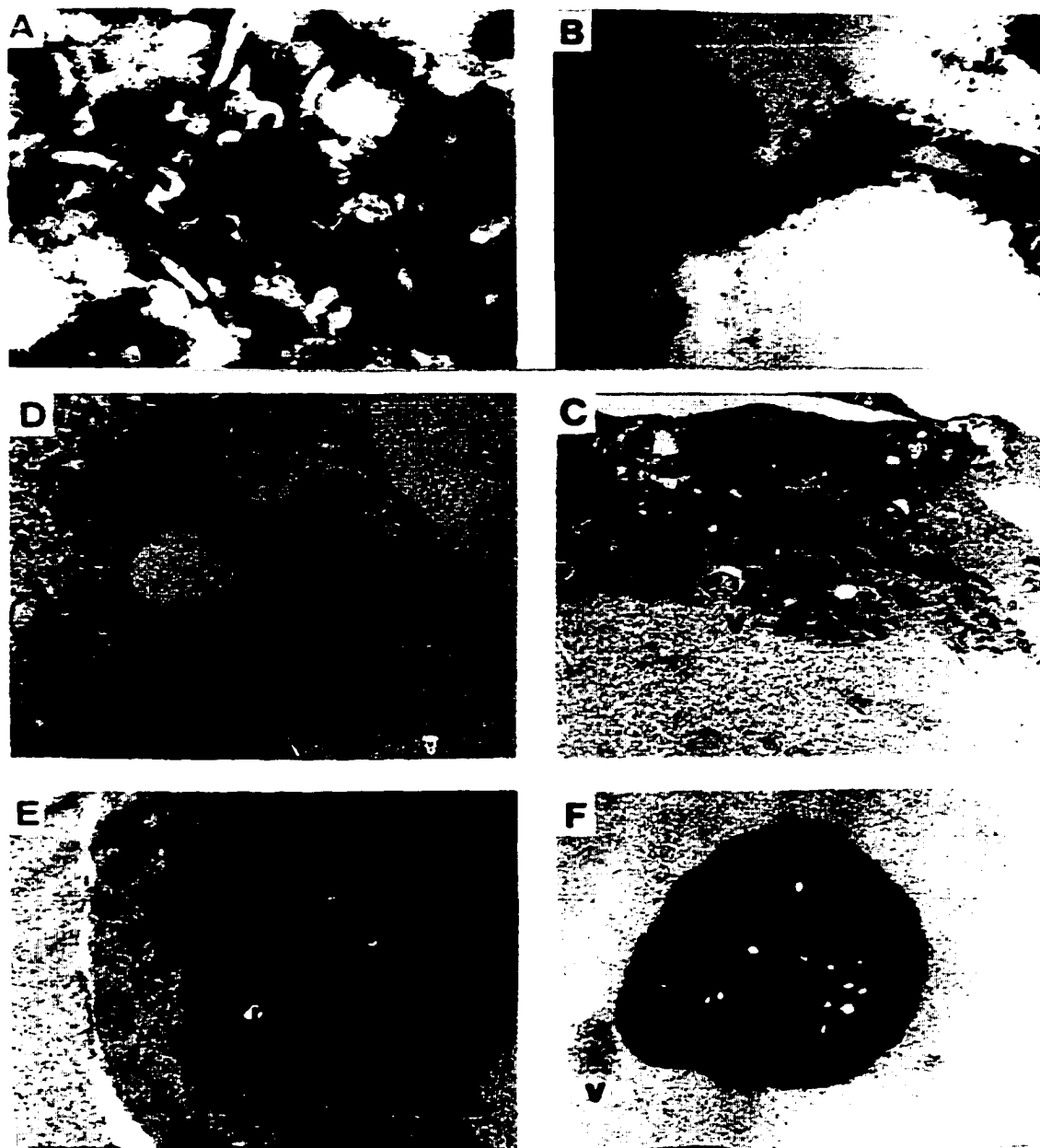


Figure 2.5. Transmission electron micrographs of SpliNPV-infected Sf9 and MSE4 cells. Budded virions from: **A)** the medium of SpliNPV-infected Sf9 cells (magnification 50,000X); and **B)** the medium of SpliNPV-infected MSE4 cells (100,000X). Cytopathic effects and virions within: **C)** SpliNPV-infected Sf9 cell (15,000X); and **D)** SpliNPV-infected MSE4 cell (15,000X). Virions within and clustered at the edge of polyhedral inclusion bodies (PIB): within **E)** SpliNPV-infected Sf9 cell (30,000X); and **F)** SpliNPV-infected MSE4 cell (15,000X).

2.5. Discussion

SpliNPV infects larvae of *S. littoralis* and *S. litura* and replicates well in cell lines derived from *S. frugiperda* (Sf9 and Sf21) and *S. littoralis* (CLS79) but does not grow in cell lines derived from *T. ni* (TN368) or *B. mori* (BmN) (Maeda *et al.*, 1990). Published reports have suggested that SpliNPV can infect two species of locust, *L. migratoria migratorioides* and *S. gregaria* (Bensimon *et al.*, 1987), as well as species of wood-dwelling termites, *K. flavicollis*, although the virus detected in the latter study was not characterized genetically (Fazairy & Hassan, 1988). Faktor and Raviv (1996) recently reported that they were unable to detect viral DNA after infection in *L. migratoria* with SpliNPV occlusion bodies. The pH of orthopteran insects, however, is characteristically slightly acidic to neutral pH 5.6-7.2 (House, 1974), which is not sufficiently alkaline to induce dissolution of viral polyhedra and release of virions. Thus, it is not surprising that no infection was detected and it leaves the question unresolved as to whether SpliNPV can infect locusts *in vivo*.

My results, however, suggest that SpliNPV can infect cells derived from an orthopteran insect, at least *in vitro*. Infection of MSE4 cells, derived from the western migratory grasshopper, *M. sanguinipes*, resulted in a time-dependent increase in intracellular viral DNA, as indicated by dot blot analysis. While the intensity of hybridization of SpliNPV was considerably lower in virus- infected MSE4 cells than in virus-infected Sf9 cells, it was more consistent over the course of the experiment than the hybridization observed in virus-infected Md210 cells, which declined steadily from 4 to 48 hpi. I interpret the data as suggesting that SpliNPV replication occurred in MSE4 cells at a low, but detectable level.

A SpliNPV-specific PCR product was detected in both intracellular and extracellular viral DNA from SpliNPV-infected MSE4 cells. While it may be argued that the virus-specific PCR product detected in the cell culture medium could be due to contamination from the virus originally used to infect the cells, I would point out that the cells were washed extensively at 1 hpi and that no PCR products were detected in Md210 cells treated in the identical manner. Moreover, I detected budded virions by electron microscopy in the cell culture supernatant from MSE4 cells but not from that of Md210 cells. Finally, infection of Sf9 cells with supernatant from SpliNPV-infected MSE4 cells

resulted in the production of viable virus progeny, while infection of Sf9 cells with supernatant from virus-infected Md210 cells did not support the production of virus progeny. The most significant evidence was the detection of the presence of virions in the virus infected MSE4 cells that made the infection of SpliNPV in the orthopteran cell line conclusive.

It is highly unusual for a lepidopteran baculovirus to replicate in cells derived from an orthopteran insect. Phylogenetic analysis based on the *polh* sequences suggested that NPVs cluster into at least two distinct groups or clades (Zanotto *et al.*, 1993). Previous studies have shown that the genomes of SpliNPV and AcMNPV are not closely related (Kislev, 1985; Kislev & Edleman, 1982; Croizier *et al.*, 1989) and phylogenetic analyses based on the *polh* and *egt* suggested that SpliNPV is distantly related to NPVs from Clade I NPVs (Levin *et al.*, 1997; Hu *et al.*, 1997; Smith & Goodale, 1998). Nucleotide sequence analysis of five SpliNPV genes and their flanking regions [*polh* (Croizier, 1994; Faktor *et al.*, 1997a), *egt* (Faktor *et al.*, 1995), *p10* (Faktor *et al.*, 1997b), *lef-3* (Wolff *et al.*, 1998), and *lef-8* (Faktor & Kamensky, 1997)] has revealed that the virus has a number of unique genetic features.

The ability of SpliNPV to replicate in MSE4 cells may reflect the unusual genetic composition of this virus. However, it may be that MSE4 cells are very aberrant. MSE4 cells appear to be a heterogeneous population of hemocytes, some of which may stop dividing after several rounds of cell division (Tim Kurtti, per. Comm.). Thus, the ability of SpliNPV to productively infect these cells *in vitro* does not necessarily reflect an ability to infect locusts or grasshoppers *in vivo*.

Acknowledgments

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Chapter 3. Molecular Characterization of The *Spodoptera littoralis* Nucleopolyhedrovirus Infection in Permissive, Semi-permissive and Non-permissive Cell Lines

3.1. Abstract

I have examined *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV) infections in CLS79, Sf9, and Se1 cells derived from lepidopteran insects of the genus *Spodoptera* (Family: *Noctuidae*), Ld652Y cells from *Lymantria dispar* (Family: *Lymantriidae*), and Md210 cells from *Malacosoma disstria* (Family: *Lasiocampidae*). CLS79, Sf9, and Se1 cells were permissive for SpliNPV infection as these cell lines supported complete viral DNA replication, virus-specific transcription, and production of viable progeny. Neither Ld652Y nor Md210 cells supported production of viable SpliNPV progeny. Ld652Y cells supported limited viral DNA replication and displayed reduced and delayed transcription of viral-specific RNAs. Md210 did not support viral DNA replication and displayed dramatically reduced transcription of viral-specific RNAs. I used transient expression assays as an indirect measure of the translation of SpliNPV early gene products in Sf9, Ld652Y, and Md210 cells. While transactivation of viral promoter-mediated luciferase expression occurred in SpliNPV-infected Ld652Y cells, little to no transactivation activity was detected in SpliNPV-infected Md210 cells. My data indicated that the block to productive SpliNPV infection in Ld652Y and Md210 cells may be at the level of viral RNA transcription and further suggested that host factors play an important role in productive SpliNPV infection.

3.2. Introduction

The best-characterized baculovirus, AcMNPV, has a genome of 134 kb and encodes approximately 150 genes (Ayres *et al.*, 1994). Studies of AcMNPV replication in cultured insect cells have indicated that while some cell lines can fully support viral DNA replication and production of viable progeny (permissive cells), other cell lines support only limited replication of the viral genome without (or with very limited) production of viable progeny (semi-permissive cells). Finally, there are cell lines that support neither viral replication nor production of viable progeny (non-permissive cells). Studies with recombinant AcMNPVs bearing reporter genes have demonstrated that although these viruses do not replicate in non-permissive insect cells, they are able to enter and express some viral encoded genes (Carbonell *et al.*, 1985; Morris & Miller, 1992). Thus, the block to productive infections in semi-permissive and non-permissive insect cells occurs subsequent to viral entry and uncoating (Guzo *et al.*, 1992; Thiem *et al.*, 1996).

SpliNPV replicates well in cell lines established from *S. frugiperda* (Sf9 and Sf21) and *S. littoralis* (CLS79) but not in cell lines derived from *T. ni* (TN368) or *B. mori* (BmN) (Maeda *et al.*, 1990). I previously demonstrated that SpliNPV can infect an orthopteran cell line, MSE4, derived from the western migratory grasshopper, *M. sanguinipes*. While these studies evaluated the ability of SpliNPV to replicate in different cultured insect cells, questions concerning the nature of the block to productive virus infection in semi-permissive or non-productive cell lines have not been addressed. In an attempt to gain insight into the cellular and molecular events that permit productive SpliNPV infections, I examined the process of SpliNPV infection in five insect cell lines (CLS79, Sf9, Se1, Ld652Y, and Md210). Three of these cell lines (CLS79, Sf9, and Se1) are derived from noctuid moths of the genus *Spodoptera* (Family: *Noctuidae*). The other two cell lines are derived from unrelated lepidopteran insects. Ld652Y cells are derived from the gypsy moth, *L. dispar* (Family: *Lymantriidae*), and Md210 cells are derived from the forest tent caterpillar, *M. disstria* (Family: *Lasiocampidae*).

3.3. Materials and Methods

3.3.1. Cells and viruses

S. littoralis cells (CLS79) were provided by Dr. S. Kamita, Department of Entomology, University of California, Davis. *S. frugiperda* (Sf9) cells were obtained commercially (PharMingen). *S. exigua* (Se1) cells were provided by Dr. B. Federici, Department of Entomology, University of California, Riverside. *L. dispar* cells (Ld652Y) were obtained from Dr. D. Theilmann, Agriculture and Agri-food Canada Research Station, Summerland, British Columbia. The *M. distria* cell line, Md210, was obtained from Dr. A. Keddie, Department of Entomology, University of Alberta, Edmonton, Alberta. Sf9, CLS79, Se1, Md210, and Ld652Y cells were cultured according to described procedures (O'Reilly *et al.*, 1992; Summer & Smith, 1987).

AcMNPV strain E2 was obtained from Dr. M. Summers, Texas A & M University, College Station, Texas. AcMNPV stocks were propagated in Sf9 cells following standard procedures (O'Reilly *et al.*, 1992). The *S. littoralis* NPV, isolate M-2 (SpliNPV) was obtained from Dr. G. Croizier, Station de Recherches de Pathologie Comparée INRA-CNRS, F-30380 Saint-Christol-Les-Ales, France. SpliNPV stocks were propagated in CLS79 cells by infecting cells at an MOI of 1.0 to minimize production of few polyhedra mutants. Virus titers were determined by end-point dilution titration in CLS79 cells following standard procedures (O'Reilly *et al.*, 1992).

3.3.2. Infection of cells with SpliNPV

Cells (10^6) were mock-infected or infected with SpliNPV at an MOI of 10. After incubation for one hour at 28 °C, the infectious medium was removed, the cells were washed twice with phosphate buffered saline (PBS), and then overlaid with fresh medium. Cells were incubated and harvested at various time points post-infection. To evaluate the ability of each cell line to support production of viable SpliNPV progeny, the medium was removed from mock-infected or virus-infected cells at 48 hpi, and the titer of virus in the media was determined in CLS79 cells, because they produce large polyhedral inclusion bodies.

3.3.3. Plasmids

Construction of the plasmids *pAcIE2-luc*, *pAcPE38-luc* and *pAcP39-luc*: Restriction endonucleases were purchased from New England BioLabs (NEB). Details of the construction of *pAcIE2-luc* and *pAcPE38-luc* were described by Liu (1995) and are outlined briefly here. The *ie-2* and *pe-38* genes of AcMNPV are adjacent on the AcMNPV genome and transcribed in opposite directions from divergent promoters (Krappa & Knebel-Morsdorf, 1991). A 455 bp polymerase chain reaction (PCR) product containing the *ie-2* and *pe-38* promoters was obtained after PCR amplification of the genome of AcMNPV with Vent[®] DNA Polymerase (NEB). The “forward” primer (5'-TTTGCTTATGGCAGCTCG-3') was complementary to positions 94 to 116 in the 5'-untranslated leader region of the *pe-38* gene (Krappa & Knebel-Morsdorf, 1991). The “reverse” primer (5'-GGCTGGGCTGGTAGGATACTGTT-3') was complementary to positions 311 to 333 of the 5'-untranslated leader region of the *ie-2* gene (Carson *et al.*, 1988). In the AcMNPV genome, the *ie-2* and *pe-38* promoters are separated by a *Pst*I restriction endonuclease (REN) site. Cleavage of the 455 bp PCR fragment with *Pst*I yielded a 336 bp fragment containing the *ie-2* promoter and a 119 bp fragment containing the *pe-38* promoter. These fragments were isolated and cloned into pBluescript II KS(+) (Stratagene) cleaved with the *Pst*I and *Sma*I. The resulting recombinant plasmids were called *pAcIE2* and *pAcPE38*. Plasmid pGL2-Basic (Promega) was digested with *Bam*HI and *Bgl*II. A 2.7 kb fragment containing the firefly (*Photinus pyralis*) luciferase (*luc*) gene (Gould & Subramani, 1988) was excised and cloned into plasmids *pAcIE2* and *pAcPE38* that had been cleaved with *Bam*HI, resulting in plasmids *pAcIE2-luc* and *pAcPE38-luc*, respectively (Figure 3.1), in which the luciferase gene was now under the transcriptional control of the respective promoters.

Details of the construction of *pAcP39-luc* were described by Wolff (Wolff, 1998). Briefly, the plasmid *pAcP39-luc* (Figure 3.1) was created by cloning the 2.7 kb *luc* gene *Bam*HI/*Bgl*II fragment into the *Bam*HI site of plasmid *p39CAT-Q*⁻, which contains the promoter region of the AcMNPV 39K gene (Guarino & Summers, 1986a). The *p39CAT-Q*⁻ plasmid was provided by Dr. D. Theilmann, Agriculture and Agri-food Canada, Summerland, British Columbia.

Construction of the plasmids *pAcPy-luc* and *pAcP10-luc*: The plasmids *pAcGP67A* and *pAcUW1* are commercial transfer vectors (PharMingen) designed for construction of

recombinant AcMNPVs that hyper-express exogenous gene products from the AcMNPV *polh* and *p10* loci, respectively. The pAcGP67A and pAcUW1 vectors were subjected to cleavage with *Bam*HI and ligated with the 2.7 kb *luc* gene *Bam*HI/*Bgl*II fragment. The resulting recombinant plasmids were called pAcPy-luc and pAcP10-luc, respectively (Figure 3.1).

Construction of the plasmid pSlPy-luc: A 305 bp fragment, containing the putative SpliNPV *polh* promoter (TAAG sequence) and the upstream 27 bp direct repeats, immediately 5' of the TAAG sequence (Croizier & Croizier, 1994), was amplified from SpliNPV genome by PCR, using *Pfu* Polymerase (Stratagene). The “forward” primer (5'-GCGCGAATCATGAACATGTTCCACAATATGC-3') contained an *Eco*RI restriction endonuclease recognition (REN) site plus a GC-clamp at the immediate 5'-end (underlined). The “reverse” primer (5'-GCGCAGATCTAAGGGATATTTGATTTTCACTACTAACG-3') contained a *Bgl*II REN site plus a GC-clamp (underlined) and terminated immediately before the *polh* gene translation start codon. The 305 bp PCR product was cloned into the *Sma*I site of pBluescript II KS(+). A recombinant plasmid with the fragment in the correct orientation was identified by REN and DNA sequence analysis, and the resulting plasmid was called pSlPy. The 2.7 kb *Bam*HI/*Bgl*II *luc* fragment was cloned into the *Bam*HI site of pSlPy and the resulting plasmid was called pSlPy-luc, in which the *luc* gene was placed under the control of the SpliNPV *polh* promoter (Figure 3.1). Details of the construction of plef3-luc were previously described (Wolff *et al.*, 1998).

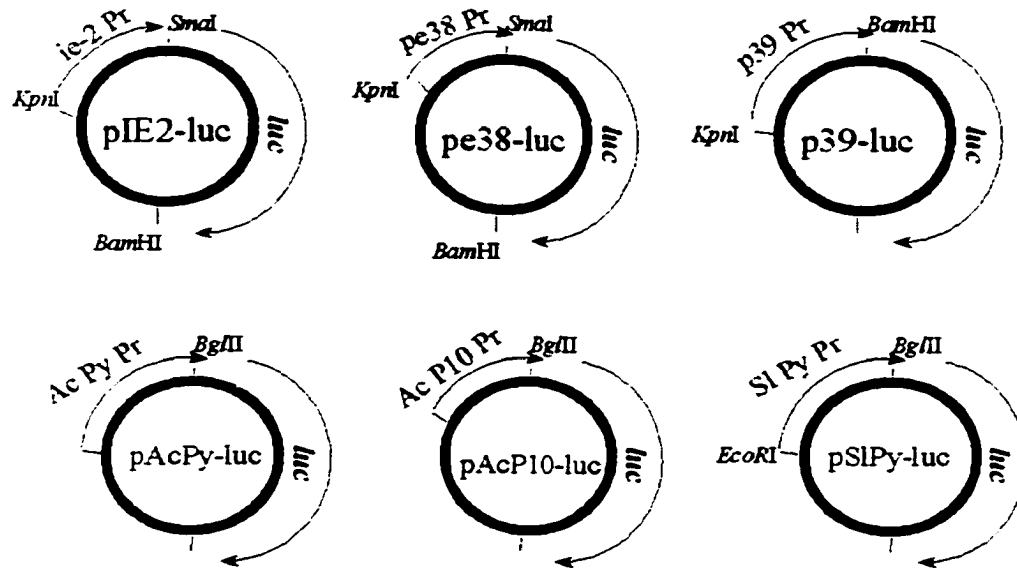


Figure 3.1. Plasmid construction. Reporter plasmids in which the firefly (*Photinus pyralis*) luciferase gene was placed under the transcriptional control of the AcMNPV *ie-2* (pAcIE2-luc), *pe-38* (pAcPE38-luc), *39K* (pAcP39-luc), *polh* (pAcPy-luc), and *p10* (pAcP10-luc) promoters, and the SpliNPV *lef-3* (plef3-luc) and *polh* (pSlPy-luc) promoters.

3.3.4. Dot blot analysis

Cells (10^6) were mock-infected or infected with SpliNPV at an MOI of 10, and harvested at various time points post-infection ($t = 4, 8, 12, 16, 20, 24, 28$, and 32 hpi). Cells (2.0×10^5) were added to Dot blot lysis buffer (0.4 M NaOH, 10 mM EDTA), heat denatured at 100°C for 10 min, and then placed on ice. Samples were applied to a nylon hybridization membrane (Hybond-N, Amersham) contained within a Dot-blot manifold (BRL Omni-blot) under vacuum. Samples applied to the membrane were washed twice with 0.4 M NaOH. The membrane was rinsed twice in $2X$ SSC, 5 min each time, dried at 65°C , and cross-linked with ultra-violet (UV; 312 nm) light for 2 min. Dot blots were hybridized with gel purified total SpliNPV genomic DNA. Probe DNAs were labeled with $\alpha^{32}\text{P}$ -dCTP by random primed PCR, according to the protocols specified in the Tag-It[®] Kit (Bios Corporation). The results of hybridization were visualized by exposing the blots to Kodak XAR x-ray film, and then quantified by integrating the density of each spot on the autoradiogram using the Eagle-Eye II (Stratagene) photodocumentation system equipped with Eagle-Sight[™] software. Data derived from this analysis was plotted using Microsoft Excel 7.0. The Dot blot replication assays were repeated three times.

3.3.5. RNA isolation and Northern blot Analysis

Cells (10^7) were mock-infected or infected with SpliNPV at an MOI of 10, and incubated at 28°C . At $4, 8, 16, 24$, and 48 hpi, the cells were harvested following standard procedures (O'Reilly *et al.*, 1992). Total RNAs were extracted from all samples with TRIzol reagent (Gibco/BRL) following the manufacturer's protocol. Total RNAs (10 ug per lane) were separated by electrophoresis in 1.25% agarose gels containing 2.2 M formaldehyde, and $1X$ MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA) at 3 volts/cm (Selden, 1987). Separated RNAs were transferred to nylon membrane (Hybond-N) by capillary blotting. Hybridization was carried out using standard protocol (Selden, 1987). Northern blots were hybridized with gel purified fragments of the SpliNPV genomic DNA, the SpliNPV *lef-3* gene, or the SpliNPV *polh* gene. Probe DNAs were labeled with $\alpha^{32}\text{P}$ -dCTP by random primed PCR according to

the protocols specified in the Tag-It[®] Kit (Bios Corporation). An RNA size ladder was co-electrophoresed and stained separately as a size marker (Gibco/BRL).

3.3.6. Transient expression assays

Cells (10^6) were infected with SpliNPV at an MOI of 10 for 2 h and then transfected with 2 μ g of plasmid containing a luciferase gene by calcium phosphate precipitation. At 4 h post-transfection, the culture medium was removed, the transfected cells were washed twice with PBS, and the cells were refed with fresh medium. At 48 h post-transfection, cells were harvested in 1 ml PBS and pelleted at 4000g for 5 min at 4 °C. The cell pellets were resuspended in 100 μ l 1X Lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol [DTT], 2 mM 1, 2 diaminocyclohexane-N,N,N',N'-tetraacetic acid [DCTA], 10% glycerol, and 1% Triton X-100) and incubated at room temperature for 10 min. In a separate tube, 20 μ l of the room temperature cell lysate were mixed with 100 μ l of the luciferase assay reagent (20 mM Tricine, 1.07 mM $(\text{MgCO}_3)_4$ $\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 2.67 mM MgSO_4 , 0.1 mM EDTA, 33.3 mM DTT, 270 μ M Coenzyme A, 470 μ M luciferin, 530 μ M ATP, pH 7.8), and the mixture was assayed for light detection using a Liquid Scintillation Counter.

3.4. Results

3.4.1. SpliNPV DNA replication

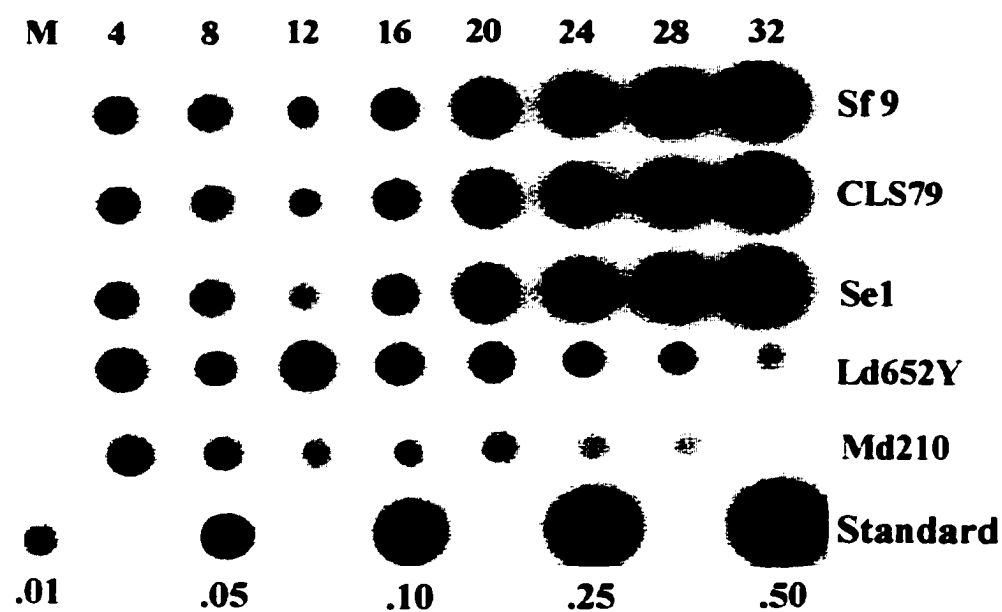
To determine the extent of SpliNPV DNA replication in each of the five cell lines, 2×10^5 cells were infected with SpliNPV, harvested at various time points post-infection, and subjected to Dot blot analysis. Substantial increases in the amounts of viral DNA were evident in Sf9, CLS79, and Se1 cells (Figure 2a and 2b), which demonstrated a time-dependent increase in the amount of intracellular viral DNA. SpliNPV DNA was detected in all permissive cell lines within 4 hpi. The intensity of DNA hybridization in virus-infected CLS79, Sf9, and Se1 cells appeared to decrease from 4 to 12 hpi, then increased significantly at 16 hpi, and continued to increase thereafter.

SpliNPV DNA was also detected within Ld625Y and Md210 cells within 4 hpi (Figure 2a). However, neither Ld625Y nor Md210 displayed a pattern of time-dependent increase in the amount of SpliNPV DNA. An increase in the intensity of viral DNA hybridization was observed in Ld652Y cells from 8 to 12 hpi, and the amount of viral DNA observed in Ld652Y cells at 12 hpi was slightly greater than the amount seen in CLS79, Sf9, and Se1 cells at 16 hpi. However, the intensity of hybridization in Ld652Y cells decreased steadily thereafter, suggesting that while some viral DNA replication may have occurred, significant replication did not continue after 12 hpi. In Md210 cells, the intensity of SpliNPV DNA hybridization decreased consistently from 4 to 32 hpi, suggesting that SpliNPV did not replicate in these cells (Figure 3.2a and 3.2b).

3.4.2. Production of viable progeny

The titer of budded virus (BV) in the media of each SpliNPV-infected cell line collected at 48 hpi was determined using CLS79 cells. Sf9 cells produced higher titers of budded virions (1.4×10^8) than did CLS79 (4.7×10^7) or Se1 (2.5×10^7) cells (Table 3.1) when titered on CLS79 cells by the end-point dilution method. Incubation of CLS79 cells with supernatants from mock-infected and SpliNPV-infected Ld652Y and Md210 cells did not result in infection, indicating that these cells did not support production of viable progeny.

(a)



(b)

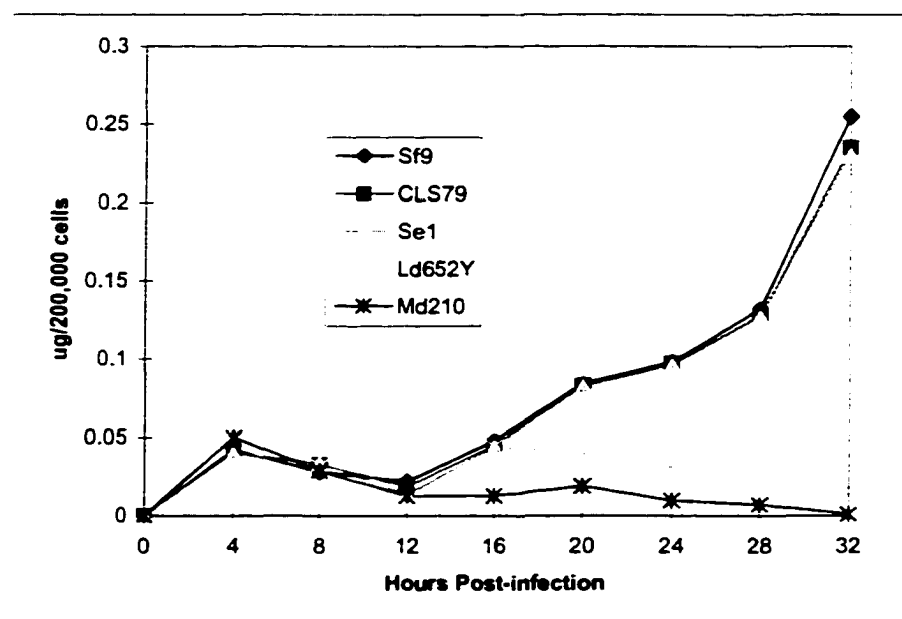


Figure 3.2. Dot blot analysis of the extent of SpliNPV DNA replication. a) SpliNPV-infected Sf9, CLS79, Se1, Ld652Y, and Md210 cells. Dot blot of total cellular DNA from 2.0×10^5 mock-infected or infected Sf9, CLS79, Se1, Ld652Y, and Md210 cells with SpliNPV at an MOI of 10, harvested at indicated time points, probed with labeled SpliNPV DNA. Known amounts of SpliNPV DNA as indicated were used as standards.

b) Graphic representation of the data after integration of the density of each spot on the Dot blot autoradiogram. Quantities of viral DNA (ug/200,000 cells) were calculated using known amounts of SpliNPV DNA as standards in the Dot blot.

Table 3.1. SpliNPV budded virus production in selected cell lines

Cell line	TCID ₅₀ at 48 hpi*
CLS79	4.7×10^7
Sf9	1.4×10^8
Se1	2.5×10^7
Md210	no productive infection
Ld652Y	no productive infection

*Media were removed from SpliNPV-infected cells (of the type indicated) at 48 hpi and the titers were determined using fresh CLS79 cells. Indicated values (TCID₅₀) were obtained from the average of three separate titration experiments for each cell line.

3.4.3. Northern blot analysis of global SpliNPV transcription in infected lepidopteran cell lines

The Northern blots revealed a complex pattern of global SpliNPV transcription in virus-infected Sf9, Se1, and CLS79 cells (Figure 3.3). The pattern of viral transcription was very consistent among the three permissive cell lines. The number and intensity of RNAs increased with the time of infection. While equal amounts of RNA were loaded in each lane of each gel, the intensity of the bands observed in the Northern blot from virus-infected Se1 cells was somewhat lower than that observed in the Northern blots of virus-infected Sf9 and CLS79 cells, suggesting that transcription of viral genes occurred at a slightly lower level in Se1 cells compared with CLS79 and Sf9 cells.

In contrast, hybridization of the radiolabelled SpliNPV genomic DNA probe with RNAs extracted from Ld652Y cells revealed a significantly reduced level of global viral transcription. Hybridization with RNAs extracted from Md210 cells revealed that most bands present in RNAs extracted from SpliNPV-infected permissive cell lines were completely absent in virus-infected Md210 cells. Only a few faint bands were detected at 48 hpi. Thus, Northern blot analysis revealed a different pattern of viral transcription in Ld652Y and Md210 cells, with Ld652Y cells displaying more bands and higher intensity of transcripts than Md210 cells, but significantly less transcription activity than that observed in the permissive cell lines (Figure 3.3).

3.4.4. Levels of SpliNPV-specific RNAs in three infected lepidopteran cell lines

Having observed the global patterns of SpliNPV transcription in the different cell lines, I then looked at the levels of specific virus transcripts in these cell lines. Since I observed that Sf9, CLS79, and Se1 cells were approximately equal in their patterns of the extent of SpliNPV DNA replication, global patterns of RNA transcription, and production of viable progeny, I chose Sf9 cells to examine the levels of specific SpliNPV transcripts in productive cell lines. The levels of mRNA of two specific SpliNPV genes: an early SpliNPV gene (*lef-3*) and a very late SpliNPV gene (*polh*) were examined in Sf9, Ld652Y,

and Md210 cells. The *lef-3* gene encodes a late expression factor that is expressed early in the virus infection cycle (Li, *et al.*, 1993; Wolff *et al.*, 1998), which is essential for viral DNA replication, and is believed to encode a putative single-stranded DNA binding protein (Li, *et al.*, 1993). The *polh* gene encodes a major matrix protein, polyhedrin, whose expression in AcMNPV is characterized by a burst in expression beginning around 24 hpi, which continues at high levels up to 72 hpi (Rohrmann, 1986).

Northern blot analysis revealed that the pattern of transcription of the *lef-3* gene was different in the three cell lines tested. The 1.5 kb *lef-3* transcript was detected at a low level by 8 hpi in SpliNPV-infected Sf9 cells and increased significantly with the course of infection (Figure 3.4a). In contrast, transcription of SpliNPV *lef-3* in virus-infected Ld652Y cells was considerably reduced. The *lef-3* transcript was detected at a low level only after 16 hpi and its level did not increase with the time of infection. No trace of the SpliNPV *lef-3* transcript was observed in virus-infected Md210 cells.

Transcription of the SpliNPV *polh* gene was observed only in SpliNPV-infected Sf9 cells (Figure 3.4b). As the probe used in this analysis was a double-stranded DNA molecule, I observed two major transcripts; one of approximately 0.98 kb, which corresponds to the expected size of the SpliNPV *polh* open reading frame, and one of approximately 2.5 kb, which may correspond to an open reading frame that overlaps the *polh* gene on the opposite strand. The *polh* transcript was initially observed at 8 hpi in SpliNPV-infected Sf9 cells, increased at 16 hpi, and was maximally expressed by 24 hpi. The 2.5 kb transcript, also first detected at 8 hpi, increased significantly at 16 hpi, and then appeared to decrease at 24 and 48 hpi. No evidence of very late transcription was detected in virus-infected Ld652Y and Md210 cells.

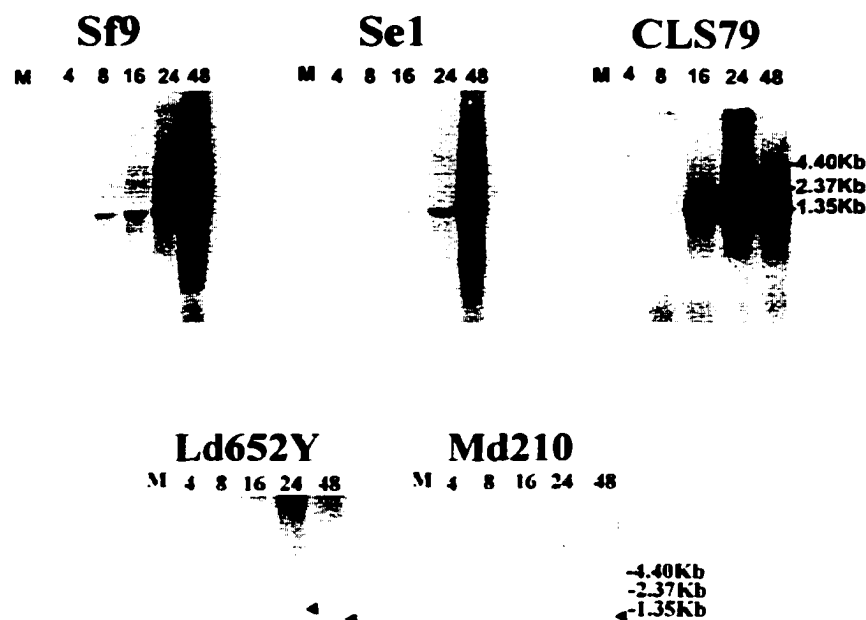


Figure 3.3. Global SpliNPV transcription in infected cell lines. Northern blots of total RNAs extracted from mock-infected (M) and SpliNPV-infected Sf9, Se1, CLS79, Ld652Y, and Md210 cells at 4, 8, 16, 24, and 48 hpi. RNAs (10 ug per lane) were fractionated on formaldehyde (2.2 M)-1.25% agarose gels and hybridized with the SpliNPV genomic DNA. RNA size standards (1.35 kb, 2.37 kb, and 4.40 kb) are indicated to the right of the blots. Arrows indicate prominent viral transcripts in Ld652Y and Md210 cells.

Figure 3.4. Levels of SpliNPV-specific RNAs in infected cell lines. Northern blots of total RNAs extracted from mock-infected and SpliNPV-infected Sf9, Ld652Y, and Md210 cells at 4, 8, 16, 24, and 48 hpi. RNAs (10 ug per lane) were fractionated on formaldehyde (2.2 M)-1.25% agarose gels and hybridized with the radiolabelled SpliNPV gene-specific probes, **a)** *lef3* and **b)** *polh*. RNA size standards (1.35 kb and 2.37 kb) are indicated to the right of the blots.

3.4.5. Transcriptional activation of SpliNPV early and very late promoters

Previous studies have demonstrated that AcMNPV enters a wider range of insect cells than can be productively infected. Although replication and production of viable progeny may be impaired in these cells, expression of reporter gene products under the transcriptional control of viral promoters was detected in transient expression assays and recombinant virus analysis (Carbonell *et al.*, 1985; Morris & Miller, 1993). The ability of a virus to stimulate early gene expression over basal levels, or to transactivate very late gene expression in a transient expression assay, is an indirect measure of the expression of viral encoded transactivating gene products (at either the level of transcription or translation, or both). Thus, since no transcriptional activating genes of SpliNPV have yet been identified and characterized, I used the ability of SpliNPV to transactivate early and late viral promoters as a measure of differences in viral gene expression in the different cell lines. I constructed plasmids in which the luciferase gene is under the transcriptional control of the SpliNPV *lef-3* or *polh* promoter. Sf9, Ld652Y, and Md210 cells were infected with SpliNPV at an MOI of 10. At 2 hpi the infected cells were transfected with a reporter plasmid.

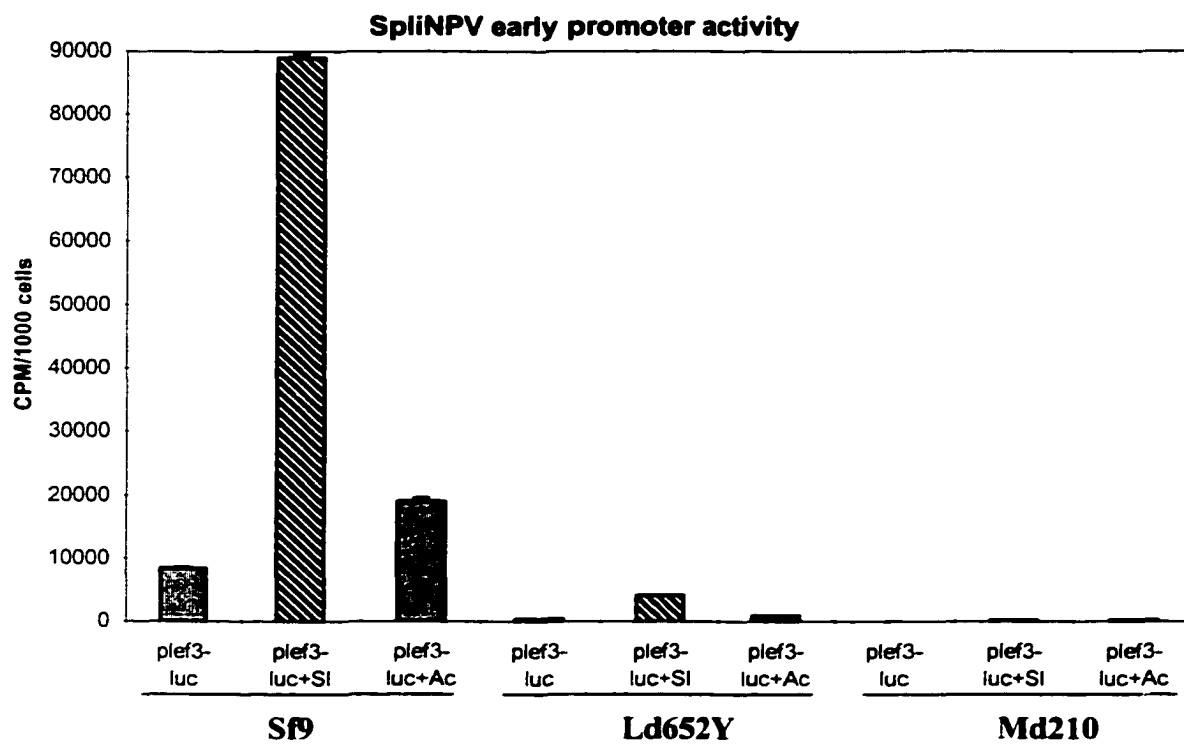
The results of the transient expression assays indicated that the SpliNPV *lef-3* promoter was expressed in uninfected Sf9 and Ld652Y cells, resulting in a basal level luciferase activity that was characteristic for each cell line. The basal level of luciferase activity was significantly higher (20-fold) in Sf9 cells than in Ld652Y cells (Figure 3.5a). The level of luciferase activity in Md210 cells was equivalent to background luciferase activity (luciferase activity detected after transfection of cells with a plasmid [pGL2-Basic] that contains only the luciferase gene, without a promoter element), suggesting that the SpliNPV *lef-3* promoter was not transcriptionally active in these cells.

lef-3 promoter-mediated luciferase activity was strongly stimulated in SpliNPV-infected Sf9 and Ld652Y cells, although again, the level of luciferase activity in virus-infected Ld652Y cells was significantly lower (20-fold) than that detected in virus-infected Sf9 cells (Figure 3.5a). While some stimulation of luciferase activity was observed in virus-infected Md210 cells, the level was just above background, and therefore not significant.

The reduced level of virus promoter-mediated luciferase expression in Ld652Y cells, and the lack of significant luciferase expression in Md210 cells, was not due to reduced transfection efficiency of these cells compared to Sf9, CLS79, and Se1 cells. Dot blot analysis of transfected cells indicated approximately equal amounts of plasmid DNA in each cell line (data not shown).

The very late *polh* promoter-mediated luciferase activity was detected only in SpliNPV-infected Sf9 cells (Figure 3.5b), and was not detected in Ld652Y or Md210 cells, even in the presence of SpliNPV. These results are consistent with studies of AcMNPV, which indicate that very late gene expression does not occur in the absence of viral DNA replication. These results further confirm my Northern blot analysis of SpliNPV *polh* transcription in virus-infected Ld652Y and Md210 cells.

(a)



(b)

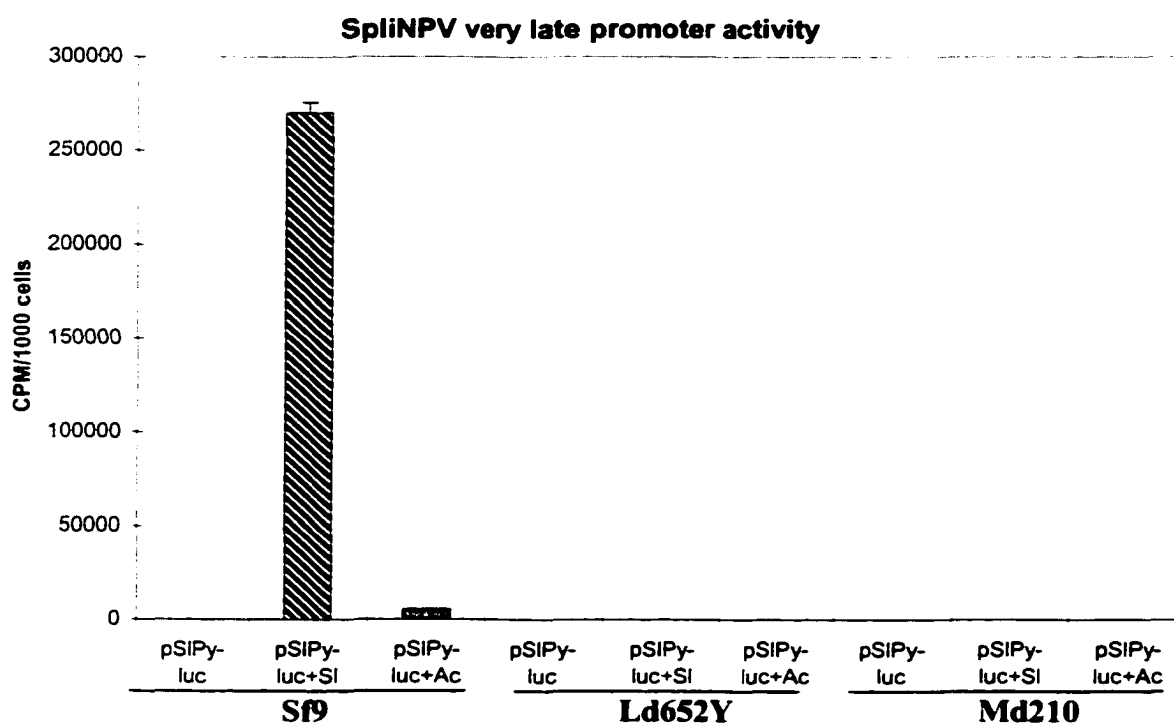


Figure 3.5. Analysis of SpliNPV promoter activity in permissive and non-permissive cells by transient expression assays. Sf9, Ld652Y, and Md210 cell lines were mock-infected or infected with either SpliNPV or AcMNPV virus at an MOI of 10, and transfected with 2 ug of recombinant plasmid in which the luciferase gene was under the transcriptional control of **a)** the SpliNPV early *lef-3* promoter or **b)** the very late polyhedrin promoter. The graphs indicate the luciferase activities from cells harvested at 48 hpi. Each column represents the average of three independent transfections with the standard deviation indicated.

3.4.6. Comparison of the SpliNPV and AcMNPV promoter activity in the presence of heterologous virus

Previous studies have suggested that the promoter chosen to drive foreign gene expression may affect the timing and level of expression of insecticidal genes expressed from recombinant NPVs, as well as the range of insects infected by recombinant baculoviruses (Morris & Miller, 1993). Analysis of gene expression from heterologous virus promoters is of interest for both the use of SpliNPV as a biological control agent and for the analysis of host-cell-virus interactions. Since the SpliNPV promoter classes available for this analysis were limited to the *lef-3* and *polh* promoters, and since AcMNPV promoters have been characterized extensively, I examined the ability of SpliNPV to stimulate or activate luciferase expression from five AcMNPV promoters, representing the early (*ie-2* and *pe-38*), late (*39K*), and very late gene classes (*polh* and *p10*), and the ability of AcMNPV to stimulate luciferase expression from SpliNPV *lef-3* and *polh* promoters in three cell lines (Sf9, Ld652Y, and Md210).

IE-2 is a 47 kDa nuclear-associated protein that stimulates transcription of several early baculovirus promoters (Carson *et al.*, 1988). The immediate early gene, *pe-38*, encodes a 38 kDa nuclear protein which is also a transregulator (Krappa & Knebel-Morsdorf, 1991; Lu & Carstens, 1993). Both *ie-2* and *pe-38* are immediate early genes that are transcribed by the host RNA Polymerase II (Wu *et al.*, 1993). The levels of *ie-2* and *pe-38* transcription are augmented early in the infection cycle by the immediate early 1 (*ie-1*) gene product (IE-1) (Friesen, 1997). I detected basal levels of AcMNPV *ie-2* and *pe-38* promoter-mediated luciferase expression in Sf9, Ld652Y, and Md210 cell lines (Figure 3.6). As observed previously, however, the basal levels of luciferase activity in the three cell lines were significantly different. The basal level of *ie-2* promoter-mediated luciferase activity in Sf9 cells was approximately 5-fold greater than that observed in Ld652Y cells, and 1000-fold greater than the basal level observed in Md210 cells, which was just above background, but detectable (Figure 3.6). Similarly, the basal level of *pe-38* promoter-mediated luciferase activity in Sf9 cells was greater than that observed in Ld652Y cells, while luciferase activity in Md210 cells was at background level.

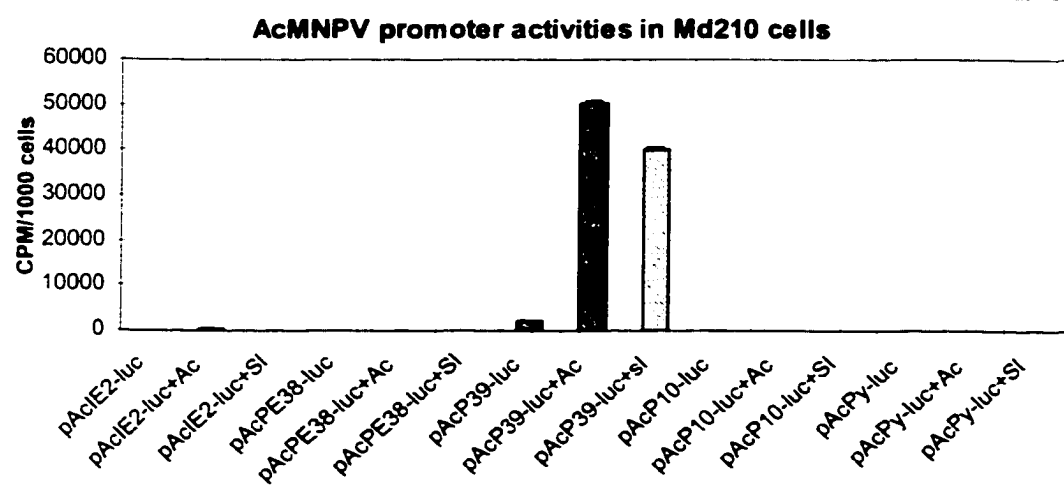
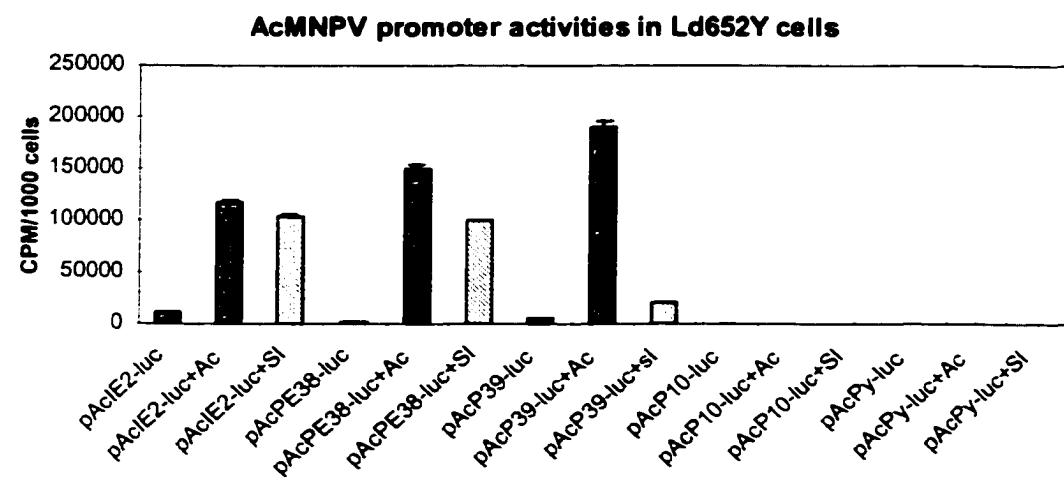
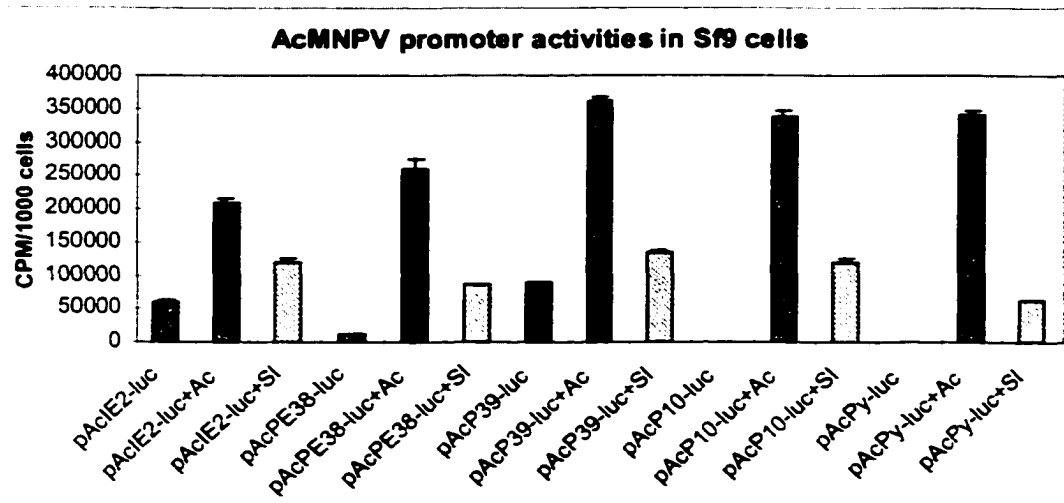


Figure 3.6. Analysis of AcMNPV promoter activity in permissive and non-permissive cell lines by transient expression assays. Sf9, Ld652Y, and Md210 cells were mock-infected or infected with virus (AcMNPV or SpliNPV) at an MOI of 10, transfected with 2 ug of reporter plasmid in which the luciferase gene was under the transcriptional control of the AcMNPV early *ie-2* or *pe-38* promoters (pAcIE2-luc or pAcPE38-luc), the AcMNPV *39K* promoter (pAcP39-luc), and very late *polh* or *p10* promoters (pAcPy-luc or pAcP10-luc). The graphs indicate the luciferase activities from cells harvested at 48 hpi. Each column represents the average of three independent transfections with the standard deviation indicated.

The levels of *ie-2* promoter-mediated luciferase activity were significantly elevated in both Sf9 cells and Ld652Y cells infected with AcMNPV or SpliNPV (Figure 3.6). While the level of *ie-2* promoter activity was approximately 2-fold greater in AcMNPV-infected Sf9 cells than that in SpliNPV-infected Sf9 cells, the levels of luciferase expressed from this promoter were almost the same in AcMNPV- or SpliNPV-infected Ld652Y cells. The levels of *ie-2* promoter-mediated luciferase activity were only marginally stimulated by AcMNPV or SpliNPV in Md210 cells.

The levels of *pe-38* promoter-mediated luciferase activity were also significantly elevated in both Sf9 cells and Ld652Y cells infected with AcMNPV or SpliNPV (Figure 3.6). Again, the level of *pe-38* promoter activity was greater in AcMNPV-infected Sf9 cells than in SpliNPV-infected Sf9 cells, but SpliNPV still stimulated the AcMNPV *pe-38* promoter to a significant level. As observed with the *ie-2* promoter, the levels of *pe-38* promoter-mediated luciferase activity were only marginally stimulated by AcMNPV or SpliNPV in Md210 cells.

AcMNPV 39K encodes a 31 kDa phosphoprotein (pp31) that is synthesized during both the early and late phases of virus infection, and is a component of virogenic stroma (Guarino *et al.*, 1992). While basal level of 39K transcription can be detected early in infection, maximal expression of 39K is dependent on the expression of IE-1 (Guarino & Summers, 1986a). In my transient expression assays, the levels of AcMNPV 39K promoter-mediated luciferase activity displayed the same general patterns as observed with the AcMNPV immediate early gene promoters. Basal levels of luciferase activity were observed in uninfected Sf9, Ld652Y, and Md210 cells with Sf9 cells displaying a greater level of basal activity than Ld652Y cells, and Md210 cells displaying a low, but detectable basal level (Figure 3.6). The levels of 39K promoter-mediated luciferase activity were significantly stimulated over the basal levels by AcMNPV or SpliNPV, but the level of stimulation by SpliNPV was far less than that accomplished by AcMNPV. Surprisingly, the levels of 39K promoter-mediated luciferase activity were significantly stimulated by AcMNPV or SpliNPV in Md210 cells. This was the only virus promoter with late phase activity that functioned in virus-infected Md210 cells.

In contrast, the levels of SpliNPV *lef-3* promoter-mediated luciferase activity were stimulated over basal levels by SpliNPV or AcMNPV, but the level of stimulation by AcMNPV was far less than that accomplished by SpliNPV in Sf9 cells. SpliNPV *lef-3* promoter-mediated luciferase activity in AcMNPV-infected Ld652Y cells was just marginally stimulated over basal level (Figure 3.5a). Neither SpliNPV nor AcMNPV could activate SpliNPV *lef-3* promoter-mediated luciferase activity in Md210 cells (Figure 3.5a).

Late in the infection cycle, NPVs expressed two proteins at very high levels; polyhedrin (*polh*), the major component of polyhedra, and p10, a 10 kDa protein which is involved in the formation of fibrillar structures and in the release of polyhedra (van Oers *et al.*, 1994). As observed with the AcMNPV *ie-2*, *pe-38* and *39K* promoters, SpliNPV could transactivate the AcMNPV very late promoters in Sf9 cells, but at a much reduced level (Figure 3.6). Interestingly, SpliNPV was able to activate the AcMNPV *polh* promoter to a greater level than AcMNPV was able to activate the SpliNPV *polh* promoter (Figure 3.5b and 3.6). Very late promoter activity was neither detectable in uninfected Sf9, Ld652Y, and Md210 cells nor in virus-infected Ld652Y and Md210 cells (Figure 3.5b and 3.6).

3.5. Discussion

The outcome of viral infection in a host cell is determined by the nature of the interactions between the virus and the host cell constituents. During replication within permissive cells, NPVs exploit cellular processes at the expense of the host cells, resulting in coordinated expression of virus-encoded genes, viral DNA replication, and packaging of viral progeny. SpliNPV infection of *S. littoralis* and *S. litura* larvae and replication in cell lines derived from *S. frugiperda* (Sf9 and Sf21) and *S. littoralis* (CLS79) have been reported (Maeda *et al.*, 1990). I observed evidence of SpliNPV-specific transcription, gene expression, DNA replication, and production of viral progeny by Dot blot, Northern blot, and transient expression assays in CLS79 and Sf9 cells and further determined that SpliNPV replicates well in a cell line derived from *S. exigua* (Se1).

Dot blot assays indicated a transient decline in the amount of viral DNA in the three permissive cell lines (Sf9, CLS79, and Se1) between 4 and 12 hpi. These experiments were repeated three times and the same results were observed. Thus, I am confident that my

observations are not an artifact of the procedure. Moreover, I have determined that SpliNPV DNA synthesis begins approximately 6 to 8 hpi using a bromodeoxyuridine (BrdU) incorporation assay (data not shown). Thus, the viral DNA detected at 4 hpi could not be replicated viral DNA and may represent viral DNA from virions that have entered the cells and/or uncoated within the cells. The apparent decrease in viral DNA between 4 and 12 hpi may be due to a response by the host cell to viral infection, such as degradation of viral genomes during the eclipse phase of viral infection. Transient decline of intracellular virus during the very early stages of virus infection was observed in studies of the AcMNPV infection cycle. Time course of infection of *S. frugiperda* cells by AcMNPV revealed a transient decrease in the titer of intracellular virus between 1 and 10 hpi (Carstens *et al.*, 1979; Knudson & Tinsley, 1978).

The Ld652Y and Md210 cell lines did not support productive SpliNPV infection. Ld652Y cells, derived from the gypsy moth, *L. dispar* (Family: *Lymantriidae*), were found to be semi-permissive for SpliNPV infection. This cell line supported transient viral DNA replication, displayed reduced and delayed global viral mRNA synthesis, and did not produce viable SpliNPV progeny. Northern blots also revealed that the levels of SpliNPV *lef-3* transcription were both reduced and delayed. The late expression factor, *lef-3*, has been demonstrated to be essential for viral replication in transient replication assays with AcMNPV (Li *et al.*, 1993). In a previous study of SpliNPV, I demonstrated that the SpliNPV *lef-3* gene plays an important role in SpliNPV replication (Wolff *et al.*, 1998). In the absence of SpliNPV *lef-3* specific antibodies, I was unable to determine whether the *lef-3* gene product was poorly or inappropriately expressed due to a deficiency in translation of virus-encoded gene products in Ld652Y cells. However, transient expression assays indicated that SpliNPV transactivating factors were expressed in sufficient amounts to stimulate reporter gene expression from both SpliNPV (*lef-3*) and AcMNPV (*ie-2*, and *pe-38*) early promoters and from the AcMNPV 39K gene promoter, which is active during both the early and late phases of infection, suggesting that translation of (at least some) viral-specific gene products was occurring in Ld652Y cells.

In AcMNPV, the primary transcriptional activating factor, IE-1, is encoded by the *ie-1* gene, and is essential for viral DNA replication. IE-1 is a transcriptional activator that stimulates basal expression of some immediate early genes (such as *ie-2* and *pe-38*) and

plays a role in the transactivation of late genes. Some of these early genes (such as *lef-3*) are also involved in viral DNA replication (Friesen, 1997). The ability of SpliNPV to stimulate expression of a reporter gene (luciferase) under the transcriptional control of AcMNPV early and late gene promoters, in addition to the SpliNPV *lef-3* gene promoter, suggests that SpliNPV encodes an *ie-1* like gene (or genes) that also has broad promoter recognition capability and that this gene product was translated in sufficient amounts in Ld652Y cells.

While the block to AcMNPV infection in Ld652Y cells has been clearly demonstrated to occur at the level of translation (McClintock *et al.*, 1986), my preliminary data suggested that the block to SpliNPV infection in Ld652Y cells may occur at the level of transcription of viral-specific genes required for viral DNA replication. Thus, the mechanisms that restrict the ability of AcMNPV and SpliNPV to productively infect Ld652Y cells appear to be different. However, SpliNPV is a poorly characterized virus and other genes essential for the viral infection process have not yet been identified. Thus, it is unclear whether other SpliNPV genes were appropriately expressed in Ld652Y cells.

Md210 cells, derived from the forest tent caterpillar, *M. disstria* (Family: *Lasiocampidae*) were found to be non-permissive for SpliNPV. While SpliNPV clearly entered these cells, they supported neither viral DNA replication, nor did they extrude viable extracellular progeny. Md210 cells also displayed extremely low levels of viral specific transcription, and did not support significant levels of SpliNPV-specific transactivating activity. Thus, the block to SpliNPV infection of Md210 cells clearly appears to reside at the level of viral-specific transcription.

In the cascade of baculovirus regulatory events, successive stages of virus replication are dependent on the proper expression of genes within the preceding stage. Thus, the appropriate expression and regulation of viral early genes is critical to baculovirus reproductive success. The products of early viral genes function to both accelerate replicative events and to prepare the host cell for virus multiplication which represents an enormous tax on cellular biosynthetic capacity. Several lines of evidence indicate that host cell transcriptional machinery (the host RNA polymerase II and associated host transcription factors) plays an important role in the early events of virus transcription, and therefore, is essential for the successive baculovirus replication (Friesen, 1997). By Northern blot and transient expression assays, I have demonstrated that SpliNPV exhibits host-specific

patterns of early gene expression. The SpliNPV *lef-3* promoter was recognized by the transcriptional machinery of permissive Sf9 cells, resulting in the basal reporter gene expression in the absence of SpliNPV infection. In contrast, *lef-3* promoter-mediated reporter gene expression was impaired in the non-productive Ld652Y and Md210 cell lines. Luciferase expression was 20-fold lower in Ld652Y cells compared with that observed in Sf9 cells and no luciferase activity was detected in Md210 cells, indicating that the SpliNPV *lef-3* promoter was not recognized by the Md210 cell transcriptional machinery.

Using heterologous promoters in transient expression assays, I characterized AcMNPV early, late, and very late promoter expression patterns in the presence and absence of homologous virus (AcMNPV) or heterologous virus (SpliNPV) in Sf9, Ld652Y and Md210 cell lines. These results revealed host-specific characteristics of SpliNPV infection and gene expression. Studies of AcMNPV infection in different cell lines suggest that the virus interacts with each host cell line in a unique way (Morris & Miller, 1992; Morris & Miller, 1993). My studies with SpliNPV are consistent with these results and further suggest that the mechanisms that determine host-specificity for AcMNPV in Ld652Y cells may be different from those that determine host-specificity for SpliNPV. Thus, my data demonstrated that host factors play an important role in SpliNPV infection.

Another application of baculovirus-host-cell interaction analysis in permissive and non-permissive cell lines is in assessing the safety and improving the efficacy of baculoviruses as biological control agents for insect pests. Baculoviruses have been engineered genetically to produce foreign proteins, such as insect-selective toxins, so that pest insects are quickly incapacitated after infection (Cory *et al.*, 1994). Previous studies with AcMNPV (Carbonell *et al.*, 1985; Morris & Miller, 1993) have suggested that promoter choice may influence the range of insects affected by a recombinant virus expressing an insecticidal gene. Although AcMNPV and SpliNPV are distantly related, they both can transactivate heterologous promoters to certain levels, especially the early viral promoters, suggesting that genetically distant baculoviruses may retain functionally conserved transactivating gene products. Very late promoters, however, direct high-level expression of exogenous genes only in permissive host insect cells, limiting the effectiveness of recombinant viruses to their natural hosts, but providing a powerful tool for avoiding non-target impacts. In contrast, the use of early viral promoters may allow

expression of exogenous genes in a wider range of insects that can not be productively infected by the virus. My studies have demonstrated expression of some baculovirus early gene promoters in nonproductive cells and thus confirm the importance of genetic design in mitigating the impact of recombinant baculovirus on nontarget populations.

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Chapter 4. Identification and Functional Analysis of a Putative Non-*hr* Origin of DNA Replication from the *Spodoptera littoralis* Nucleopolyhedrovirus

4.1. Abstract

A putative non-*hr* origin of DNA replication was identified in the *Spodoptera littoralis* multinucleocapsid nucleopolyhedrovirus (SpliNPV) genome by transient replication assays. The putative SpliNPV *ori* was mapped to the *Pst*I-J fragment between 75.1-77.9 map units in the SpliNPV genome. While the DNA sequence of the putative SpliNPV *ori* aligned with regions within the non-*hr oris* of AcMNPV, OpMNPV, and SeMNPV, it has limited DNA sequence identity with these elements. The sequence of the putative SpliNPV non-*hr ori* fragment contains a unique distribution of imperfect palindromes, multiple direct repeats, and putative transcription factor binding sites. Transient expression assays indicated that the putative SpliNPV *ori* fragment repressed SpliNPV *lef-3* promoter-mediated luciferase reporter gene expression. However, the putative SpliNPV *ori* fragment itself was capable of directing luciferase expression in the absence of a recognizable baculovirus promoter element in an orientation-independent fashion, suggesting that DNA sequence motifs within its sequence can activate transcription. Gel mobility shift analyses confirmed that proteins within nuclear extracts from both uninfected and virus-infected cells bound with specificity to the putative SpliNPV *ori* fragment.

4.2. Introduction

Studies on DNA replication revealed that AcMNPV contains a set of closely related sequences known as homologous regions (*hrs*), which are interspersed throughout the genome (reviewed by Lu *et al.*, 1997). The *hrs* consist of one to eight copies of a repeated sequence composed of 30 bp palindromes flanked by 20 bp direct repeats and separated by approximately 80 to 120 bp of non-repetitive DNA. Some *hr* sequences have been demonstrated to act as origins of DNA replication in transient replication assays in which recombinant plasmids carrying these elements replicate when introduced into NPV-infected insect cells (Kool *et al.*, 1995; Lu *et al.*, 1997). The identification of similar DNA elements in the genomes of OpMNPV (Ahrens *et al.*, 1995), BmNPV (Majima *et al.*, 1993), CfMNPV (Xie *et al.*, 1995), LdMNPV (Pearson & Rohrmann, 1995), and SeMNPV (Broer *et al.*, 1998) suggests that *hrs* perform an essential function during the replication cycle of these viruses. Currently, there is no direct evidence that *hrs* function as origins of replication in the context of a virus infection (Lu *et al.*, 1997). Some *hrs* from AcMNPV and OpMNPV have been demonstrated to function as *cis*-acting enhancers of IE-1-mediated early gene expression (Guarino *et al.*, 1986b; Rodems & Friesen, 1993; Kool *et al.*, 1995; Leisy *et al.*, 1995; Theilmann & Stewart, 1992).

A second type of putative baculovirus origin of replication, referred to as non-*hr* origins (non-*hr oris*), has been described in AcMNPV (Kool *et al.*, 1994b), OpMNPV (Pearson *et al.*, 1993), and SeMNPV (Heldens *et al.*, 1997). Non-*hr oris* contain unique palindromic and repetitive sequences that are not found in baculovirus *hr* sequences and are relatively complex in organization. Only one copy of a non-*hr* sequence was identified in the genome of AcMNPV (Kool *et al.*, 1994b; Lee & Krell, 1994). Sequences in the AcMNPV *Hind*III-K region, also referred to as *oriK* and located between 84.9 to 87.3 m.u. of the AcMNPV genome, support replication of plasmids in transient replication assays (Kool *et al.*, 1994b) and become enriched in defective AcMNPV genomes (Lee & Krell, 1994). Deletion analysis of the *Hind*III-K fragment indicated that the sequences required for optimal replication are contained within a relatively large region within the *p94* gene. The function of *oriK* *in vivo* is unknown, but its conservation in defective AcMNPV genomes (Lee & Krell, 1994) and in the genome of BmNPV,

which is closely related but lacks the *p94* gene (Kool *et al.*, 1994b), suggests that non-*hr* elements may play an important role in the replication of NPVs.

Deletion analysis of the OpMNPV non-*hr* sequence, located within the *Hind*III-N fragment (7.0-11.3 m.u. of the OpMNPV genome), revealed a complex organization, since deletion of any portion of the *Hind*III-N fragment resulted in reduced replication efficiency, suggesting that sequences affecting *ori* activity were distributed throughout the fragment. Sequence analysis identified a variety of direct and inverted repeat sequences and palindromic sequences (Pearson *et al.*, 1993). The non-*hr* sequence of SeMNPV (Heldens *et al.*, 1997) was mapped to a 1052 bp within the *Xba*I-F fragment (60.7-62.3 m.u. of the SeMNPV genome). Sequence analysis revealed a unique distribution of six different imperfect palindromes, several polyadenylation consensus motifs, multiple direct repeats, and several putative transcription factor binding sites. In this study I describe the identification and characterization of a putative non-*hr* origin of SpliNPV DNA replication.

4.3. Materials and Methods

4.3.1. Cells and virus

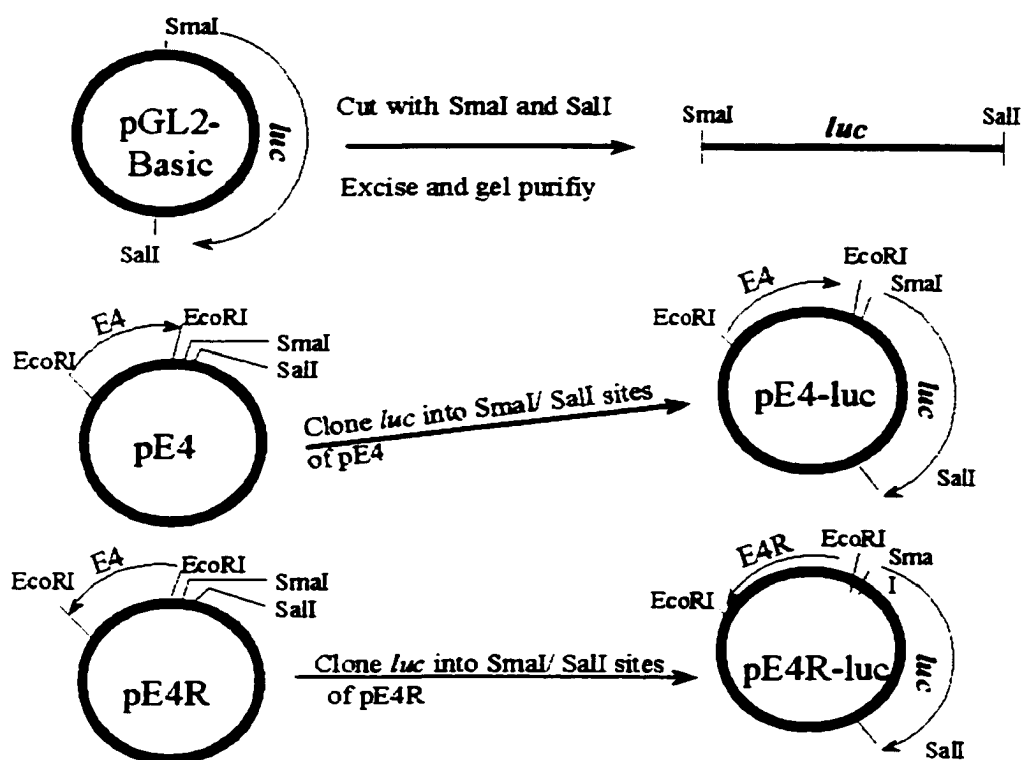
S. littoralis cells (CLS79) were kindly provided by Dr. S. Kamita, Department of Entomology, University of California, Davis. *S. frugiperda* (Sf9) cells were obtained commercially (PharMingen). Both Sf9 cells and CLS79 cells were cultured according to described procedures (O'Reilly *et al.*, 1992). *S. littoralis* NPV, isolate M-2 (SpliNPV) was obtained from Dr. G. Croizier, Station de Recherches de Pathologie Comparée INRA-CNRS, F-30380 Saint-Christol-Les-Ales, France. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (O'Reilly *et al.*, 1992).

4.3.2. Recombinant plasmids

The SpliNPV DNA was prepared as described by O'Reilly *et al.* (1992) and digested with the restriction endonucleases *EcoRI*, *SacI*, *XbaI*, *PstI*, and *HindIII* (New England Biolabs [NEB]) individually. The viral DNA fragments were cloned into the *EcoRI*, *SacI*, *XbaI*, or *HindIII* sites of plasmid pUC18 (NEB) to generate a partial SpliNPV genomic library. Plasmid pE4 was found to contain a 344 bp *EcoRI* fragment (the “E4 fragment”) that conveyed the ability of the plasmid to replicate in SpliNPV-infected cells by transient replication assays (see below). Plasmids with the E4 fragment in both “forward” and “reverse” orientations were recovered and designated pE4 and pE4R, respectively (Figure 4.1a). In the forward orientation, the *HinfI* restriction endonuclease sites within the E4 sequence are proximal to the *SmaI* and *SalI* sites of pUC18. In the reverse orientation, the *HinfI* sites are distal to the *SmaI* and *SalI* sites of pUC18. The plasmid pGL2-Basic was purchased from Promega. The luciferase gene (bp positions 18 to 2744) was excised from pGL2-Basic after cleavage with *SmaI* and *SalI*, and inserted into pE4 and pE4R, creating the plasmids pE4-luc and pE4R-luc, respectively (Figure 4.1a).

The E4 fragment was removed from pE4 by cleavage with *EcoRI* and sub-cloned into the *EcoRI* site of pBluescript II KS+ (Stratagene) to create a plasmid called pBS-E4. The E4 fragment was then excised from pBS-E4 after cleavage with *EcoRV* and *SmaI* and cloned into the *SmaI* site of p lef3 -luc, a plasmid that contains the *lef3* promoter driving the luciferase gene (Wolff *et al.*, 1998), creating the expression plasmids, pE4-*lef3*-luc (forward orientation) and pE4R-*lef3*-luc (reverse orientation; Figure 4.1b). In the forward orientation, the *HinfI* sites of the E4 fragment are proximal to the 5' end of the *lef-3* promoter element. In the reverse orientation, the *HinfI* sites of the E4 fragment are distal to the 5' end of the *lef-3* promoter element. DNA isolation, restriction digestion, agarose gel electrophoresis, and Southern blot analysis were carried out according to standard protocols (Sambrook *et al.*, 1989).

(a)



(b)

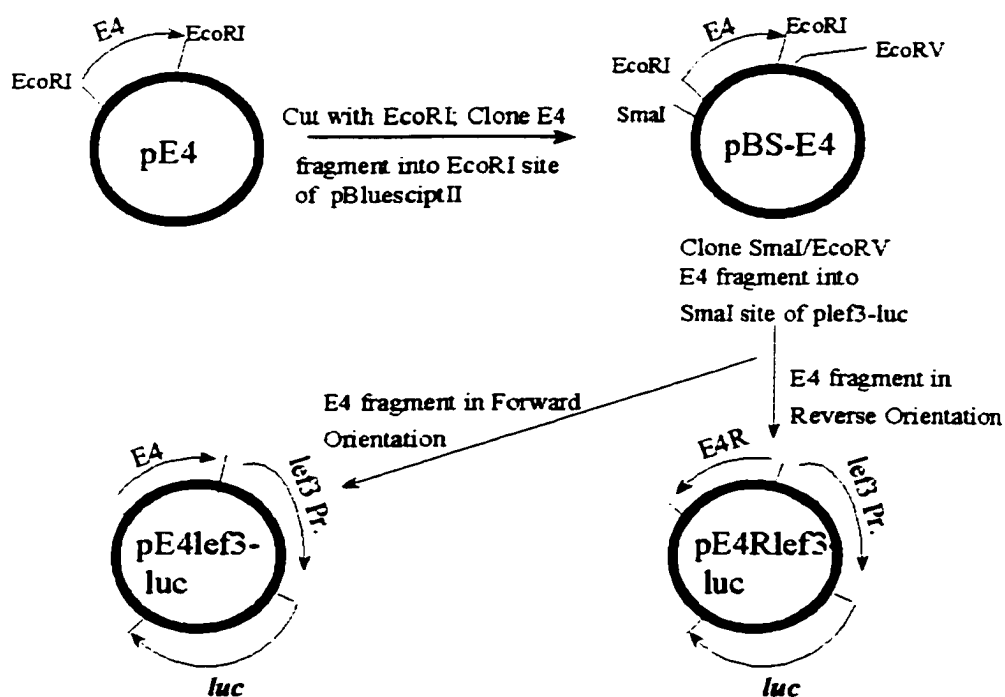


Figure 4.1. Plasmid constructs. a) pE4-luc, in which the E4 fragment was cloned immediately 5' of the luciferase gene (*luc*); and pE4R-luc, in which the E4 fragment was cloned immediately 5' of the luciferase gene in the reverse orientation to that of pE4-luc. b) plef3-luc, in which the luciferase gene was placed under transcriptional control of the SpliNPV *lef-3* promoter (plef3); pE4-lef3-luc, in which the E4 fragment was cloned immediately 5' of the *lef-3* promoter; pE4R-lef3-luc, in which the E4 fragment was cloned immediately 5' of the *lef-3* promoter, in the reverse orientation to that of pE4-lef3-luc.

4.3.3. Transient replication assay

Five (5) μ g of plasmid DNA were transfected into 5×10^6 cells by calcium phosphate precipitation. After 24 h at 27°C, cells were mock-infected or infected with SpliNPV at an MOI of 10 for 2 h. The infectious media were then replaced by fresh medium and the cultures were further incubated at 27°C for 72 h. The cells were harvested and the DNA was extracted following the protocol of Sarisky & Hayward (1996). To test for replication in cells, 10 μ g DNA were digested in a 100- μ l reaction volume, overnight at 37°C, with 30 U of *Hind*III to linearize the plasmid, and with 30 U of *Dpn*I, which cleaves in the sequence 5'-CAGT-3' only if the A is methylated. To monitor the *Dpn*I activity, 5 μ l of the *Dpn*I reactions were removed and incubated simultaneously with 500 ng of pUC18 DNA overnight at 37°C. Complete cleavage of the pUC18 DNA indicated that experimental DNA was also completely digested. After electrophoresis in 0.7% agarose gel, the DNA was transferred to nylon membranes (Hybond-N, Amersham Life Science) and hybridized with 32 P-dCTP labeled pUC18 plasmid DNA. The plasmid DNA was labeled by a random primed PCR according to the protocols specified in the Tag-It[®] Kit (Bios Corporation). All transfection replication assays were repeated at least three times. Replicated plasmid DNAs were subjected to partial digestion with *Hind*III followed by electrophoresis on agarose gels to determine if a step-ladder of fragments, indicative of high molecular weight concatameric DNAs, were detectable.

4.3.4. DNA sequence analysis

The E4 fragment (in pBS-E4) was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). DNA sequencing reactions were performed with the *fmo*I[®] DNA Sequencing System (Promega) according to the manufacturer's protocol. Sequences of both strands were obtained by bidirectional sequencing of plasmid DNA using T3 and T7 primers. Sequences were assembled and analyzed with the aid of computer programs from the Lasergene Package (DNASTAR Inc). The E4 sequence was compared with DNA and amino acid sequences in Genbank using the BLAST and FASTA

network service programs (Altschul *et al.*, 1990). Nucleotide sequence alignments were performed using the Clustal W multiple sequence alignment program (Thompson *et al.*, 1994). Direct and inverted repeat sequences were identified by using the Align program (DNASTAR Inc.). A weight matrix search program, MatInspector (Quandt *et al.*, 1995), was used to search for putative transcription factor binding sites. The helical stability of the E4 fragment sequence was analyzed by use of the algorithm Oligo program (National BioSciences). The $-\Delta G$ values across the entire sequence were plotted using MicroSoft Excel.

4.3.5. Luciferase assays

Mock-infected and SpliNPV-infected (MOI = 10) Sf9 and CLS79 cells (10^6) were transfected at 2 hpi with two (2) ug of plasmid containing luciferase gene, by calcium phosphate precipitation. At 4 h post-transfection, the culture medium was removed, the cells were washed twice with Phosphate-buffered Saline (PBS), and then provided with fresh medium. Cells were harvested at 48 h post-transfection. The cell pellets were resuspended in 100 ul Lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol [DTT], 2 mM 1,2 diaminocyclohexane-N,N,N,N'-tetraacetic acid [DCTA], 10% glycerol, 1% Triton X-100) and incubated at room temperature for 10 min. Luciferase assays were conducted using the Luciferase Assay System (Promega Corporation), following the manufacturer's protocol. Assays were performed using a Liquid Scintillation Counter (Wallac, model 1410). All samples were measured within 2 minutes of the addition of the luciferase assay reagent.

4.3.6. Preparation of nuclear extracts and gel mobility shift assays

Extracts of nuclear protein were prepared following the methods of Parker and Topol (1984). Protein concentrations of 5-20 mg/ml were determined by the method of Bradford (1976), with bovine serum albumin as a standard (Bio-Rad). Gel-purified, double-stranded DNA probes were radioactively labeled by the use of Klenow and $\alpha^{32}\text{P}$ -ATP. Unlabeled, double-stranded DNAs were used as specific (E4 DNA) and non-

specific (poly[dI-dC]) competitors in the gel mobility shift assays. Binding reactions with nuclear extracts from uninfected and infected cells were carried out by incubating various amounts of nuclear extract with 1 ng of radio-labeled E4 DNA, in a buffer containing 20 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM MgCl₂, 0.1% BSA, 6% glycerol, 1 mM DTT, and 3 ug poly[dI-dC] (Pharmacia) at 25 °C for 40 minutes. Two types of labeled probe were used. The preliminary experiments were conducted with intact labeled E4 fragment. Later experiments were conducted with *HinfI* cleaved E4 fragment, to observe the pattern of protein-DNA complex formation with different regions of the E4 sequence. *HinfI* cleaves the E4 fragment at bp 176 and bp 274, generating fragments of 176, 98, and 70 bp. Protein-DNA complexes were separated by electrophoresis in 6% polyacrylamide (37.5:1 acrylamide-bisacrylamide) gels prepared and run in 0.5X Tris-borate-EDTA buffer at 4 °C

4.4. Results

4.4.1. Identification of an origin of SpliNPV DNA replication

I screened 25 clones, representing 15% of the viral genome (data not shown), by transient replication assays in my search for a putative SpliNPV origin of DNA replication. Southern blot hybridization revealed virus-dependent replication of one plasmid containing a small SpliNPV *EcoRI* fragment in both Sf9 and CLS79 cells (Figure 4.2a, lanes 4 and 10, respectively). The putative SpliNPV origin fragment was called the “E4 fragment,” as it was the fourth *EcoRI* fragment tested, and the plasmid was subsequently called the pE4. *DpnI* resistant-*HindIII* sensitive pE4 plasmids, derived from SpliNPV-infected Sf9 or CLS79 cells, were detected as linearized molecules of approximately 3.0 kb (2.7 kb pUC18 + 0.34 kb insert) by Southern blot analysis. Control experiments, in which DNA extracted from transfected/virus-infected cells was cleaved with *HindIII* alone or with *HindIII* + *DpnI*, confirmed that *DpnI* was unable to cleave the replicated plasmid DNA (Figure 4.2b). Other plasmids tested did not generate a *DpnI*-resistant fragment after co-digestion, indicating lack of virus-dependent DNA replication (Figure 4.2a, lanes 3, 4, 6, and 7, respectively). The pUC18 vector, without insert, did not

replicate in the virus-dependent, transient replication assay (Figure 4.2a, lanes 3 and 7). Finally, pUC18 plasmid with or without the E4 fragment did not replicate in uninfected Sf9 cells (Figure 4.2a, lanes 1 and 2). Partial digestion of replicated plasmid DNA with *HindIII* did not result in the production of a “step-ladder” of fragments, indicating that high molecular weight, concatameric DNA replication products were not produced during replication of pE4 (data not shown).

4.4.2. Location, DNA Sequence analysis, and structural features of the origin

To map the location of the E4 fragment in the SpliNPV genome and to determine if there are other regions of the SpliNPV genome with homology to the putative SpliNPV origin fragment, radio-labeled E4 DNA was used as a probe in Southern blots of SpliNPV genomic DNA cut with several restriction endonucleases. Under high stringency hybridization conditions, the E4 probe hybridized to high molecular weight restriction fragments resulting from cleavage by *BamHI*, *HindIII*, *KpnI*, and *SacI*, to the 2.7 kb *PstI*-J fragment, and to the 344 bp *EcoRI* fragment (data not shown). Thus, the E4 fragment was localized to the *PstI*-J fragment (Figure 4.3a), between 75.1 and 77.9 map units of the SpliNPV physical map (Croizier *et al.*, 1989) and is unique in the SpliNPV genome.

DNA sequence analysis of the 344 bp E4 fragment revealed a number of perfect and imperfect palindrome sequences, direct repeat sequences (Table 4.1), and putative transcription factor binding sites (Figure 4.3b). The E4 fragment contains a small perfect palindrome of 8 bp (P1) and a large imperfect palindrome (P2) of 22 bp. There are numerous direct repeats of 6 bp to 11 bp (R1-R19), which are found from 2 to 4 times within the sequence (Table 1). Analysis of the E4 sequence with MatInspector (Quandt *et al.*, 1995) revealed a number of putative transcription factor binding sites on both strands (Figure 4.3b). This program provides three useful parameters by which putative transcription factor binding sites may be identified; a “matrix similarity,” which indicates the frequency of nucleotide identity between the test sequence and the sequence of a corresponding transcription factor binding site; a “core similarity,” which consists of the four best-conserved consecutive nucleotides of the matrix; and a threshold of “minimum

matrix similarity.” A large number of consensus transcription factor binding sites with high similarity (matrix similarity >0.85; core similarity >0.95) were well conserved and readily identified.

Two putative Stimulating Protein binding sites (SP1; at bp positions 32-40 and 56-64 of the E4 DNA sequence, respectively) and one putative Upstream Stimulating Factor (USF) binding site detected on the opposite strand (bp position 77-68) were identified on the 5' site of a 13 bp A+T-rich sequence which was shown to be a region of high helical instability, identified by plotting the $-\Delta G$ values across the E4 sequence (Figure 4.3c). A putative Nuclear Factor III (NFIII) binding site (bp position 112-125) was found to overlap the A+T-rich sequence. Two putative Nuclear Factor I (NFI) binding sites that overlap on opposite strands (bp positions 205-222 and 228-211, respectively) and two adjacent putative GATA-binding sites (also found on opposite strands at bp positions 312-299 and 314-327, respectively) were detected on the 3'-side of the A+T-rich sequence. The two NFI sites overlap the large imperfect palindrome (P2). SP1, USF, NFI, and NFIII transcription factor recognition-sequence clusters are often found in vertebrate virus origins of replication (van der Vliet, 1996). GATA-binding proteins are ubiquitous eukaryotic transcription factors and have been demonstrated to play a role in modulating transcription of both AcMNPV and SpliNPV early gene promoters (Kogan and Blissard, 1994; Wolff *et al.*, 1998).

The SpliNPV E4 sequence aligned with regions within each of the AcMNPV *HindIII*-K (bp 1190 to bp 1592; Kool *et al.*, 1994; GenBank Accession Number M16821), OpMNPV *HindIII*-N (bp 517 to bp 899; Pearson *et al.*, 1993; GenBank Accession Number D17353), and SeMNPV *XbaI*-F (bp 213 to bp 598; Heldens *et al.*, 1997) non-*hr ori* fragments. While the SpliNPV non-*hr ori* sequence shared 57% sequence identity in the region of alignment in the AcMNPV non-*hr ori*, and approximately 50% sequence identity with the regions to which it aligned in the SeMNPV and OpMNPV non-*hr* sequences, no common sequences (other than consensus putative transcription factor binding motifs) were identified. No sequence similarities to *hr oris* of other baculoviruses were detected, and no open reading frames could be identified in any of the three possible reading frames in the putative SpliNPV non-*hr* origin fragment.



Figure 4.2. Transient replication assays. **a)** Sf9 and CLS79 cells were transfected with recombinant plasmids containing SpliNPV fragments and with pUC18 (P18) as a control. Plasmids pE4, pX2, pS3 consisted of pU18 with cloned SpliNPV *EcoRI*, *XbaI*, and *SacI* fragments, respectively. The number to the left of the panel indicates the size (kb) of replicated pE4 that was linearized after digestion with *HindIII* and *DpnI*. pUC18 plasmid with or without the E4 fragment did not replicate in uninfected Sf9 cells (M1 and M2). **b)** Sf9 and CLS79 cells were transfected with pE4, and then infected with SpliNPV at 24 h post-transfection. Cellular DNA was isolated and then digested with *HindIII*, with (+) or without (-) *DpnI*.

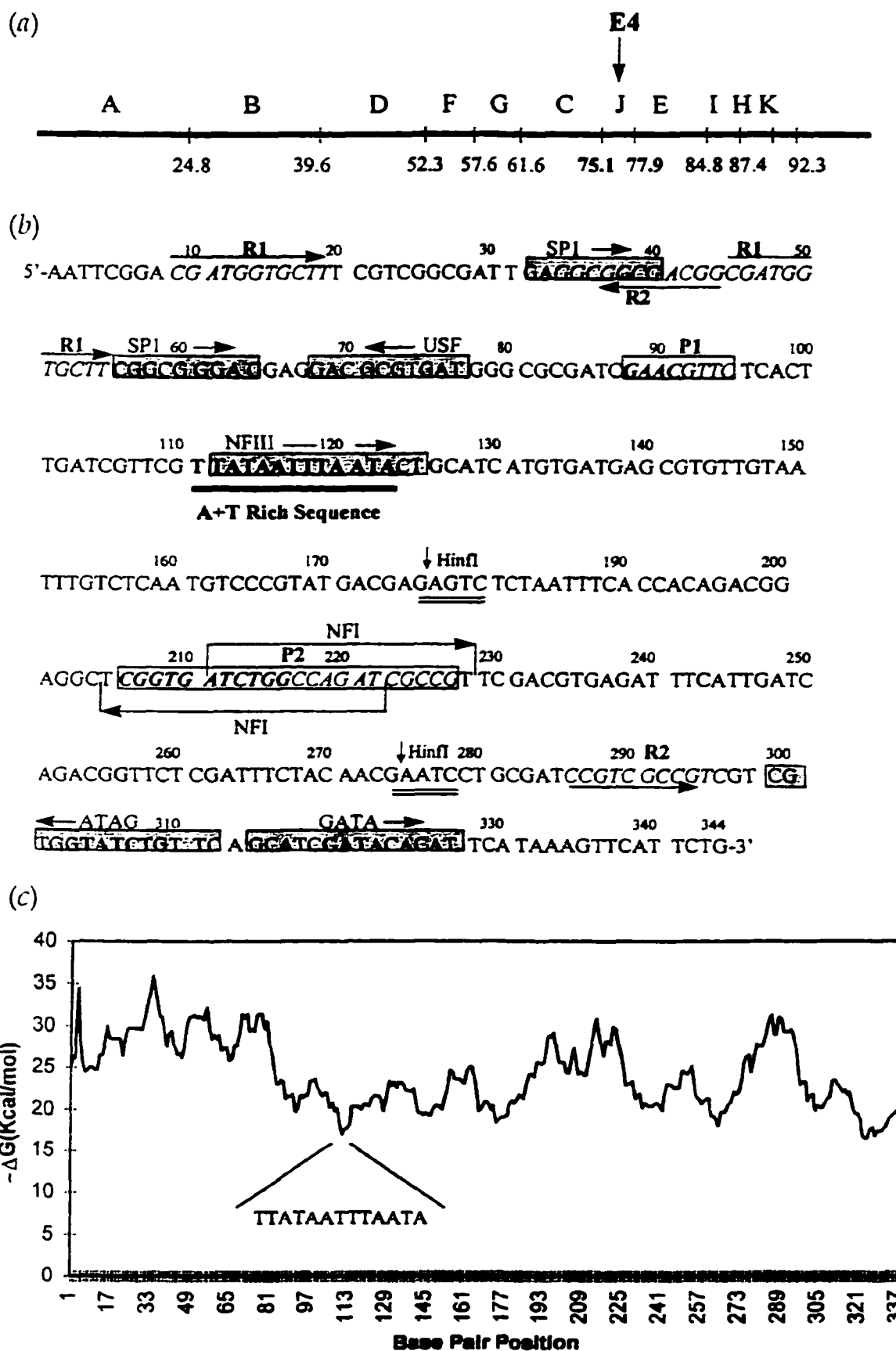


Figure 4.3. Location and DNA sequence analysis of the origin. a) Physical map of *Pst*I cleavage sites (adapted from Croizier *et al.*, 1989) and the location of the putative SpliNPV non-*hr* origin of DNA replication (E4) within the SpliNPV genome. **b)** Nucleotide sequence of the 344 bp SpliNPV *Eco*RI fragment. Numbers above the line indicate the base pair position within the E4 sequence. The A+T-rich region is indicated by bold letters and underlined. Palindrome sequences are indicated by P1 and P2 above the open boxes that surround their respective sequences. The 5'-half of each palindrome is indicated by bold, italic letters and the 3'-side is indicated by non-bold, italic letters. Repeat sequences (R1 and R2) are indicated by arrows above or below their respective sequences, shown in italic letters. Putative transcription factor binding sites are indicated by unshaded or shaded boxes; SP1- Stimulating Protein-1; USF- Upstream Stimulating Factor; NFI- Nuclear Factor I; NFIII- Nuclear Factor III; GATA- GATA-binding Protein. Arrows indicate the orientation of putative transcription factor binding sites. Locations of the *Hin*II restriction endonuclease sites are indicated by double lines. The vertical arrows indicate the actual cleavage sites. **c)** Predicted helical stability of the putative SpliNPV non-*hr ori*. DNA helical stability ($-\Delta G$) was calculated with the Oligo algorithm, and plotted by nucleotide position in the E4 fragment. The A+T-rich sequence with the lowest $-\Delta G$ region is indicated.

Table 4.1. Structural motifs within the putative SpliNPV non-*hr ori* sequence

Motif	Sequence	Position (bp)*		Length
		Top strand	Bottom strand	
Palindrome sequence†				
P1	GAACG TTC	88-95		8
P2	CGGtGATCTGGCCAGATCgCCG	206-217		22
Repeat Sequence‡				
R1	CGATGGTGCTT	9-19, 45-55		11
R2	CCGTCGCCG	286-294	44-36	9
R3	CGTTCGA	226-232	92-86	7
R4	TCGCCGT	221-227, 289-295	47-41	7
R5	CAGACGG	194-200, 250-256		7
R6	GCGATC/T	26-31, 82-87, 281-286	225-220	6
R7	GGAGGA/C	64-69, 61-66, 199-204		6
R8	GTGAGA	234-239	99-94	6
R9	TTAATTT	114-119, 148-153, 183-188		6
R10	CGTTCG	105-110	92-87	6
R11	CGCCGT/C	223-228	46-41, 40-35	6
R12	TCGCCG	289-294	29-24	6
R13	GATTTCT	238-243, 262-267		6
R14	CCGTCTG	286-191, 192-297		6
R15	CTCAAT	156-161	34-29	6
R16	AACGAA	271-276	112-107	6
R17	TACAAC	268-273	149-144	6
R18	CATTGA	243-248	162-157	6
R19	TCCGTC	285-290	201-196	6

* The base pair positions indicated refer to the nucleotide sequence of the SpliNPV E4 fragment as illustrated in Figure 4.3b.

[†] Palindromic Sequences; P1 and P2 are perfect and imperfect palindromes, respectively.

Mismatched bases in the imperfect palindrome P2 are indicated by lower case letters.

[‡] Repeat sequences; indicated as R1 to R19.

4.4.3. Functional analysis of *cis*-acting sequences in the origin

I investigated the ability of the SpliNPV E4 fragment to act as an enhancer of SpliNPV early gene promoter-mediated gene expression using transient expression assays. In both uninfected and SpliNPV-infected cells, luciferase expression from constructs containing the E4 fragment (pE4-lef3-luc and pE4R-lef3-luc; see Figure 4.1b), was lower than luciferase expression from the plef3-luc control plasmid, indicating that the E4 fragment repressed, rather than enhanced, *lef3* promoter-mediated luciferase expression in both Sf9 and CLS79 cells (Figure 4.1a).

I further tested the E4 fragment for potential ability to promote expression of the luciferase gene. In uninfected cells, transfection of the pE4-luc and pE4R-luc plasmids (Figure 4.1a) resulted in basal luciferase gene expression in an orientation-independent manner. Transfection of plasmids into SpliNPV-infected cells resulted in an approximately 10-fold increase in luciferase gene expression and was also orientation-independent (Figure 4.4b). However, the level of E4-mediated luciferase expression was much lower than SpliNPV *lef-3* promoter-mediated luciferase expression (compare the first column of Figures 4.4a and 4.4b). Transient expression assays in SpliNPV-infected Sf9 and CLS79 cells resulted in approximately the same pattern of activity, although the level of E4-mediated luciferase expression was higher in SpliNPV infected CLS79 cells than in virus-infected Sf9 cells. Transient expression assays in which pBluescript II KS+ containing the luciferase gene alone (pBS-luc) resulted in only background levels of luciferase expression (Figure 4.4b).

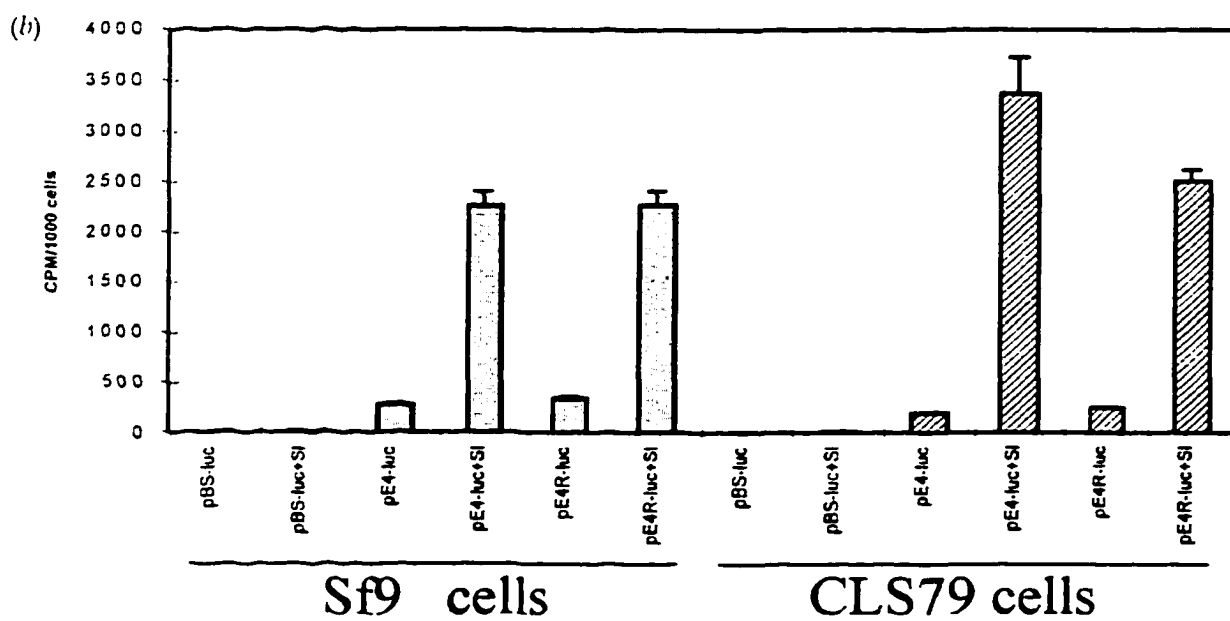
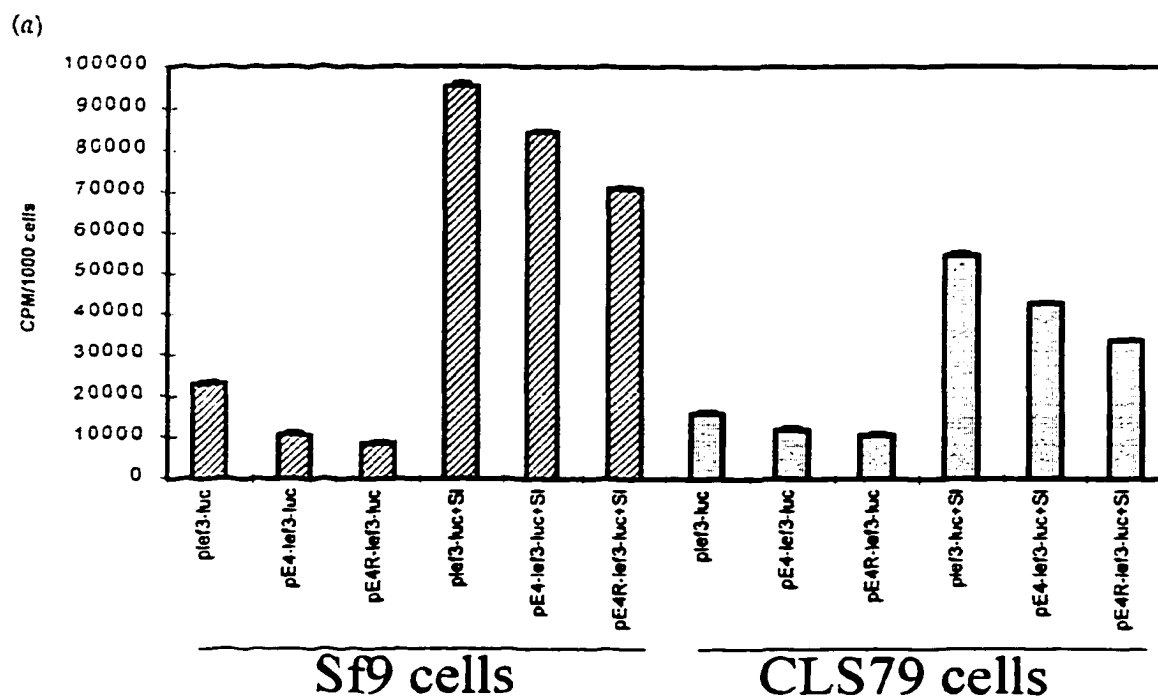


Figure 4.4. Functional analysis of *cis*-acting sequences in the origin. a) The E4 fragment repressed SpliNPV *lef-3* promoter-mediated luciferase expression. Mock-infected and SpliNPV-infected Sf9 and CLS79 cells were transfected with plasmids, pE4-lef3-luc or pE4R-lef3-luc, cells were harvested and then assayed as described in the Methods. Luciferase activities were plotted as CPM per 1000 cells. Each column represents the average of three independent transfections with the standard deviation indicated. Assays conducted with SpliNPV-infected cells are indicated as “+ Sl.” **b)** The E4 fragment alone promoted luciferase expression. Sf9 and CLS79 cells were transfected with plasmids pBS-luc, pE4-luc or pE4R-luc in the presence or absence of SpliNPV. Luciferase activities were plotted as CPM per 1000 cells and each column represents the average of three independent transfections with the standard deviation indicated.

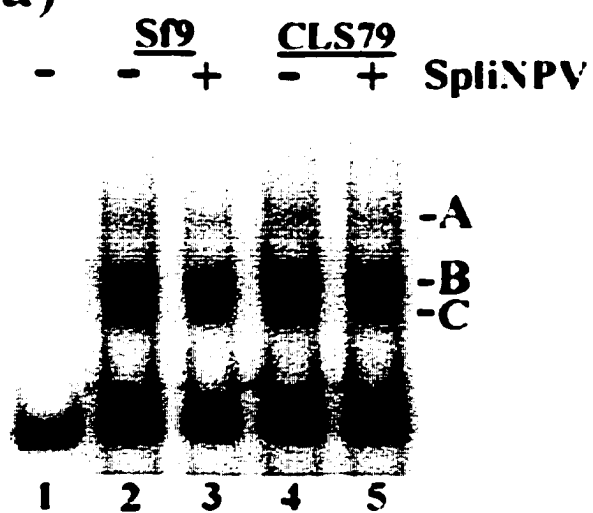
4.4.4. Cellular proteins bind to the putative SpliNPV origin sequences

Incubation of nuclear extracts from virus-infected Sf9 or CLS79 cells with the intact E4 fragment resulted in the formation of three discrete DNA-protein complexes (Figure 4.5a). The addition of increasing amounts of nuclear extract showed that the appearance of each protein-DNA complex was protein concentration dependent (Figure 4.5b). Protein-DNA complex B appeared more readily at low protein concentrations than protein-DNA complexes A and C, and protein-DNA complex A formed a clear, discrete, band only at high protein concentrations. The addition of increasing amounts of competitor DNA, in the form of intact, unlabelled E4 fragment, abolished the formation of protein-DNA complexes (Figure 4.5c).

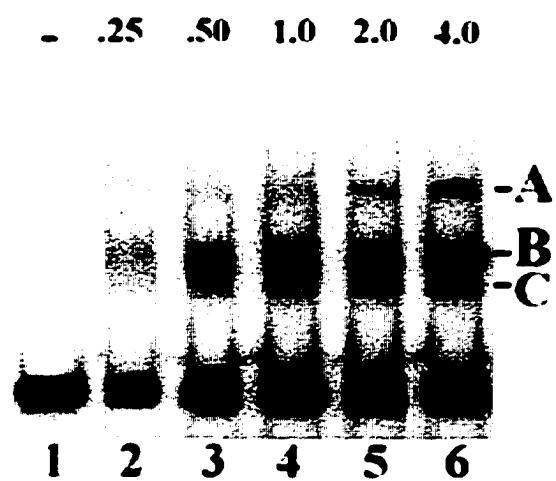
To determine the regions of the E4 sequence to which the cellular proteins bind, I conducted gel mobility shift assays with *HinfI* cleaved E4 fragment, which generates restriction fragments of 176 bp, 98 bp, and 70 bp (Figure 4.3b). The 176 bp fragment contains the A+T-rich region with its overlapping putative NFIII transcription factor binding site, flanked on the 5' end by two putative SP1 and one putative USF transcription factor binding sites. The 98 bp fragment contains two putative NFI binding sites, and the 70 bp fragment contains two putative GATA-binding sites.

Incubation of nuclear extracts from uninfected or virus-infected cells with the E4 fragment cut with *HinfI* (176 bp + 98 bp + 70 bp fragments) also resulted in the formation of three discrete DNA-protein complexes (Figure 4.5d, lanes 6-9). Incubation of nuclear extracts from uninfected or virus-infected cells with the gel purified 98 bp + 70 bp fragments resulted in the formation of only one protein-DNA complex (Figure 4.5d, lanes 2-4). I could not detect a difference in protein-DNA complex formation between the E4 fragment and extracts prepared from uninfected or virus-infected cells using the gel mobility shift assay.

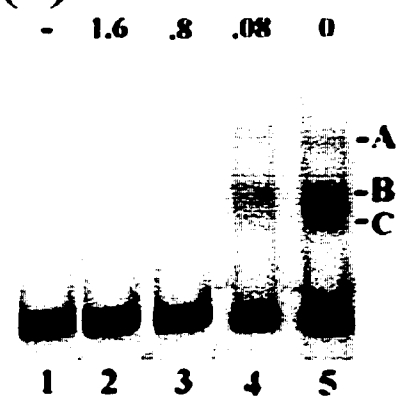
(a)



(b)



(c)



(d)

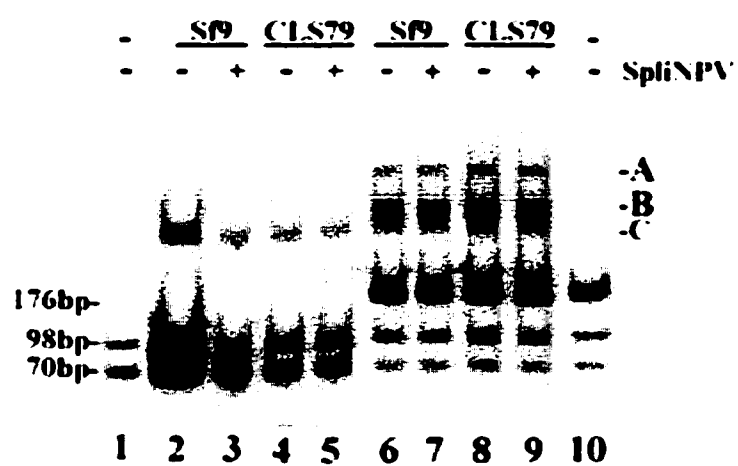


Figure 4.5. Gel mobility shift assays with the SpliNPV origin. **a)** Nuclear proteins bind to the SpliNPV E4 fragment. Nuclear extracts from Sf9 and CLS79 cells were incubated with the labelled 344 bp E4 fragment and subjected to electrophoresis in gel mobility shift assays. Three DNA-protein complexes (A, B, and C) were detected with nuclear extracts from both uninfected (lane 2 and 4) and SpliNPV-infected cells. The mobility of the free probe (no protein added to the reaction) is shown in lane 1. **b)** Host nuclear proteins interact specifically with the E4 fragment. Binding reactions were carried out with a constant amount of labelled E4 fragment, using increasing amounts of nuclear extracts from uninfected Sf9 cells. The mobility of the free probe is shown in lane 1. **c)** Addition of excess unlabelled specific competitor DNA abolished protein-DNA complex formation. Binding reactions, conducted with constant amounts of nuclear extract from uninfected Sf9 cells, labelled E4 fragment, and an excess of unlabelled, non-specific competitor DNA were challenged with different amounts of unlabelled E4 competitor DNA; lane 2- 1.6 ug, lane 3- 0.8 ug, lane 4- 0.08 ug, lane 5- no specific competitor DNA. The mobility of the free probe is shown in lane 1. **d)** Host nuclear proteins bind to different regions of the E4 fragment. The E4 fragment was cut with *HinfI*, which generates three segments (176 bp + 98 bp + 70 bp). Only one protein-DNA complex was detected when the 98 bp + 70 bp *HinfI* segments were used as probes with either SpliNPV-infected or uninfected nuclear extracts. Three DNA-protein complexes were detected when all three *HinfI* fragments (176 bp + 98 bp + 70 bp) were used simultaneously (lanes 6-9). The mobilities of the 98 bp and 70 bp fragments, as well as of all three fragments are shown in the lanes 1 and 10, respectively.

4.5. Discussion

I have identified and characterized a 344 bp *EcoRI* fragment (E4) from the genome of SpliNPV that can support both DNA replication, as determined by transient replication assays, and transcription, as determined by transient expression assays. Several lines of evidence support the conclusion that the E4 fragment contains a non-*hr* type of baculovirus origin of replication.

First, the SpliNPV E4 fragment supported SpliNPV-infection dependent plasmid replication in transient replication assays. Recombinant plasmids containing the E4 fragment did not replicate in uninfected cells, and the parent plasmid (pUC18) did not replicate in uninfected or SpliNPV-infected cells. Thus, replication of the recombinant plasmid in SpliNPV-infected cells was due to the presence of the E4 fragment. Comparison of the E4 nucleotide sequence with sequences in GenBank failed to reveal any similarities with reported baculovirus *hr* sequences and no known baculovirus promoters were detected. Baculovirus early gene promoters, such as the *ie-1* gene promoter, are known to be capable of supporting plasmid replication in transient replication assays (Wu & Carstens, 1996). Thus, the ability of the E4 fragment to support plasmid replication is not due to the presence of an *hr*-like *ori* or a baculovirus early promoter sequence.

Second, the SpliNPV E4 sequence displayed limited alignment with known non-*hr ori* sequences of other NPVs. The E4 fragment shared 57% DNA sequence identity with the AcMNPV non-*hr* element in the region of alignment, which occurred at the 3' end of the AcMNPV *HindIII*-K fragment. It also shared approximately 50% DNA identity with both the OpMNPV *HindIII*-N fragment and the SeMNPV *XbaI*-F fragment. However, the region to which the SpliNPV E4 fragment aligned in AcMNPV *oriK* is contained completely within the AcMNPV *EcoRI*-S fragment of *HindIII*-K, which by itself was shown to be unable to support plasmid replication in AcMNPV-infected cells. Deletion analyses revealed that sequences between 84.9 and 85.9 m.u. of the AcMNPV genome (approximately 1,300 bp of the *HindIII*-K fragment) were required to support plasmid replication. Thus, sequences essential for replication of AcMNPV *oriK* are distributed over a much larger region of DNA than that to which the SpliNPV E4 sequence aligned. Transient replication assays revealed that deletion clones of OpMNPV

HindIII-N were replication competent only if they contained a central region that spanned bp 1786 to bp 2342. The SpliNPV E4 sequence aligned to a location well upstream of this essential region. Thus, while the SpliNPV E4 sequence aligned with sequences within the AcMNPV and OpMNPV non-*hr ori* elements, the locations of alignment in these elements did not correspond to regions that by themselves are replication competent. The region to which the SpliNPV E4 fragment aligned in the SeMNPV non-*hr*, however, did lie within an 800 bp *SspI* fragment that was shown to be replication competent by deletion analyses (Heldens *et al.*, 1997).

The lengths of NPV non-*hr oris* range from 1052 bp (SeMNPV *XbaI*-F), to 1,300 bp (AcMNPV *HindIII*-K), to 4,000 bp (OpMNPV *HindIII*-N). Thus, the putative SpliNPV non-*hr ori* is the shortest baculovirus non-*hr ori* identified to date. The lack of common sequence elements within each non-*hr ori* (other than consensus motifs of putative transcription factor binding-sites) and the fact that sequences required for replication competence for the AcMNPV, OpMNPV, and SeMNPV non-*hr oris* are distributed over large regions of DNA suggest that the SpliNPV E4 fragment is, so far, unique among baculovirus non-*hr* elements.

Third, the complex structure of direct repeats, palindrome sequences, A+T-rich regions, and putative transcription factor binding sites in the SpliNPV E4 sequence has much in common with the non-*hr oris* identified in the AcMNPV, OpMNPV, and SeMNPV (Kool *et al.*, 1994b; Pearson *et al.*, 1993; Heldens *et al.*, 1997). Figure 6 displays the distribution of putative transcription factor binding sites and A+T-rich domains in the four non-*hr oris* within the regions of alignment of the SpliNPV sequence with the AcMNPV, OpMNPV, and SeMNPV non-*hr ori* elements. I did not show the numerous direct and inverted repeat sequences that are also found in these elements. While there are common putative transcription factor binding sites, as well as A+T rich domains, in the four non-*hr oris*, each element has a unique distribution of these sequences. The role of host proteins in the replicative ability of non-*hr oris*, and the importance of palindrome sequences, direct repeat sequences, and putative transcription factor binding sites within non-*hr* sequences, are unknown.

I used gel mobility shift assays to demonstrate that nuclear proteins from both uninfected and SpliNPV-infected cells bound with specificity to sequences within the

putative SpliNPV non-*hr ori* fragment. Much higher concentrations of non-specific, unlabeled competitor DNA were required to abolish protein-DNA complex formation than unlabeled E4 DNA, suggesting that nuclear proteins bound the E4 fragment with specificity. However, at least 50% of the labeled probe remained unbound in these experiments. This may be due to the vast excess of labeled probe DNA used in the assays or it may be that the reaction conditions used were optimal for high affinity binding, or perhaps other factors necessary for high affinity binding were not active in the extracts.

Comparison of the non-*hr oris* from AcMNPV, OpMNPV, SeMNPV, and SpliNPV with the consensus *oris* of vertebrate viruses revealed some intriguing structural similarities. Origins of replication among prokaryotes, viruses, and multicellular organisms possess a number of common features which variably include simple tri-, tetra-, or higher dispersed repetitions of nucleotides, A+T rich tracts, inverted repeats, initiator-protein binding sites, intrinsically curved DNAs, DNase I-hypersensitive sites, and/or binding sites for transcription factors (Boulikas, 1996). Analyses of viral DNA replication in vertebrate cell lines have demonstrated that the *oris* of most vertebrate viruses consist of two components: 1) an essential core sequence that recruits origin recognition proteins and specifies the replication initiation site; and 2) auxiliary regions, which contain transcription factor binding sites and modulate the efficiency of replication initiated at the core (DePamphilis, 1988; 1993; 1996). It is well established that viral replication often depends on sequence elements and proteins that also activate transcription (Herendeen *et al.*, 1989; DePamphilis, 1988; 1996).

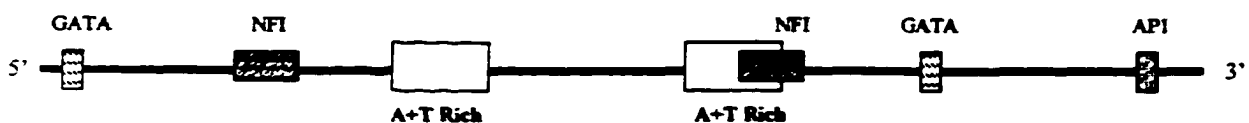
SpliNPV



AcMNPV



SeMNPV



OpMNPV

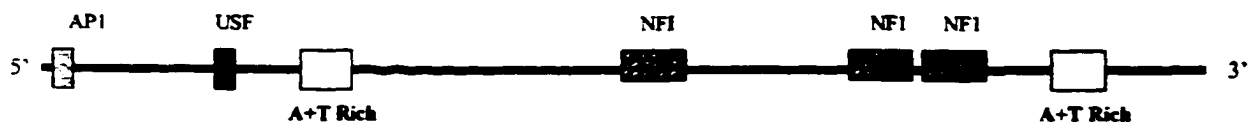


Figure 4.6. Comparison of the structural organization of selected *oris*. Schematic representations of the putative SpliNPV non-*hr ori* and corresponding non-*hr oris* of AcMNPV, SeMNPV, OpMNPV in the regions to which the SpliNPV E4 sequence aligned.

Transcription factors are known to play an essential role in replication of vertebrate viruses such as adenovirus (Jones *et al.*, 1987; Pruijn *et al.*, 1988; Mul *et al.*, 1990), SV40 (Cheng & Kelly, 1989), and herpes simplex virus (Nguyen-Huynh & Schaffer, 1998) by recruiting replication proteins to the origin and modulating the efficiency of replication initiation. The SpliNPV E4 fragment was able to activate transcription when it was cloned upstream of a reporter gene (luciferase). The absence of a known baculovirus promoter element in the E4 sequence and the observation that expression from the E4 fragment was orientation-independent, suggest that the transcriptional activity detected (indirectly as a function of luciferase activity) may have been a consequence of host and/or viral-encoded transcription factors that bind to the E4 element, in addition to an increase in the copy number of the luciferase gene as a result of infection-dependent plasmid replication. Gel mobility shift assays support the contention that there are proteins in nuclear extracts from both uninfected and virus-infected cells that bind specifically to DNA sequence elements in the E4 fragment.

In some cases, however, origins of replication have been demonstrated to act as transcription silencers (Rivier & Rine, 1992). In my transient expression assays, luciferase expression was repressed when the E4 fragment was placed immediately upstream of the *lef-3* promoter. It is possible that *lef-3* promoter-mediated luciferase expression was reduced due to competition between the E4 fragment and the *lef-3* promoter for transcription factors. An alternative explanation might be that it was due to changes in the topological state of the replicating plasmid that renders the *lef-3* promoter inactive. However, I feel this is unlikely. Some *hr* sequences have been demonstrated to act as origins of DNA replication in infection-dependent, transient replication assays, as well as *cis*-acting enhancers of early baculovirus promoters, such as *39K*, *ie-2*, *p35*, and *p145* (Guarino & Summers, 1986b; Nissen & Friesen, 1989; Carson *et al.*, 1991; Lu & Carstens, 1993). *Hr*-containing plasmids replicated in AcMNPV-infected cells were shown to consist of high molecular mass DNA, possibly in the form of a linear concatamer containing multiple copies of the plasmid (Leisy & Rohrmann, 1993). Despite this replication-dependent change in plasmid DNA structure, *hr* sequences still enhanced transcription in an orientation- and position-independent manner.

Restriction endonuclease analysis of replicated pE4 plasmid indicated that it did not form high molecular weight DNAs, suggesting that it does not form concatamers. Thus, repression of *lef-3* promoter-mediated luciferase expression by the E4 fragment is not likely to have occurred as a consequence of changes in DNA topology. Moreover, unlike transcription of the AcMNPV *lef-3* gene, which reaches its peak at 6 hpi and then decreases to a low level by 24 h (Li *et al.*, 1993), the SpliNPV *lef-3* gene is first detected approximately 4 hpi and steadily increases up to 56 hpi (Wolff *et al.*, 1998). Thus, the decreased luciferase expression observed in SpliNPV-infected cells was not due to a reduction in *lef-3* promoter activity as a consequence of the kinetics of *lef-3* transcription. Further characterization of the putative SpliNPV non-*hr ori* will resolve the role of these sequence motifs in viral DNA replication.

Acknowledgments

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Chapter 5. Identification, Transcription and Sequence Analysis of the *Spodoptera littoralis* Nucleopolyhedrovirus DNA Polymerase Gene

5.1. Abstract

Sequence analysis of a 6.4 kb DNA region from the *Spodoptera littoralis* Nucleopolyhedrovirus (SpliNPV) revealed a large open reading frame (ORF) encoding a predicted polypeptide of 998 amino acid (aa) sequences with a molecular mass of 114.93 kDa, located between 47.2-52.3 m.u. on the SpliNPV genome. Comparative sequence analyses demonstrated that the ORF encodes a DNA polymerase gene (*dnapol*) that contains conserved exonuclease domains and DNA polymerase motifs found in many prokaryotic, eukaryotic, and viral replicative DNA polymerases. A second ORF, ORF138, located between the *lef-3* and *dnapol*, encodes a 138 aa polypeptide that is homologous to ORF66 of AcMNPV. SpliNPV DNA polymerase shares an overall aa sequence identity of 39% with that of AcMNPV. A 3.0 kb SpliNPV *dnapol*-specific transcript was detected initially at 2 hpi and became abundant 48 hpi by Northern blot analysis. The transcription initiation site was mapped to a baculovirus early promoter element, ACGT. 3' RACE demonstrated that the SpliNPV *dnapol* transcript terminated at the polyadenylation signal AATAAA. Sequence analysis suggested that the SpliNPV *dnapol* and the *dnapol* of the NPV of *S. litura* (SpltNPV) are closely related.

5.2. Introduction

Like their host cells, DNA viruses require a DNA-dependent DNA polymerase to faithfully replicate their genomic information. These enzymes represent the core of the complex enzymatic machinery responsible for the faithful duplication of genomic DNA. DNA polymerases have been classified into four families based on amino acid sequence similarities to the *E. coli* DNA polymerase I, II, and III (Family A, B, and C, respectively), and to the cellular repair enzyme DNA polymerase β (Family X; Braithwaite & Ito, 1993). Eukaryotes and archaea both possess multiple genes coding for the Family B DNA polymerases that faithfully replicate their genomic information. In animals and fungi, the Family B DNA polymerases (α , δ , and ϵ), are responsible for replication of nuclear DNA (Edgell *et al.*, 1998). Large eukaryotic DNA viruses encode Family B DNA polymerases. Most of the Family B DNA polymerases possess two enzymatic activities: a DNA synthesis (polymerase) activity that enables the DNA polymerase to bind primer-template junctions and catalyze the addition of the correct nucleotides to the nascent strand; and an exonucleolytic activity that degrades single-stranded DNA in the 3'-5' direction (Blanco *et al.*, 1991). Amino acid sequence analyses have revealed that the polymerase domains consist of seven distinct consensus sequences with conserved order (IV-II-VI-III-I-VII-V). The 3'-5' exonuclease activity is associated with three amino acid sequence motifs (Exo I, Exo II, and Exo III) located in the N-terminal region of the DNA polymerase polypeptide (Knopf, 1998).

Early studies of AcMNPV-infected cells demonstrated the presence of a novel DNA polymerase activity that is distinct from host cell DNA polymerases (Miller *et al.*, 1981). A 3'-5' exonuclease activity, specific for single-stranded DNA, was shown to be associated with the DNA polymerase of BmNPV (Mikhailov *et al.*, 1986). Functional analyses demonstrated that the DNA polymerase of AcMNPV is highly processive and that the strand displacement ability of the DNA polymerase is stimulated by a single-stranded DNA-binding protein encoded by the viral gene, *lef-3* (McDougal & Guarino, 1999).

DNA sequences of eight complete baculovirus DNA polymerase genes (*dnapol*) have been identified from AcMNPV (Tomalski *et al.*, 1988), BmNPV (Chaeychomsri *et al.*, 1995), LdMNPV (Bjornson *et al.*, 1992), CfMNPV (Liu & Carstens *et al.*, 1995),

OpMNPV (Ahrens & Rohrmann, 1996), SeMNPV (Ijkel *et al.*, 1999), the NPVs of *Helicoverpa zea* (HzNPV; Cowan *et al.*, 1994; Bulach *et al.*, 1999), as well as from the *Xestia c-nigrum* GV (XecnGV) (Goto *et al.*, 1998). Recently, five partial DNA polymerase sequences from SpltNPV, the NPVs of *Buzura suppressaria* (BsNPV), *Orgyia anartoides* (OaNPV), *H. armigera* (HaNPV), and *Mamestra brassicae* (MbNPV) have become available (Bulach *et al.*, 1999). Analyses of the predicted amino acid sequences have revealed that these DNA polymerases share a number of sequence motifs, not only with one another, but also with DNA polymerases of other prokaryotes and eukaryotes (Ahrens & Rohrmann, 1996; Lu *et al.*, 1997).

Although baculoviruses have been extensively used as gene expression vectors, investigated as bioinsecticides, and studied for potential use as gene delivery systems (Kost & Condreay, 1999), most molecular information comes from the studies of the Group I NPV, such as AcMNPV. While eight complete baculovirus *dnaPol*s have been sequenced, analyses of the kinetics of *dnaPol* transcription are limited to only a few NPVs, such as AcMNPV, BmNPV, CfMNPV, and LdMNPV. Questions concerning the temporal regulation of the Group II NPV *dnaPol* transcription have not been addressed.

In this report, I present the identification, transcription, and sequence analysis of the SpliNPV *dnaPol*. Most significantly, I provide a complete comparison of fourteen available baculovirus *dnaPol* genes.

5.3. Materials and Methods

5.3.1. Cells and virus

S. frugiperda (Sf9) cells were obtained commercially (PharMingen). SpliNPV isolate M-2 was obtained from Dr. G. Croizier, Station de Recherches de Pathologie Comparée INRA-CNRS, F-30380 Saint-Christol-Lès-Alés, France. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (O'Reilly *et al.*, 1992).

5.3.2. Molecular cloning, PCR and sequencing

SpliNPV genomic DNA was digested using *EcoRI*, *SacI*, or *XhoI*; the fragments were randomly cloned into *EcoRI*, *SacI*, or *XhoI* sites of pBluescript KS+ (Stratagene). DNA sequence analysis of one clone, pE5, revealed a 992 bp *EcoRI* fragment (E5) containing a predicted amino acid sequence with significant similarity to DNA polymerase sequences present in GenBank. Subsequent Southern blot analyses determined that the putative SpliNPV *dnapol* sequence was contained on several plasmids within the library of SpliNPV clones (fragments E12, E30, E19, E5, E44, X18, X20, X24, X38, X14, X34) (see Figure 5.2a). These plasmids were sequenced by the standard Sanger dideoxynucleotide sequencing method using T3 and T7 universal primers (Sanger *et al.*, 1977). In order to sequence the gap between the *lef-3* and the region upstream of the *dnapol* (designated E10), the following primers were generated: forward primer, 5'-GAACGTGTCGACCGAGTTGCG-3', located at nucleotides (nt) 2519 to 2539 of the *lef-3* sequence (Wolff *et al.*, 1998; Accession number U77619); reverse primer, 5'-CGGCGATCGTCTCCCCGGTTCGCGTCC-3', located on the E12 fragment, complementary to nt 700 to 725 of the 6401 bp SpliNPV *dnapol* sequence reported herein. A standard polymerase chain reaction (PCR) protocol was followed using *Pfu* DNA polymerase (Stratagene). The PCR product was sequenced using the same primers. Sequences were assembled and analyzed with the aid of computer programs from Lasergene Package (DNASTAR Inc). The sequence was compared with nucleotide and amino acid sequences in GenBank using BLAST and FASTA network service programs (Altschul *et al.*, 1997). Sequence alignments were performed using Clustal W multiple sequence alignment program (Thompson *et al.*, 1994). A weight matrix search program, MatInspector (Quandt *et al.*, 1995) was used to search for putative transcription factor binding sites.

5.3.3. Southern blot analysis

To locate the putative *dnapol* within the virus genome, SpliNPV genomic DNA was digested with restriction endonucleases (REN; *HindIII*, *KpnI*, *NotI*, *PstI*, and *SacI*, respectively). Southern blots analysis followed standard procedures (Sambrook *et al.*,

1987) with a ^{32}P -dCTP labeled probe consisting of the *dnapol* using a random primer DNA labeling system (Gibco/BRL).

5.3.4. RNA isolation and Northern blot analysis

Cells (10^7) were mock infected or infected with SpliNPV at a multiplicity of infection (MOI) of 10. A protein synthesis inhibitor, cycloheximide, was added to cultured Sf9 cells at a final concentration of 100 ug/ml, 1 h before infection and maintained at this concentration thereafter. A DNA polymerase inhibitor, aphidicolin, was added to a final concentration of 5 ug/ml following virus absorption and maintained at this concentration thereafter. RNA extraction and Northern blot analyses followed the standard protocol as described previously (Huang *et al.*, 1999). Northern blots were hybridized with a SpliNPV *dnapol* gene-specific riboprobe derived from pE44, containing sequences from +1240 to +2583 relative to the putative translation initiation codon. Plasmid pE44 was linearized with *EcoRV* and subjected to *in vitro* transcription using T7 RNA polymerase in the presence of ^{32}P -UTP (Stratagene).

5.3.5. Mapping ends of the SpliNPV *dnapol* transcript

The 5' end of the DNA polymerase transcript was mapped by primer extension assays using 30 ug of total cellular RNA and a 5' end-labeled 34-base primer complementary to the SpliNPV DNA polymerase mRNA (5'-GCGGCAACCGTTTT CGGCTGCCATCTCTCGTTGC-3', position +65 to +33 relative to the putative translation initiation site). To accurately locate the transcription initiation site, DNA sequencing reactions were carried out using plasmid pE19 as template with the same primer. To determine the 3' end of the mRNA, 1 ug of total RNA extracted at various time points post-infection was hybridized with a cDNA synthesis (CDS) primer (Clontech; 5'-AAGCAGTGGTAACAACGCAGAGTACT₍₃₀₎-3') at 45 °C. First-strand cDNA synthesis was performed using AMV reverse transcriptase at 45 °C for 60 min. The resulting mixture was used for PCR amplification by adding a second primer (5'-GCGACGACGACGACGAGGACGACGACG-3', nt 5373 to 5399 of the SpliNPV

dnapol sequence, herein) for 35 cycles. The 180 bp PCR product was gel purified and sequenced.

5.3.6. GenBank accession number

The nucleotide sequence of the SpliNPV *dnapol* was submitted to GenBank. The GenBank accession number is AF215639.

5.4. Results

5.4.1. Identification, localization and sequence analysis of the SpliNPV DNA *dnapol* gene

To localize the putative *dnapol*, SpliNPV genomic DNAs were digested with different RENs and subjected to Southern hybridization analysis (Figure 5.1a). The putative *dnapol* hybridized with the *Hind*III-B, *Kpn*I-A, *Not*I-A, *Pst*I-D, and *Sac*I-B fragments, respectively (Figure 5.1b), and was mapped to 47.2-52.3 m.u. on the SpliNPV genome (Figure 5.2a). The localization of the *dnapol* indicated that it is close to *lef-3*, which maps to 42.8 to 46.8 m.u. on the genome (Wolff *et al.*, 1998). The gap region (E10) between the *lef-3* gene and the E12 fragment was obtained by PCR and sequenced. All clones containing the putative SpliNPV *dnapol* and flanking regions were sequenced at least three times on both strands and assembled (Figure 5.2a).

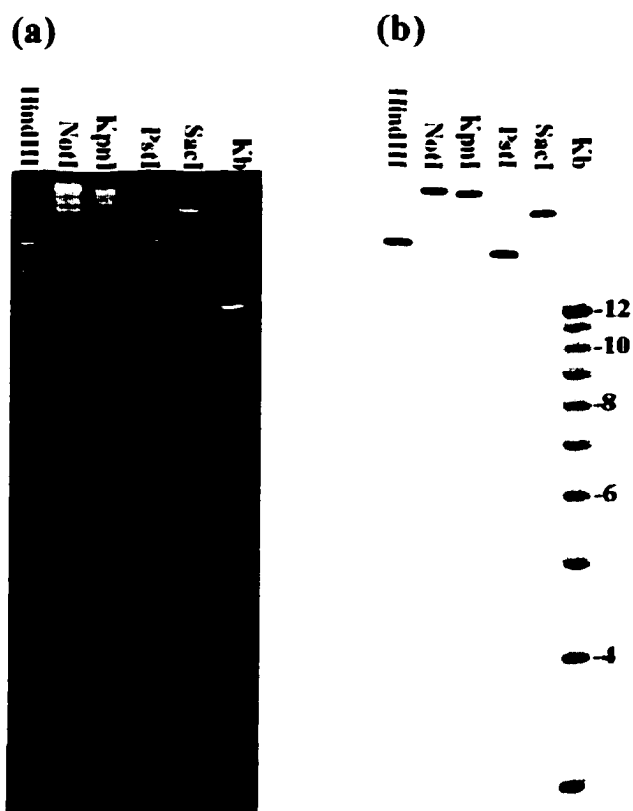


Figure 5.1. Localization of the SpliNPV *dnapol*. **a)** REN fragments of SpliNPV genomic DNA digested with *Hind*III, *Not*I, *Kpn*I, *Pst*I, and *Sac*I and separated on a 0.7% agarose gel. **b)** The digested SpliNPV genomic DNA was subjected to the Southern blot analysis and probed with SpliNPV *dnapol* gene fragment, E5, which hybridized to the *Hind*III-B, *Not*I-A, *Kpn*I-A, *Pst*I-D, and *Sac*I-B fragments.

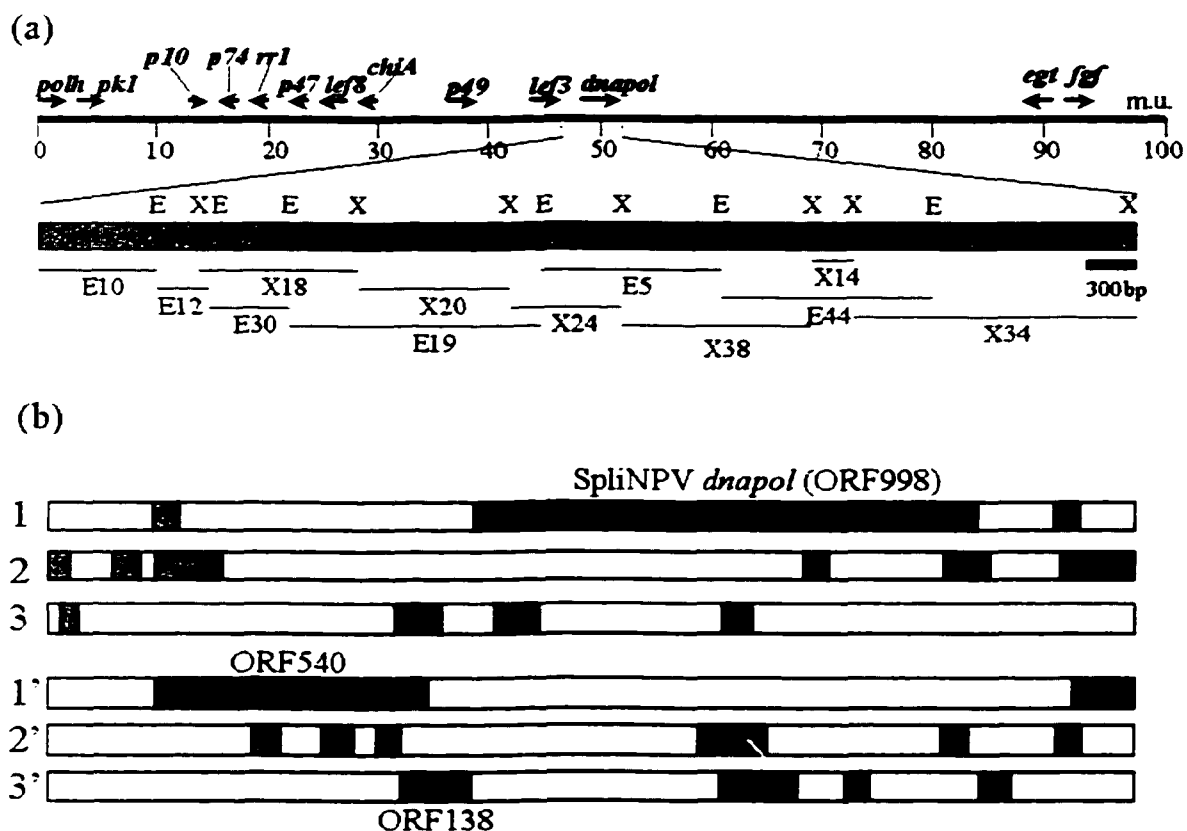


Figure 5.2. Sequencing strategy of the SpliNPV *dnapol* gene. **a)** The physical map of the SpliNPV genome (Croizier *et al.*, 1989). The map location and orientation of known SpliNPV genes are indicated. The 6.4 kb DNA sequence containing the putative SpliNPV *dnapol* gene is shown in a thick grey line. The locations of REN sites are indicated above the thick line, E (*EcoRI*); X (*XhoI*). Lines labeled as E12, E30, E19, E5, E44, X18, X20, X24, X38, X14, and X34 correspond to the *EcoRI* (E) and *XhoI* (X) fragments that were cloned into pBlueScriptII KS+ and sequenced at least three times. E10 segment is a PCR product located between the *lef-3* gene and the E12 fragment. **b)** ORFs determined by computer analysis of the DNA sequence using ORF Finder from NCBI. Open Reading Frames with dark shading are ORFs homologous to known polypeptides while unknown small ORFs are indicated with light shading.

The 6401 bp DNA sequence immediately downstream from the 2600 bp *lef-3* sequence (Wolff *et al.*, 1998) was found to contain several open reading frames (ORF) on both strands. A large ORF (designated ORF998) containing a putative DNA polymerase gene was identified (Figure 5.2b). Comparison of ORF998 with known NPV DNA polymerase genes suggested that the translation initiation codon was located at the first ATG of ORF998, resulting in a deduced SpliNPV *dnapol* of 2997 bp, encoding a putative 998 aa polypeptide with a predicted molecular mass of 114.93 kDa (Figure 5.2b). Immediately upstream and in the opposite direction of the *dnapol* is a 417 bp ORF (designated ORF138) with 57% aa sequence identity with ORF417 of HzNPV (Cowan *et al.*, 1994) and 40% aa sequence identity with ORF66 of AcMNPV (Tomalski *et al.*, 1988). Sequence analysis revealed an additional ORF (designated ORF540), located between the *lef-3* and the *dnapol*, which overlaps with ORF138 in the opposite direction of *dnapol* transcription. ORF540 predicts a 540 aa polypeptide with limited aa sequence similarities to the myosin heavy chain polypeptide from different origins (Nyitray *et al.*, 1991) and to the exonuclease subunit 2 of protein gp46 from bacteriophage RB69, which is involved in phage DNA recombination, replication and repair (Yeh *et al.*, 1998).

The complete DNA sequence of the SpliNPV *dnapol* is presented in the GenBank. BLAST analysis demonstrated that the SpliNPV *dnapol* and the SpltNPV *dnapol* share 88% nucleotide sequence identity (nt 1-2468 of the SpltNPV *dnapol* region). The SpliNPV *dnapol* shares limited homology with *dnapols* from LdMNPV (Bjornson, *et al.*, 1992), SeMNPV (Ijkel *et al.*, 1999), HzNPV, HaNPV, and MbNPV (Bulach *et al.*, 1999). No homologous region at the nucleotide sequence level was detected between the SpliNPV *dnapol* and the AcMNPV, BmNPV, OpMNPV, or CfMNPV *dnapol* genes.

The 5' flanking region of SpliNPV *dnapol* showed limited sequence similarities with those of other NPVs [44% identity with that of SeMNPV *dnapol* (Ijkel *et al.*, 1999) and 43% identity with that of AcMNPV *dnapol* (Tomalski *et al.*, 1988)]. Several putative transcription factor binding sites were detected within the 5' flanking region of the SpliNPV *dnapol* (see discussion). A 39 bp imperfect inverted repeat sequence (AAAAGTCGGCCAGGTTCTGAATCGAACGTGTCTGACTTTT), located from nt 5594 to 5632, was found downstream of the SpliNPV *dnapol* translation stop codon.

BLAST analysis revealed that similar repeat sequences occur once at the 3' end of the SpliNPV *lef-3*, twice at the 3' end of the SpliNPV *lef-8*, twice within the SpliNPV enhancer element located 1.0 kb from the polyhedrin gene, twice within a 5.7 kb *SacI* fragment located 10.7 kb from the polyhedrin gene, and four times in a 2.4 kb *SaII* fragment containing the SpliNPV *rr1* (Faktor & Kamensky, 1997).

5.4.2. Transcriptional analysis of the SpliNPV *dnapol* gene

Northern blot analysis was performed to characterize *dnapol* transcription, using a SpliNPV *dnapol*-specific riboprobe derived from the E44 fragment. The probe was complementary to the SpliNPV *dnapol* sense strand [containing 3738 to 5081 nt of the sequence (or +1240 to +2583 relative to the putative translation initiation codon)] (Figure 5.3a). A 3.0 kb SpliNPV *dnapol* transcript, detected at very low levels at 2 hours post infection (hpi), was observed to increase in intensity up to 48 hpi (Figure 5.3b). In the presence of a protein synthesis inhibitor, cycloheximide, transcription of the 3.0 kb *dnapol* mRNA remained at dramatically reduced levels from 6 to 28 hpi, but was observed to attain the same levels of intensity at 36 and 48 hpi as those of untreated samples. In the presence of a specific DNA polymerase inhibitor, aphidicolin, the 3.0 kb *dnapol* transcript was detected at very low levels at all time points.

5.4.3. Mapping ends of the SpliNPV *dnapol* transcript

Primer extension analyses revealed that only one transcription initiation site was detected (Figure 5.4a). The kinetics of RNA accumulation during the SpliNPV infection cycle suggested that *dnapol* mRNA was detectable as early as 2 hpi, increased, and accumulated during the course of infection (at least up to 48 hpi). This extension product was mapped to one of the baculovirus early promoter elements, ACGT, at position -30 nt relative to the predicted translation initiation codon. In the presence of cycloheximide, little extension product was detected from 2 to 24 hpi, and then increased to high levels at 36 and 48 hpi. While cycloheximide delayed SpliNPV *dnapol* transcription, treatment of infected cells with aphidicolin resulted in a dramatic reduction of *dnapol* transcription at

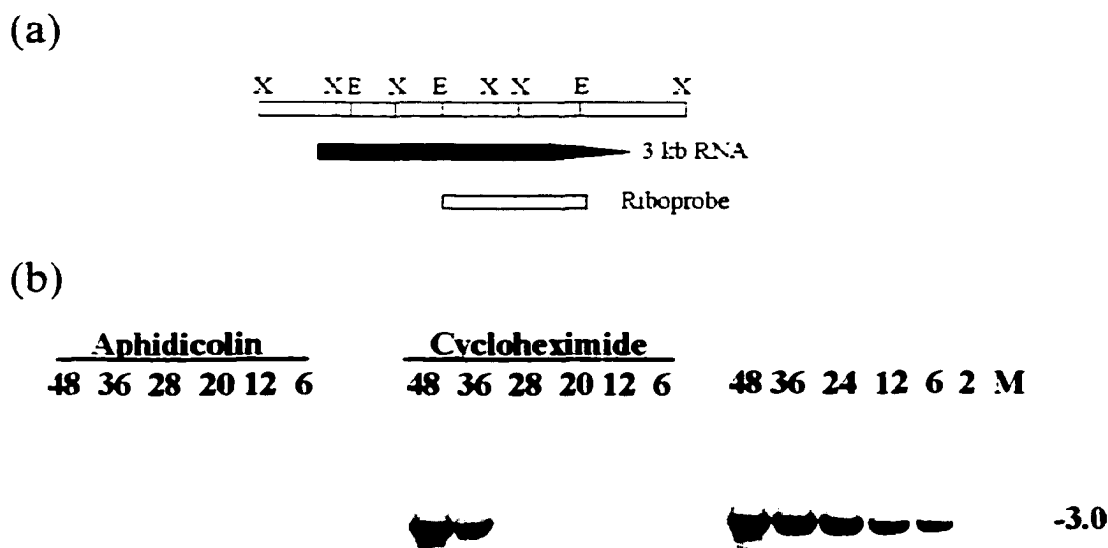


Figure 5.3. Transcriptional analysis of the SpliNPV *dnapol* gene. a). Location of riboprobe used in Northern hybridization. A ^{32}P -labeled RNA probe derived from *Eco*RI fragment (E44) of the SpliNPV *dnapol* gene (+1240 to +2583 relative to the putative translation initiation codon) was generated by *in vitro* transcription. b) Northern hybridization of the SpliNPV *dnapol* transcription. Total RNA was extracted from mock-infected or SpliNPV-infected cells at 2, 8, 12, 24, 36, and 48 hpi, as well as from SpliNPV-infected cells treated with aphidicolin or cycloheximide, and harvested at the indicated time points. The RNA was separated on a denaturing agarose gel, blotted onto a membrane, and hybridized with the riboprobe.

all time points. Primer extension analyses were consistent with the Northern blot assays and further confirmed that the SpliNPV *dnapol* is transcribed at low levels during the early phase of viral infection, and that transcripts accumulate to high levels during the course of infection.

3' Rapid Amplification of cDNA End (3'RACE) was employed to characterize the 3' end of *dnapol* mRNA. A 3'RACE amplification product of 180 bp was detected at low levels at 2 hpi and increased during the course of infection up to 48 hpi (Figure 5.4b). DNA sequence analysis of the PCR product revealed that the SpliNPV *dnapol* transcript terminated at the polyadenylation signal AATAAA, 58 nt downstream from ORF988. This is located at +3055 nt relative to the putative translation initiation codon.

5.4.4. Amino acid sequence conservation among baculovirus DNA polymerases

Amino acid sequence analysis revealed that the SpliNPV *dnapol* has an overall aa sequence identity of 51% with the predicted HzNPV *dnapol* (Cowan *et al.*, 1994); 48% with the SeMNPV *dnapol* (Ijkel *et al.*, 1999); 44% with the LdMNPV *dnapol* (Bjornson, *et al.*, 1992); 39% with the AcMNPV (Tomalski *et al.*, 1988) and BmNPV *dnapol* (Chaeychomsri *et al.*, 1995); 36% with the CfMNPV (Liu & Carstens, 1995); and 37% with the OpMNPV *dnapol* (Ahrens & Rohrmann, 1996) (Table 5.1). Although only partial sequences of the SpltNPV *dnapol* are available, the SpliNPV *dnapol* shares a 94% aa sequence identity with the deduced SpltNPV *dnapol* fragment (over 603 aa) (Bulach *et al.*, 1999). The SpliNPV *dnapol*, however, shares an overall aa sequence identity of only 29% with the XecnGV *dnapol* (Goto *et al.*, 1998). BLAST analysis also demonstrated that the SpliNPV DNA polymerase shares homology with other Family B DNA polymerases, such as *E. coli* (Pol II, Iwasaki *et al.*, 1991), herpes simplex virus (Quinn & McGeoch, 1985), vaccinia virus (Earl *et al.*, 1986), human cytomegalovirus (Kouzarides *et al.*, 1987), and other eukaryotic δ -like DNA polymerases. The aa sequence identities of these sequences range approximately from 20% to 25% (data not shown).

(a)

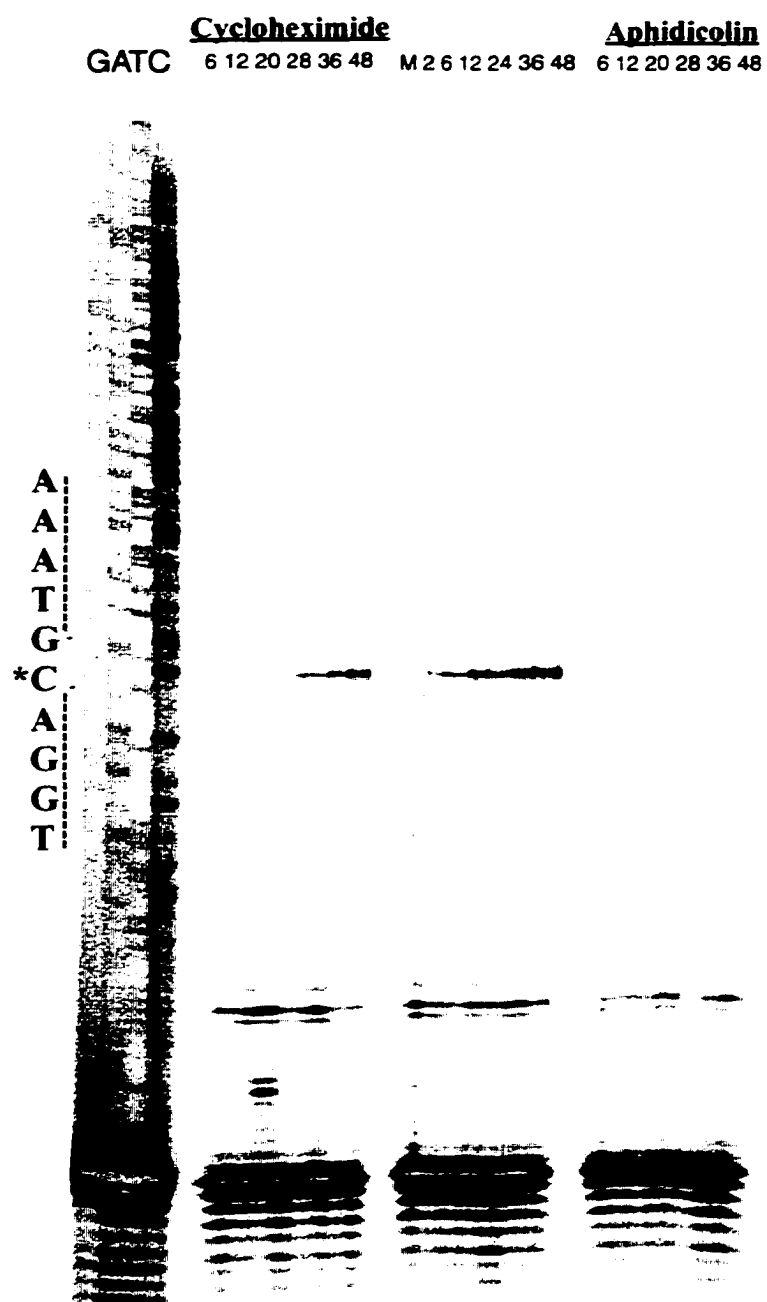




Figure 5.4. Mapping ends of the SpliNPV *dnapol* transcript. **a)** Primer extension assay. The ^{32}P end-labeled oligonucleotide (5'-GCGGCAACCGTTTTCTGGCTGCCATCTCTCGTTGC-3') was annealed to 30 ug RNA isolated from mock-infected cells (M) or SpliNPV-infected cells harvested at 2, 6, 12, 24, 36, and 48 hpi. RNA extracted at various time points post infection in the presence of aphidicolin or cycloheximide as indicated, was also subjected to primer extension analysis. The same radiolabeled primer was used to derive a sequencing ladder from the plasmid pE19 containing the primer extension region. The extension products and sequencing ladder were resolved on a 6% sequencing gel. The sequence complementary to the transcriptional initiation site is shown on the left and indicated with a star. **b)** 3' RACE analysis. RNA isolated from mock-infected or SpliNPV-infected cells at 2, 6, 12, 24, 36, and 48 hpi was hybridized to a cDNA synthesis (CDS) primer [5'-AAGCAGTGGTAACACGCAG AGTACT₍₃₀₎-3'] and subjected to the first strand cDNA synthesis. The cDNA was amplified by using a second primer located at nt 5373 to 5399 of the SpliNPV *dnapol* sequence (5'-GCGACGACGACGACGAAGACGACGACG-3'). The 180 bp PCR product was separated on a 1.0% agarose gel and sequenced.

An optimal multiple alignment of the putative SpliNPV DNA polymerase with 13 other complete or partial baculovirus *dnapol* was generated with Clustal W (Figure 5.5). There are clearly conserved regions distributed throughout the sequences, as well as a number of aa sequence insertions and deletions. Most notably, the N-terminal region of the SpliNPV DNA polymerase contains three conserved exonuclease domains which are present in the Family B DNA polymerases (Morrison *et al.*, 1991): the Exo I domain (aa 236-248), the Exo II domain (aa 330-346), and the Exo III domain (aa 464-478). Like true replicases, all DNA-dependent DNA polymerases possess an associated 3'-5' exonuclease acting as a proofreading function. The putative metal binding residues identified in *E. coli* Pol I are completely conserved in baculovirus DNA polymerases and those of other Family B DNA polymerases (Polesky *et al.*, 1992). The motifs of Exo I (D1E), Exo II (N3YD), and Exo III (Y3D) present in baculovirus DNA polymerases are found in colinear arrangement in both Family A and Family B DNA polymerases (Knopf, 1998). A set of seven sequence motifs, which are conserved among the Family B DNA polymerases, and which exhibit a conserved linear-spatial arrangement (IV-II-VI-III-I-VII-V) were also identified in baculovirus DNA polymerases (Figure 5.5). These motifs are believed to function as the polymerase domain and are involved in substrate binding, primase interaction, and pyrophosphate hydrolysis (Wang *et al.*, 1991). Although the overall aa sequence identities are low between GV and NPVs, the exonuclease domains and polymerase motifs are very conserved in these viruses.

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      20      40      60
*-----*-----*-----*
HsGLLNNV : -----MKVFIQNFYQLQREMAAENGCRKGDVYISTRDVFRIIRMYR : 40
HsGLLNNV : -----
HsGLLNNV : -----
HsGLLNNV : -----
HsGLLNNV : -----MTSSSSSSSEMETTTTFALLDWDQMQRKVN-FGTARGLTIGADDVFRIIRMYR : 54
HsGLLNNV : -----
HsGLLNNV : -----MDIALLTNGLIGOLLR-FGNRQHRVIEPEDVFRIIRMYR : 41
HsGLLNNV : -----MSSVNLMEWSALEKTLQAGRDAGKARVSIQPADTARITRMYA : 43
HsGLLNNV : -----MKIYSYNELKTRFAEYAKPGEFSITSADTFRIIRLHYD : 38
HsGLLNNV : -----MKIYSYNELKTRFAEYAKPGEFSITSADTFRIIRLHYD : 38
HsGLLNNV : -----MKIYTNQLQEAAPROYS-PROFAISSRONVFRVMRIYV : 37
HsGLLNNV : -----MKIYTNQLQEAAPROYA-PROFAVSRDDAERVMRIYV : 37
HsGLLNNV : -----MMGDQVEDFDIADYEEIDWCIREEEBKLPTSPGGRVCTNVLESEMSEMYSRQSTVPPQNTVYRITRLHYD : 71

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      80      100      120      140
*-----*-----*-----*
HsGLLNNV : -----NKOLYVFMGTG-----YLESDDPRVFQFYMRSSCVLYSYRSCYNVHMN--SSCQ-CRSYKTLVVPGLSGF : 103
HsGLLNNV : -----
HsGLLNNV : -----
HsGLLNNV : -----
HsGLLNNV : -----DNYLIVFFTGYLNNNNNNSSNGTELYQFYMETKCDLYSYRKCFGTHANTTCYNK-CISYKTMVMPGLRGV : 104
HsGLLNNV : -----
HsGLLNNV : -----DNCLIFFTG-----YVSSDPRKIFQFYMETKCDLYSYRSCYNVHONNECRYK-CKSYKTFVVPGLRGS : 104
HsGLLNNV : -----DNHIVFMWAPLAKEN-----HE-LYQFYAEVRCDLYSYKSCYGTASATCHRN-CTSYKTFVMPGLRGV : 106
HsGLLNNV : -----EFQGGCLFAFON-----TNIEKRVLQFYFKVKNLYSYKQCYDKHIFPSCRNK-CISYTFVAPGVVGN : 100
HsGLLNNV : -----EFQGGCLFAFON-----TNIEKRVLQFYFKVKNLYSYKQCYDKHIFPSCRNK-CISYTFVAPGVVGN : 100
HsGLLNNV : -----ENVGQLVAFON-----TQLAGRLAQFYFTIKMDLYSYKQCYNSHIFATCRNF-CSSYNTFVAPGVKNV : 99
HsGLLNNV : -----EGGGNLVAFON-----AQLGGRLAQFYFVMKMDLYSYKPCNSHIFATCRNF-CSSYNTFVAPGVKNV : 99
HsGLLNNV : -----NQRLYIFLTG-----VGNVQFYCKIFCPYISYKLCITNHRVVRQCRNAGCSTYKMMVVTGLKNY : 109

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      160      180      200
*-----*-----*-----*
HsGLLNNV : -----GDDKINVIKYARYEPSSCRNGSAAGRGKNDGADYFLKDINRVEMQLG : 175
HsGLLNNV : -----
HsGLLNNV : -----
HsGLLNNV : -----
HsGLLNNV : -----RGERINIIKYKKEPMN-----AHYKKEFLDSEFLNDINRVETQTN : 176
HsGLLNNV : -----
HsGLLNNV : -----YNERINIVHYKRTGEHD-----RNNKNCFLDSEFLKDINRVEMQTD : 176
HsGLLNNV : -----HTDKLHVVKFRSDER-----RDKNCLDGYLADVNRVEMQTD : 176
HsGLLNNV : -----CLNKINVIKYERNKAA-----PNDNAACLDKFLHNVRVHMOT : 176
HsGLLNNV : -----CLNKINVIKYERNKAS-----PNDNAACLDKFLHNVRVHMOT : 176
HsGLLNNV : -----YMDKINVIKFRNGSS-----FGEKAAALDKFLHNANRVHMOT : 176
HsGLLNNV : -----YMDKINVIKFRNGSS-----FGEKTHALDKFLHNANRVHMOT : 176
HsGLLNNV : -----CCERVHVVKVIRTLAN-----ANERFLLDEFCDVNRVQMOS : 176

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Exo I

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      220      240      260
*-----*-----*-----*
HsGLLNNV : -----KOLLVDAEREREERAS : 220
HsGLLNNV : -----KOLLVDAEREREERAS : 220
HsGLLNNV : -----ELTSDM-VNKMFEYEMESEREERAS : 220
HsGLLNNV : -----NACDADA-LKRLFKFPVEEREREERAS : 220
HsGLLNNV : -----TOFEA-IRNLENTDDEEREREERAS : 220
HsGLLNNV : -----SNFCA-AQSMFEYDDEEREREERAS : 220
HsGLLNNV : -----SNONT-FNDIFDDEEREREERAS : 220
HsGLLNNV : -----SNONT-FNDIFDDEEREREERAS : 220
HsGLLNNV : -----K-DVHE-FESMLERQVSEEREREERAS : 220
HsGLLNNV : -----TRMENLOHFEYEDFEEDEREREERAS : 220
HsGLLNNV : -----TRMENLOHFEYEDFEEDEREREERAS : 220
HsGLLNNV : -----APPELEAFADDFQENFAEREREERAS : 220
HsGLLNNV : -----APPELEAFADDFQENFAEREREERAS : 220
HsGLLNNV : -----N-----VMSCLAEEREREERAS : 220

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[illegible][illegible]

	1000	1020	1040	1060	
1	VSWKKHIGIMCSFINELIQVIGD--RGEFDSVENEICNYNSKVMYDVRVPULKEITESVAPKIKOGYPER				958
2	-----				
3	S-WLKHVGIMCTFFNELIQIIFGD--RKEFFYYFQNICRYYMQNQ-LFDVKYPLKLTVNHIKKSKKKFCWAEBQD				1007
4	-----				
5	INWIKHMGILCTFFNELIEVFGD--RETFQYVEGQITSVEMAQQSYDKYPLNTI-----NPKKLTQADD				962
6	ISWLKHMNILCTFMNELIQVFGN--SEETFEYVGAIVDEYTSAGQYDVRYPVLVSTR-----RANAG				967
7	ISWIKHMGILCTFMNELIEIFGDEQKDNLAKECTAIMQKYMQNGLYDRKEEPLVKIKINJEKKQYVE-----R				944
8	ISWIKHMGILCTFMNELLEIFGDEQKDNLAKECTAIMQKYMQNGLYDRKEEPLVKIKINJEKKQYVE-----R				944
9	ISWLKHMILTILTFMNELLEIFGDEHTSALNKCYDAILTKYMQHQYADKKRYTLIRIKNAARYNKAHSSDCAAR				940
10	ISWLKHMILTILTFMNELLEIFGDEHKDLALACYSAILKYMCHQYADKKRAALYKIATKKKAPSACASGK				940
11	LQWKKHLSIICSEFLNLMISMGN--SCAFYVAFEGICVYQKKNQNDITWETLKELTDSKKKDLKHEEMNI				916

[illegible]

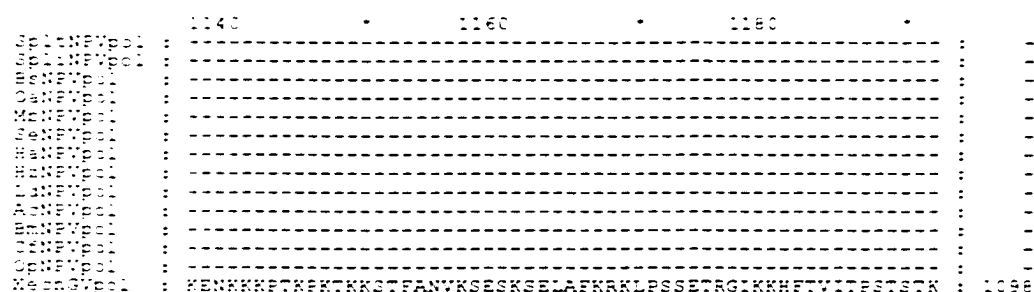


Figure 5.5. Amino acid sequence conservation among baculovirus DNA polymerases. The DNA polymerase aa sequences of AcMNPV (Tomalski *et al.*, 1988), BmNPV (Chaeychomsri *et al.*, 1995), CfMNPV (Liu & Carstens, 1995), OpMNPV (Ahrens & Rohrmann, 1996), LdMNPV (Bjornson *et al.*, 1992), HzNPV (Cowan *et al.*, 1994), SeMNPV (Ijkel *et al.*, 1999), BsNPV, OaNPV, HaNPV, MbNPV, and SpltNPV (Bulach *et al.*, 1999), and XecnGV (Goto *et al.*, 1998) were obtained from GenBank. The SpliNPV *dnapol* aa sequence is reported herein. Amino acid sequence alignments were generated with Clustal W. Identical nucleotides are indicated by white letters on a black background; those conserved in more than 50% of the sequences are shown on a grey background. Also labeled above the sequence are exonuclease domains (Exo I-III) and polymerase motifs (Regions I-VII) as defined by Wang *et al.* (1989) and Knopf (1998).

Table 5.1. Percentage of aa sequence identity of baculovirus DNAPOLs

	SpliNPV	BsNPV	OaNPV	LdMNPV	HaNPV	HzNPV	MbNPV	SeMNPV	AcMNPV	BmNPV	CtMNPV	OpMNPV	XecnGV
SpliNPV*	56	53	50	32	58	36	57	32	26	26	25	26	20
SpliNPV		33	31	44	36	51	35	48	39	39	36	37	29
BsNPV*			62	32	55	35	55	34	26	26	25	24	20
OaNPV*				32	57	36	54	33	25	25	23	23	19
LdMNPV					34	49	33	49	39	39	36	37	27
HaNPV*						60	62	37	28	27	26	26	20
HzNPV							39	56	41	41	38	37	29
MbNPV*								43	28	28	25	26	21
SeMNPV									40	40	36	37	30
AcMNPV										99	60	60	27
BmNPV											60	60	27
CtMNPV												82	26
OpMNPV													27

*Sequences are partial aa sequences from GenBank.

5.5. Discussion

I have sequenced a 6.4 kb DNA region from the SpliNPV genome that contains an ORF of 2997 bp and encodes a putative 998 aa DNA polymerase. The predicted SpliNPV *dnapol* is located at 47.2-53.2 m.u. on the genome, downstream from the *lef-3* gene. Primer extension and Northern blot analyses revealed that the 3.0 kb SpliNPV *dnapol* transcript initiates at a baculovirus gene promoter element, ACGT. Transcription of the SpliNPV *dnapol* was detected at low levels at 2 hpi and the transcripts were observed to accumulate to high levels during the course of infection. The SpliNPV *dnapol* is similar to the SpltNPV *dnapol* at both nucleotide and aa sequence levels, suggesting that SpliNPV and SpltNPV are closely related.

All known viral DNA polymerases belong to the Family A or Family B DNA polymerases. While Family A DNA polymerases share nine distinct consensus sequences, the Family B DNA polymerases contain seven DNA polymerase domains, and only two of these domains are homologous to those of the Family A DNA polymerases (Knopf, 1998). The conserved linear-order (IV-II-VI-III-I-VII-V) of the seven DNA polymerase domains in the SpliNPV *dnapol* suggested that the SpliNPV DNA polymerase is a member of the Family B DNA polymerases. On the basis of the crystal structure and mutational analysis of *E. coli* Pol I Klenow enzyme, it was demonstrated that the conserved Regions II and III are critical for binding and positioning DNA within the active site (Joyce *et al.*, 1994; Polesky *et al.*, 1992). Mutations of the analogous Region I ("YGD TD") in bacteriophage $\Phi 29$ were shown to affect the metal binding and pyrophosphorolytic activity (Polesky *et al.*, 1992), while mutations in this conserved motif in HSV-1 DNA polymerase resulted in the abolition of the DNA synthesis (polymerase) activity (Dorsky & Crumpacker, 1990). Region VII, which is also conserved in baculovirus DNA polymerases, has been implicated in recognition and binding of nucleotides and pyrophosphate substrates in other Family B DNA polymerases (Hwang *et al.*, 1992). The Family B DNA polymerases are further divided into two subfamilies: the protein-primed DNA polymerase subfamily and the RNA-primed DNA polymerase subfamily (Braithwaite & Ito, 1993). The SpliNPV DNA polymerase belongs to the RNA-primed DNA polymerase subfamily.

Early transcription of baculovirus is believed to be mediated by host RNA polymerase II. Functional analyses of transcription suggest that many baculovirus early promoters contain a functional TATA box, and/or conserved transcription initiation sequences, CAGT or ACGT, at or near the transcription start sites (Friesen, 1997). The *dnapol* promoter regions from AcMNPV (Tomalski *et al.*, 1988), BmNPV (Chaeychomsri *et al.*, 1995), CfMNPV (Liu & Carstens, 1995), and LdMNPV (Bjornson *et al.*, 1992) have been identified and functionally characterized.

The AcMNPV *dnapol* promoter region lacks the conventional TATA motif and CAGT start sites. It contains two sets of transcription start sites, located -120 to -124 nt and -200 to -216 nt, upstream of the translation initiation codon (Tomalski *et al.*, 1988). Although the 5' flanking nucleotide sequences of the BmNPV and AcMNPV *dnapol* are highly conserved (94% identity in 340 nt), the transcription initiation sites of BmNPV *dnapol* were mapped to different positions, -57 to -67 nt and -252 to -262 nt, upstream of the translation initiation codon (Chaeychomsri *et al.*, 1995). In CfMNPV, the major transcription initiation site was mapped to -76 nt upstream of the translation initiation codon at CGTGTC/AA, which is a consensus nucleotide motif found in the AcMNPV *dnapol* proximal transcription start site (Tomalski *et al.*, 1988; Liu & Carstens, 1995). There are at least two other transcription initiation sites in CfMNPV, mapped to CAGT motifs at -108 and -302 nt (Liu & Carstens, 1995). In contrast to AcMNPV, the LdMNPV *dnapol* contains both TATA and CAGT motifs in its 5' flanking sequence. *In vitro* run-off transcription suggested that the CAGT motif was used as the promoter element (Bjornson *et al.*, 1992).

While TATA and CAGT sequences are present upstream of the putative translation initiation codon of the SpliNPV *dnapol*, primer extension analysis revealed that the *dnapol* transcript is initiated at -30 nt upstream of the putative translation initiation codon, at the consensus sequence TACGTTC. The "CGT" motifs, with consensus sequence A(A/T)CGT(G/T), are present within the upstream activation regions (UAR) of the *p35*, *p94*, *39K* (*pp31*), *p143*, and *gp64* in baculoviruses (Friesen, 1997). In AcMNPV, the CGT motif alone provides an approximately five-fold stimulation of *p35* basal promoter activity (Dickson & Friesen, 1991). The ACGT motif of the SpliNPV *dnapol* bears a striking resemblance to the CACGTG motif first identified in the

OpMNPV *gp64* UAR that was shown to be recognized by a family of eukaryotic transcription factors known as the upstream stimulatory factor, USF (Kogan & Blissard, 1994).

In order to compare these *dnapol* 5' flanking regions, an optimal alignment of six *dnapol* promoter regions was generated. While the AcMNPV and BmNPV 5' flanking regions are very conserved, the 300 bp 5' flanking regions are highly variable among other *dnapol* genes (Figure 5.6a). The *dnapol* 5' flanking region of CfMNPV is similar to those of AcMNPV and BmNPV, but the 5' flanking region of OpMNPV *dnapol* is distinct from these and is presumably distantly related. The 5' flanking region of SpliNPV *dnapol* is very different from those of all other NPVs, sharing only 44% sequence identity with that of SeMNPV and 43% with that of AcMNPV.

Sequence structure analyses of the 5' flanking regions of NPV *dnapol* genes, however, reveal some intriguing structural similarities (Figure 6b). The 5' flanking regions contain putative binding sites for the Deformed (DFD) and Hunchback (HB) insect transcription factors, as well as other eukaryotic transcription factors, such as AP1, GATA, NFI, and NFIII. DFD is a homeotic selector gene product that binds to the core sequence "ATTA" and plays an important role in the transcriptional regulation of insect development (Egger *et al.*, 1992). HB transcription factor is a zinc finger protein that binds to the core sequence "AAAA" and regulates transcription in cultured *Drosophila* cells (Zou *et al.*, 1991). GATA-binding proteins are ubiquitous eukaryotic transcription factors and function in modulating transcription of AcMNPV early gene promoters (Kogan & Blissard, 1994). AP1, NFI and NFIII transcription factor recognition-sequence clusters are often found in eukaryotic and viral promoter regions and play very important roles in gene expression and regulation (van der Vliet, 1996). Differences in the organization of transcription factor binding sites in the 5' flanking regions of these NPVs may account for the observed differences in gene expression and regulation.

Of the eight complete baculovirus *dnapols*, the AcMNPV *dnapol* is the best characterized. The AcMNPV *dnapol* transcripts were detected as early as 2 hpi (Tomalski *et al.*, 1988) and declined to very low levels by 12 hpi. The BmNPV *dnapol*, whose product shares a 99% aa sequence identity with that of the AcMNPV *dnapol*, was shown to have a similar pattern of transcription kinetics, although the BmNPV *dnapol*

transcripts persist for a longer period of time post infection (Chaeychomsri *et al.*, 1995). It was suggested that the decline in AcMNPV *dnapol* transcription might be due to the presence of a transcription inhibitor during the late phase of infection (Ohresser *et al.*, 1994).

In contrast, CfMNPV, an NPV closely related to AcMNPV, encodes a *dnapol* gene whose expression was reported to initiate at 6 hpi and became abundant during the late phase of infection. It has been demonstrated that the regulation of CfMNPV *dnapol* transcription is different from that of the AcMNPV *dnapol* (Liu & Carstens, 1995). Although SpliNPV and CfMNPV are distantly related, the kinetics of their respective *dnapol* transcription appears similar. Northern hybridization and primer extension assays revealed that both the CfMNPV and SpliNPV *dnapol* genes are transcribed at low levels at early viral infection and that the transcripts become abundant toward the late phase of infection. The patterns of transcription kinetics of different NPV *dnapol* genes might reflect the differences between virus species during the replication cycle.

Several lines of evidence indicate that the SpliNPV *dnapol* is transcribed as an early gene. First, studies of NPV gene expression revealed that the transition from the early to late phase of the virus infection cycle is characterized by replication of viral DNA and activation of an α -amanitin resistant DNA-dependent RNA polymerase activity (Blissard & Rohrmann, 1990). With AcMNPV, this transition occurs approximately 6 to 8 hpi, when viral DNA synthesis begins in AcMNPV-infected *S. frugiperda* (Sf) cells (Huang *et al.*, 1999). In contrast, viral DNA synthesis, and the transition from the early to late phase of infection, begins 12 to 16 hpi in SpliNPV-infected Sf cells (Huang *et al.*, 1999). In replication time course assays, DNA synthesis products accumulated to detectable levels at 16 hpi and continued to increase to high levels of up to 48 hpi (Huang *et al.*, 1999). The kinetics of SpliNPV *dnapol* transcription have observed in the present study are entirely consistent with the kinetics of SpliNPV DNA replication. SpliNPV *dnapol* transcripts appear by 2 hpi, increase dramatically about 12 hpi, and continue to increase to up to 48 hpi. Thus, SpliNPV *dnapol* transcription precedes viral DNA replication and *dnapol* transcripts continue to accumulate to high levels while viral replication is occurring.

Second, the transcription initiation sequence, ACGT, resembles the baculovirus early promoter element, CAGT, which is believed to be transcribed by the host transcription machinery (Friesen, 1997). Current evidence suggests that the structure of NPV early promoters mimic that of promoters responsive to the host RNA polymerase II. To enhance RNA polymerase II-mediated transcription, NPVs encode transactivators that stimulate transcription in a promoter-specific manner. The baculovirus early promoters can be categorized as TATA-containing, initiator-containing, or composite (containing both) promoters. The TATA element consists of a consensus TATAA motif located upstream from the +1 RNA start site (Friesen, 1997). Sequence analysis revealed a TATAA motif located 104 nt upstream from the SpliNPV *dnapol* transcription initiation site, ACGT. Moreover (as indicated above), the ACGT motif of SpliNPV *dnapol* is very similar to motifs found within the UARs of a number of NPV genes (*p35*, *39K* (*pp31*), *p143*, and *gp64*) that are classified as early to late genes (i.e. they are initially transcribed in the early phase and continue to be transcribed in the late phase of infection) (Friesen, 1997).

Third, SpliNPV *dnapol* transcription is delayed in the presence of cycloheximide, suggesting the *dnapol* can be classified as a “delayed” early gene (Crawford & Miller, 1988). It is expressed prior to DNA replication and requires virus encoded transcription activators for its transcription. The delay of SpliNPV *dnapol* transcription in the presence of cycloheximide is consistent with studies of the effects of cycloheximide on early gene expression in AcMNPV (Tomalski *et al.*, 1988) and BmNPV (Chaeychomsri *et al.*, 1995). Previous studies have suggested that baculovirus *dnapols* require transcription activator proteins for their timely and efficient expression (Glocker *et al.*, 1992). A concentration of 100 ug/ml cycloheximide has been demonstrated to significantly reduce transcription of the major transcription activator proteins, IE1 and IE0, in AcMNPV-infected Sf9 cells (Ross & Guanrio, 1997). Translation is not, however, completely inhibited by cycloheximide at this concentration, and the proteins that are expressed accumulate overtime sufficiently to activate transcription of genes that are dependent upon them for expression (Ross & Guanrio, 1997). Treatment of NPV-infected cells with higher concentrations of cycloheximide (250 ug/ml) results in the induction of apoptosis and reduced cell viability [(Ross & Guanrio, 1997), our unpublished data]. Thus, the

mechanism of the delay of the SpliNPV *dnapol* transcription by cycloheximide may be due to low levels of viral transcription activator proteins required for the maximum *dnapol* expression. While I cannot make this conclusion with certainty, my results are consistent with observations of AcMNPV and BmNPV *dnapol* transcription in the presence of cycloheximide.

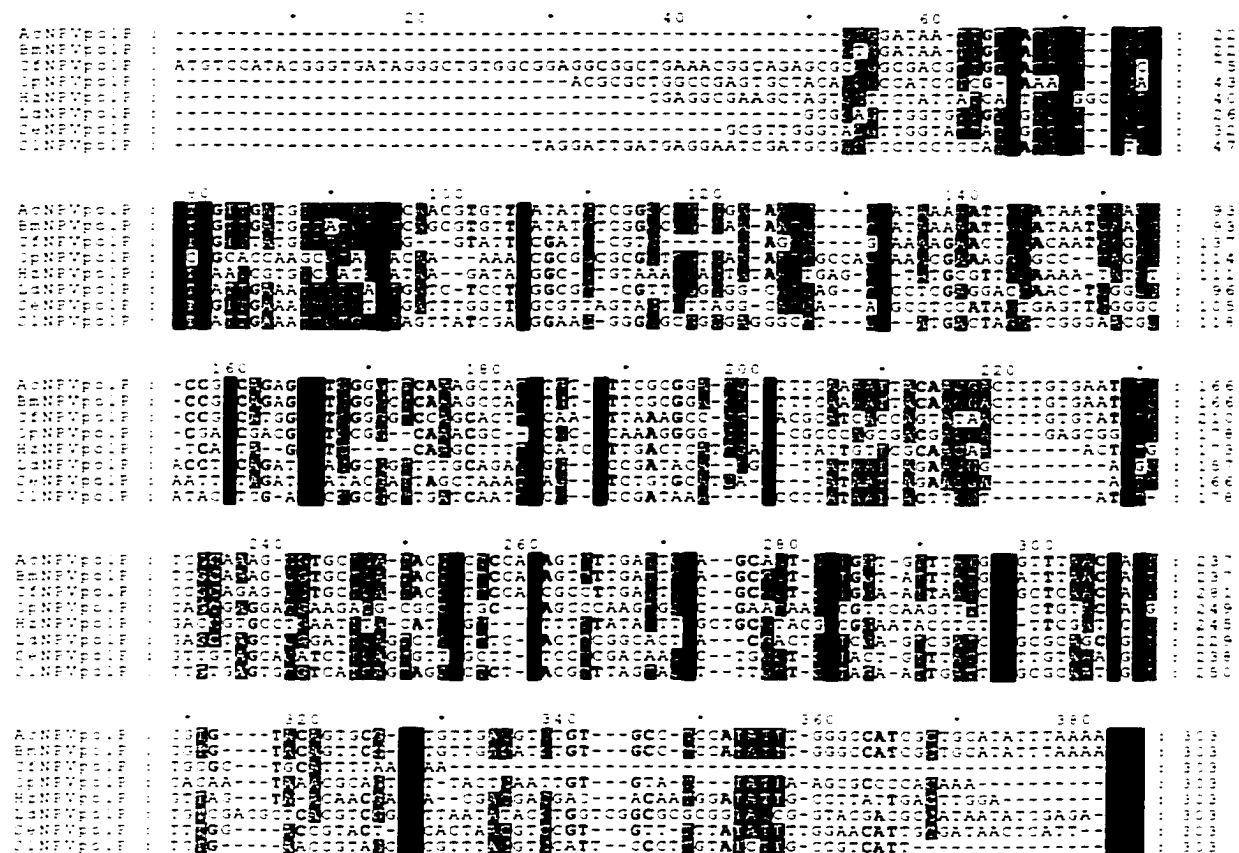
The results of inhibition of the SpliNPV *dnapol* transcription by aphidicolin were rather intriguing, but not surprising. Early studies demonstrated that aphidicolin inhibits the transcription of AcMNPV *dnapol* (Tomalski *et al.*, 1988). The inhibitory effect was exerted on both the distal and proximal transcription initiation sites in AcMNPV. In a more recent study, steady-state levels of BmNPV *dnapol* transcripts in infected cells were greatly reduced by treatment with aphidicolin (Chaeychomsri *et al.*, 1995). My results are in agreement with these observations. Studies on inhibition of the DNA polymerase protein by aphidicolin revealed a mechanism in which inhibition occurs at very specific template positions, through ternary complex formation and interfere with the binding of nucleotides by sterically blocking part of the nucleotide binding site (Sheaff *et al.*, 1991). How aphidicolin affects viral *dnapol* transcription is still unknown and needs to be addressed at the molecular level.

The SpliNPV *dnapol* gene represents the ninth such gene to be fully sequenced and the fifth such gene to be subjected to transcriptional analyses. Over half of the *dnapol* genes examined are derived from NPVs that belong to the Group I NPV phylogenetic clade (AcMNPV, BmNPV, CfMNPV, and OpMNPV). SpliNPV belongs to the Group II NPV clade (along with SeMNPV, HzNPV, and LdMNPV). Of the Group II NPVs, functional analyses of transcription, and sequence-structural analyses of sequences in the 5' flanking region, have been previously conducted only with LdMNPV. My analyses demonstrate that SpliNPV *dnapol* transcription initiation, termination, and kinetics have features that are different from other NPVs thus far examined, and provide further evidence of unique genetic characteristics of SpliNPV.

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(a)



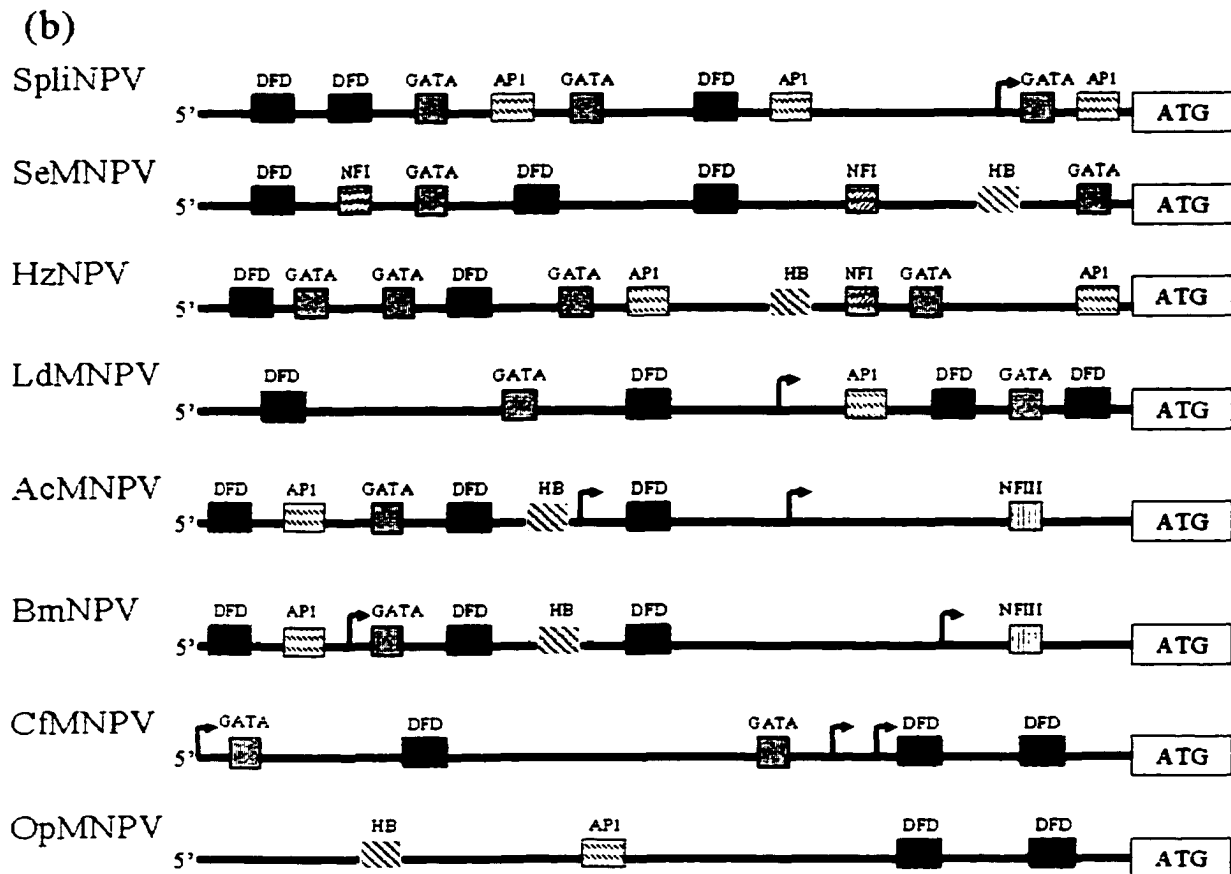


Figure 5.6. Comparison of 5'flanking region of the NPV *dnapol* genes. a) Sequence comparison of the 300 bp 5'flanking region of the SpliNPV *dnapol* gene along with those of other baculovirus *dnapol* genes [AcMNPV (Tomalski *et al.*, 1988), BmNPV (Chaeychomsri *et al.*, 1995), CfMNPV (Liu & Carstens, 1995), OpMNPV (Ahrens & Rohrmann, 1996), LdMNPV (Bjornson *et al.*, 1992), HzNPV (Cowan *et al.*, 1994), and SeMNPV (Ijkel *et al.*, 1999)]. The sequence alignment was performed using Clustal W. Identical nucleotides are indicated by white letters on a black background; those conserved in more than 50% of the sequences are shown on a grey background.

b) Comparison of the structural organization of the *dnapol* 5'flanking region in different NPVs. Transcription factor binding sites are indicated with the corresponding transcription factors. DFD: Deformed protein; HB: Hunchback protein; Ap1: Activating Protein 1; GATA: GATA-binding protein; NFI: Nuclear Factor I; and NFIII: Nuclear Factor III. Transcription initiation sites are indicated with bent arrows.

Chapter 6. Expression and Characterization of the *Spodoptera littoralis*

Nucleopolyhedrovirus DNA Polymerase

6.1. Abstract

Having previously identified and analyzed both the nucleotide and the amino acid sequences of a *Spodoptera littoralis* nucleopolyhedrovirus DNA polymerase gene, I here report on the expression, purification, and characterization of the DNAPOL protein. The 110 kDa full-length DNAPOL was expressed both in *E. coli* and in baculovirus expression systems. The DNAPOL was purified to near homogeneity and exhibited high DNA polymerase activity with gapped-duplex DNA as the substrate. A 3'-5' exonuclease activity was detected as an intrinsic property of the enzyme. Deletion of the 80 amino acid residues from the N-terminal of DNAPOL did not dramatically affect DNA polymerase and exonuclease functions, suggesting that the N-terminal region of the DNAPOL may not be required for the activities. Replication products from single-stranded M13 DNA demonstrated that the DNA synthesis activity of SpliNPV DNA polymerase is highly processive.

6.2. Introduction

In eukaryotic cells, three polymerases, Pol α , Pol δ and Pol ϵ , are essential for DNA replication. While Pol α contains primase activity and initiates DNA synthesis on both leading and lagging strands, Pol δ and Pol ϵ are believed to be involved in elongation of DNA synthesized by Pol α (Schumacher *et al.*, 2000). Like their hosts, DNA viruses require DNA polymerases to replicate their genomic information. While large DNA viruses encode a single DNA polymerase gene in each genome, bacteriophage DNA polymerases are found in family A or B, and eukaryotic viral DNA polymerases exclusively in family B DNA polymerase (Knopf, 1999). Three-dimensional structural studies, together with biochemical and mutagenesis analyses on DNA polymerases, have led to the identification of the catalytic residues responsible for polymerization and exonuclease activity, as well as substrate binding, processivity, fidelity and nucleotidyl transfer (Kiefer *et al.*, 1998; Brautigam & Steitz, 1998; Wang *et al.*, 1997).

Baculovirus genomes sequenced to date contain a single DNA polymerase gene in their genomes (Ayres *et al.*, 1994; Gomi *et al.*, 1999; Ahrens *et al.*, 1997; Kuzio *et al.*, 1999; Ijkel *et al.*, 1999). Previous sequence comparison studies demonstrated that baculovirus DNA polymerases share significant amino acid sequence structure similarity with family B DNA polymerases (Huang & Levin, 2000). In AcMNPV, DNA polymerase was purified either from virus-infected cells or from cells infected with recombinant viruses (Hang & Guarino, 1999; McDougal & Guarino, 1999). Functional analyses of the native, or recombinant DNA polymerases revealed the presence of exonuclease and polymerase activities. The recombinant protein was shown to possess processivity and moderate stranded-displacement activity (McDougal & Guarino, 1999). In this report, I have expressed the SpliNPV *dnapol* gene product, DNAPOL, using both prokaryotic and eukaryotic expression systems, and characterized the functional activities of the full-length DNAPOL and of a deletion mutant.

6.3. Materials and Methods

6.3.1. Cells and virus

S. frugiperda (Sf9) cells were obtained commercially (PharMingen). *S. littoralis* NPV, isolate M-2 (SpliNPV) was obtained from Dr. G. Croizier, Station de Recherches de Pathologie Comparée INRA-CNRS, F-30380 Saint-Christol-Les-Ales, France. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (O'Reilly *et al.*, 1992).

6.3.2. PCR and cloning of SpliNPV *dnapol* gene

Based on DNA sequence analysis of SpliNPV *dnapol* (Huang & Levin, 2000), I designed two primers that amplify the full-length SpliNPV *dnapol* (Table 6.1). In order to amplify a deletion mutation without the first 80 amino acids of the full-length DNAPOL, a 39-mer forward primer was used (Table 6.1). These primers were designed to create an insert with a *Bam*HI and a *Not*I site on the amino and carboxyl termini of the DNA polymerase gene, respectively. All amplifications were carried out using *Pfu* DNA polymerase following the manufacturer's instruction (Stratagene). The amplification products (3.1 kb for full length and 2.8 kb for Δ 80 mutation) were gel purified, digested with *Bam*HI and *Not*I, and ligated into vectors pGEX-5X-1 (*E. coli* GST gene fusion system, Pharmacia) or pFastBacHTc (Bac-To-Bac baculovirus expression system, Gibco/BRL) following the manufacturers' instructions. Southern blot analysis followed standard procedures (Sambrook *et al.*, 1987) with a 32 P-dCTP labeled probe consisting of the *dnapol* using a random primer DNA labeling system (Gibco/BRL).

Table 6.1. Primers used for PCR amplification

Primer	Length	Sequence
DNAPOL-F	26	5'-GACTGGATCCCATTTATGAAGGTGAAG-3'
DNAPOL-R	36	5'-GACGTGACGCGGCCGCTGGCCGACTTTTGATTGG-3'
DNAPOL-F Δ 80	39	5' GACTGGATCCGAGAACAGAAATTCGTCGTGC CAATGTCGC-3'

6.3.3. Expression and purification of SpliNPV DNAPOL in *E. coli*

The full-length SpliNPV *dnapol* and the $\Delta 80$ mutant were introduced into pGEX-5X-1 (Pharmacia) to create fusion proteins in which the amino termini of the DNAPOL polypeptides were linked, in frame, with the carboxyl termini of glutathione S-transferase (GST). The expression construct was transformed into *E. coli* BL21 cells. The transformed BL21 cells were grown to an A_{600} of 0.6 in LB media containing 50 $\mu\text{g}/\text{ml}$ kanamycin. Protein production was then induced by the addition of isopropyl β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. After 1 h of induction at 37 °C, the cells were harvested by centrifugation for 5 min at 1000g in a Sorvall GSA rotor at 4 °C. Cell pellets were then frozen in liquid nitrogen.

All procedures were carried out at 4 °C unless otherwise specified. The bacterial cell pellets were thawed and resuspended in 10 volumes (mass to volume) of lysis buffer (20 mM Tris-HCl pH 8.0, 250 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, 60 $\mu\text{g}/\text{ml}$ lysozyme, 0.2 mM phenylmethylsulfonyl fluoride, and 0.7 $\mu\text{g}/\text{ml}$ leupeptin). The suspension was stirred at room temperature for 30 min to lyse the cells. The lysate was sonicated on ice until it lost viscosity and then centrifuged for 1 h at 4000g in a Beckman SS34 rotor at 4 °C. The pellet and supernatant were analyzed via 8% SDS-PAGE. Fusion proteins were purified from bacteria lysates by affinity chromatography using Glutathione Sepharose 4B contained in the GST Purification Modules (Pharmacia). Cleavage of the desired proteins from GST were achieved using a site-specific protease Factor Xa, whose recognition sequence is located immediately upstream from the multiple cloning site on the vector plasmid, pGEX-5X-1. Fusion proteins were detected using an immunoassay provided in the GST Detection Module following the manufacturer's protocol.

6.3.4. Expression and purification of SpliNPV DNAPOL in Sf9 cells

pFastBacHT-DNAPOL and pFastBacHT- $\Delta 80$ DNAPOL were constructed by subcloning the full-length *dnapol* and the $\Delta 80$ mutant into the *Bam*HI and *Not*I sites of pFastBacHTc. Recombinant bacmids and baculovirus were subsequently prepared

according to the manufacturers' instructions. Viral titers were optimized for protein expression. Sf9 cells were infected at a multiplicity of infection of 10 and cells were harvested at various time points between 24 and 72 hpi. The recombinant proteins, designated as HisDNAPOL and His-del80DNAPOL, respectively, were purified using Ni-NTA agarose from Sf9 cells infected with recombinant baculovirus. Expression and purification of the fusion proteins were monitored by SDS-PAGE analysis (Laemmli, 1970). The purified polymerases were stored at -80°C in buffer containing 65% glycerol.

6.3.5. SDS-PAGE and Western blot analysis

Protein concentrations were determined by the Bradford method (1976) with bovine serum albumin as a standard. For western blotting, proteins were electrophoresed by SDS-PAGE and transferred to immobilon-NC membranes (Millipore). The membranes were blocked with 5% nonfat milk and probed with a 1:5000 dilution of primary rabbit anti-His polyclonal antibodies. Membranes were then incubated with a 1:2000 dilution of goat anti-rabbit IgG-horseradish peroxidase conjugates, and developed using Enhanced Chemiluminescence (Amersham) substrates.

6.3.6. DNA templates

Singly primed single-stranded M13mp18 was made by annealing 25 pmol of M13 universal forward primer (5'-TGTAACGACGGCCAGT-3') to 2.5 pmol of single-stranded M13 DNA. The mixtures, containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, and 100 mM NaCl in 50 μl , were heated to 100°C and slowly cooled to room temperature. To prepare exonuclease substrate, reaction mixtures (50 μl) containing 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 5 mM MgCl₂, 0.4 mM each of dCTP, dGTP, and dTTP, 20 μCi of [α -³²P] dATP (3000 Ci/mmol), 500 $\mu\text{g/ml}$ activated calf thymus DNA, and 10 units of Klenow enzyme were incubated at 37°C for 30 min, quenched by the addition of 2.5 μl of 0.5 M EDTA, and chilled in ice. DNA substrate was extracted twice with phenol/chloroform/isoamyl alcohol and purified by gel filtration using TE buffer.

6.3.7. DNA polymerase assay

Reaction mixtures contained 50 mM Tris-Cl, pH 8.0, 50 ug of bovine serum albumin, 0.5 mM dithiothreitol, 15 mM MgCl₂, 200 mM KCl, 0.1 mM each of dCTP, dGTP, and dTTP, 0.0125 mM dATP, 0.5 uCi of [α -³²P]dATP (3000 Ci/mmol), 25 ug of activated calf thymus DNA (Sigma), and purified DNA polymerase in a reaction volume of 100 ul. After incubation at 37 °C for 30 min, the reaction mixtures were stopped by the addition of 100 ul of 25 mM EDTA, 25 mM sodium pyrophosphate and 50 ug/ml of salmon sperm DNA followed by 1 ml of 10% trichloroacetic acid (TCA). After 10 min on ice, the mixture was filtered through GF/C glass filters. The filters were washed twice with 2 ml of 1 M HCl, 0.05 M sodium pyrophosphate, rinsed with ethanol, dried and counted in a liquid scintillation counter. One unit of DNA polymerase activity was defined as the amount of enzyme required to incorporate 1 nmol [α -³²P]dNTP into acid-insoluble material/min at 37 °C. When inhibition of DNA polymerase activity by aphidicolin was measured, the concentration of dCTP was lowered to 10 uM.

For DNA synthesis on singly-primed M13 single-stranded circular template, reaction mixtures (50 ul) contained 20 fmol substrate DNA, 20 mM Tris-acetate (pH 7.3), 75 mM potassium acetate, 5 mM magnesium acetate, 1mM DTT, 0.5 mM ATP, 60 uM each dGTP, dATP and dTTP, 20 uM [α -³²P]dCTP (3000 Ci/ mmol), 50 ug/ml BSA and 200 fmol purified DNA polymerase. The reaction was incubated at 37 °C and terminated by the addition of an equal volume of stop buffer (1% SDS, 40 mM EDTA, 60 ug/ml sonicated calf thymus DNA). The reaction products were precipitated with ethanol, resuspended in 20 ul sample buffer (0.1 M NaOH, 5% glycerol, 1mM EDTA, 0.025% bromocresol green) and separated on a 1% alkaline agarose gel as described (Sambrook *et al.*, 1989). Dried gels were exposed and visualized by autoradiography.

6.3.8. Exonuclease Assay

Exonucleolytic activities were determined in the absence of dNTPs under the conditions of the DNA polymerase assay. Reaction mixtures (100 ul) were incubated for 30 min 37 °C with 25 ug of activated calf thymus DNA containing 0.15 ug (6.7×10^5 cpm/ug) of ³²P-labeled exonuclease substrate. Reactions were terminated by chilling on

ice and by adding 20 μ l of a mixture of 0.25 M EDTA, pH 8.0 and 5 mg/ml bovine serum albumin, and 20 μ l of 100% trichloroacetic acid. After centrifugation (13,000g, 30 min, 4 °C) the radioactivity of supernatant fractions (100 μ l) was determined in a liquid scintillation counter. One unit of 3'-5' exonuclease activity was defined as the amount of enzyme required to release 1 pmol [α -³²P]dCTP into acid-soluble material in 30 min at 37 °C.

6.4. Results

6.4.1. Cloning the full-length SpliNPV *dnapol* and the Δ 80 mutant

The complete sequence of the SpliNPV DNA polymerase gene (Huang & Levin, 2000) was submitted to GeneBank (accession number AF215639). Based on the sequence data, I designed two primers to amplify the full length of *dnapol*. Gel electrophoresis analysis revealed a single band with 3.1 kb (Figure 6.1a), which was gel purified, digested with *Bam*HI and *Not*I, and cloned into pGEX-5X-1 for expression in *E. coli* or into pFastBac-HTc for expression in Sf9 cells using the baculovirus expression system. The 3.1 kb fragment, containing the full length of *dnapol* ORF, includes 9 nucleotides upstream of the ATG start codon and 107 nucleotides specifying the 3'untranslated region. The ORF encodes a 998 amino acid polypeptide with a predicated molecular mass of 116 kDa. A polyadenylation signal AATAAA is located 60 nucleotides downstream of the DNAPOL stop codon.

In order to express an N-terminal truncated DNAPOL (Δ 80DNAPOL), a forward primer was designed to amplify the *dnapol* gene with deletion of the first 80 amino acid residues. The resulting PCR product (Figure 6.1a) was gel purified and cloned into either the *E. coli* expression vector or the baculovirus expression vector. The Δ 80DNAPOL mutant contains 2.8 kb encoding 918 amino acids. The *dnapol* and the deletion mutant were inserted into the *Bam*HI/*Not*I sites of pGEX-5X-1 vector permitting expression of fusion proteins with GST-tagged N-terminus. The bacterial expression constructs were confirmed by the REN assays (Figure 6.1b). The baculovirus expression constructs were transfected into Sf9 cells. Recombinant virus DNAs compassing the expected constructs

were subjected to Southern blot analysis to confirm the presence of the full-length *dnapol* and the $\Delta 80$ mutant (Figure 6.1 c).

6.4.2. Over-expression and purification of SpliNPV DNAPOL and the $\Delta 80$ mutant proteins

The expression of SpliNPV DNAPOL and its $\Delta 80$ mutant counterpart from bacterial expression system was visualized by SDS-PAGE and Western blot analysis. The proteins have molecular masses of 110 kDa and 96 kDa, respectively (Figure 6.2), which correspond to the molecular masses predicated from the amino acid sequences. The concentrations of the DNAPOL and $\Delta 80$ DNAPOL proteins, purified from 500 ml bacterial cultures [determined by Bradford assay (1976)], were 0.5 and 0.7 mg, respectively. The specific activities of the proteins were 6532 U/mg and 6063 U/mg (Table 6.2), respectively, with activated calf thymus DNA as a template.

The baculovirus expression system was used to obtain larger and more concentrated amounts of DNA polymerase with potentially higher specific activity. The *dnapol* gene was cloned into pFastBacHTc (Gibco/BRL), which incorporates an amino terminal extension of 20 amino acids containing a six-histidine affinity tag. Approximately 3.5 mg of recombinant proteins were purified, in a one-step Ni-resin affinity chromatography, from a 500-ml Sf9 culture. The specific activities of the full-length His-DNAPOL and His-del80DNAPOL were 6890 U/mg and 6743 U/mg (Table 6.2), respectively.

Western blot analyses show the time course of recombinant proteins expression in Sf9 cells (Figure 6.2a). Proteins from recombinant virus infected Sf9 cells were transferred to membrane filter and reacted with antisera directed against 6XHis-antibody. The antisera detected an immunoreactive species in the cell extracts of about 110 kDa and 96 kDa in good agreement with the predicted full-length and the deletion mutant protein molecular masses. The polypeptide species were abundant in the recombinant virus infected cell extracts from 36 to 48 hpi (Figure 6.2a). The purification systems yielded single coomassie blue-stained species of 110 kDa and 96 kDa on SDS-PAGE for

proteins expressed both in *E coli* (Figure 6.2b), and in Sf9 cells (Figure 6.2c), respectively. While less protein with the same specific activity was obtained from the *E. coli* expression system, the expressed proteins from two different expression systems showed essentially the same biochemical characteristics (see below).

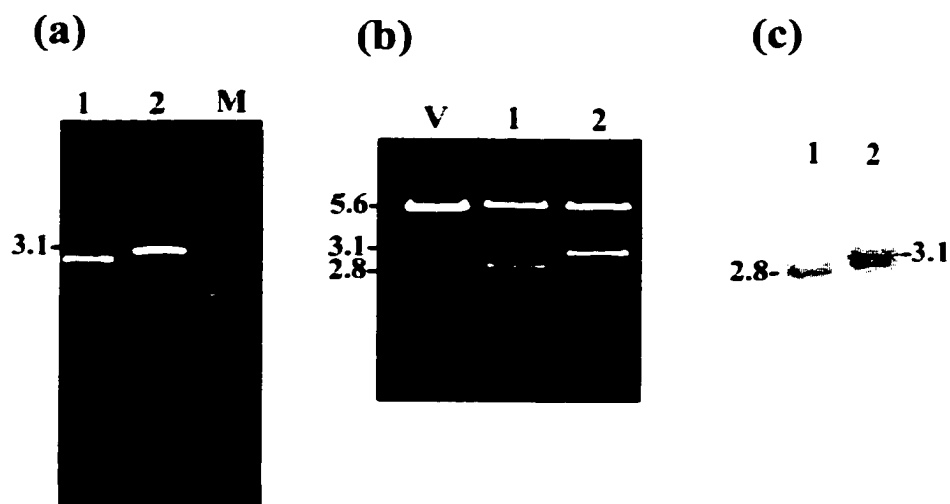


Figure 6.1. Cloning the full-length SpliNPV *dnapol* and the $\Delta 80$ mutant. **a)** PCR amplification of the 2.8 kb fragment containing the $\Delta 80$ mutant *dnapol* (lane 1), and the 3.1 kb fragment containing the full-length *dnapol* (lane 2) were carried out and separated in a 0.8% agarous gel with 1-kb ladder (M). **b)** Recombinant plasmids containing the 2.8 kb $\Delta 80$ mutant fragment and the 3.1 kb full-length *dnapol* were cloned into pGEX-5X-1, a GST fusion expression vector, and confirmed by REN analysis; V) the 5.6 kb vector plasmid linearized after cleavage by *Bam*HI and *Not*I; 1) the 2.8 kb $\Delta 80$ mutant clone cleaved by *Bam*HI and *Not*I, 2) the full-length *dnapol* clone. **c)** The full-length *dnapol* and 2.8 kb $\Delta 80$ mutant were cloned into a baculovirus expression vector, pFastBacHTc. The recombinant baculovirus DNAs were extracted, digested with *Bam*HI and *Not*I, and subjected to Southern blot analysis using the SpliNPV *dnapol* as a probe; 1) recombinant baculovirus containing the 2.8 kb $\Delta 80$ mutant *dnapol* fragment; 2) recombinant baculovirus containing the full-length 3.1 kb *dnapol* fragment.

Table 6.2. Specificity of SpliNPV DNAPOL

Expression system	DNA polymerase activity	3'-5' exonuclease activity
<i>E. coli</i>	U/mg	U/mg
DNAPOL	6532	489
del80DNAPOL	6063	480
Baculovirus		
His-DNAPOL	6890	500
His-del80DNAPOL	6743	490

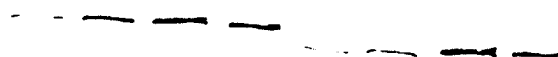
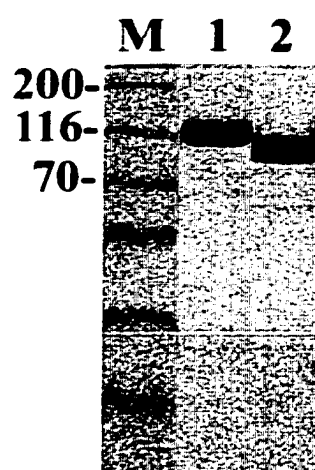
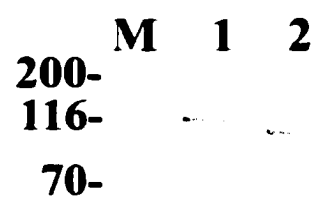
(a)**24 36 48 72 24 36 48 72****(b)****(c)**

Figure 6.2. Expression of the SpliNPV DNAPOL and the $\Delta 80$ mutant. a) Western blot analysis demonstrated the time course expression of recombinant proteins in Sf9 cells harvested at indicated hpi (the full-length His-DNAPOL shown at first four lanes; the mutant His-del80DNAPOL at last four lanes); b) Coomassie brilliant blue R-250 stained SDS-PAGE analysis of the recombinant full-length DNAPOL (lane 1) and the $\Delta 80$ mutant ($\Delta 80$ DNAPOL) (lane 2) proteins over-expressed in *E. coli* and purified to near homogeneity after digested with Factor Xa; c) Coomassie brilliant blue R-250 stained SDS-PAGE analysis of the recombinant full-length DNAPOL (His-DNAPOL, lane 1) and the $\Delta 80$ mutant (His-del80DNAPOL) (lane 2) proteins over-expressed in Sf9 cells, purified to near homogeneity using Ni-NTA agarose. M, molecular weight standard (kDa).

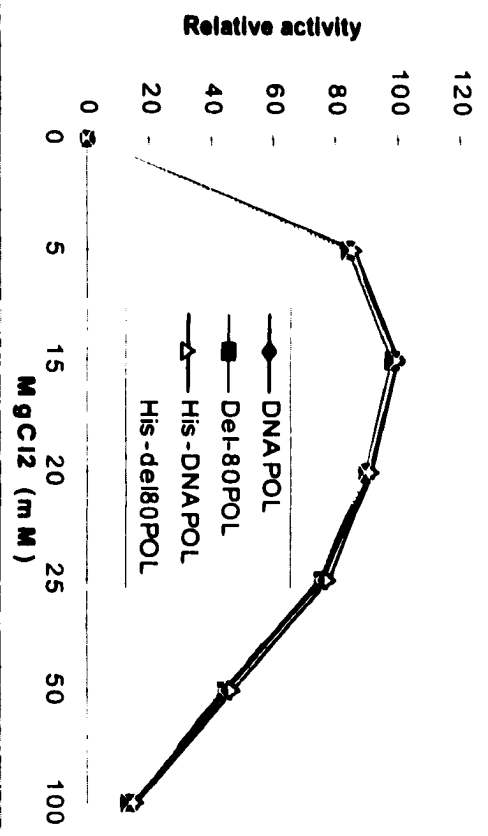
6.4.3. DNA polymerase activity

Amino acid sequence analysis suggested that SpliNPV *dnapol* encodes a DNA polymerase, which catalyzes template-directed incorporation of deoxyribonucleotides into DNA from 3'-OH primer termini. Comparison studies revealed that the specific activities of proteins prepared using the bacterial and baculovirus expression systems were indistinguishable (Table 6.2). Optimal conditions for the DNA polymerase activity were tested in the presence of activated calf thymus DNA as a template. The DNAPOLs were most active at pH 7.5 (data not shown). The enzyme required moderate concentrations of divalent cations for activity; a concentration of 0.1 mM MgCl₂ is inhibitory (Figure 6.3a). No activity was detected in the absence of divalent cations. The SpliNPV DNAPOL polymerase activity was optimal at 200 mM KCl and was only inhibited at much higher salt concentrations (Figure 6.3b). Only 20% of residual activity could be detected in the presence of 400 mM KCl, and 40% could be detected in the presence of 300 mM (NH₄)₂SO₄ (Figure 6.3c). Higher concentrations of ammonium ions strongly inhibited the enzymes. Thus, maximum DNA polymerase activity was observed with 15 mM MgCl₂, 200 mM KCl, and 100mM (NH₄)₂SO₄.

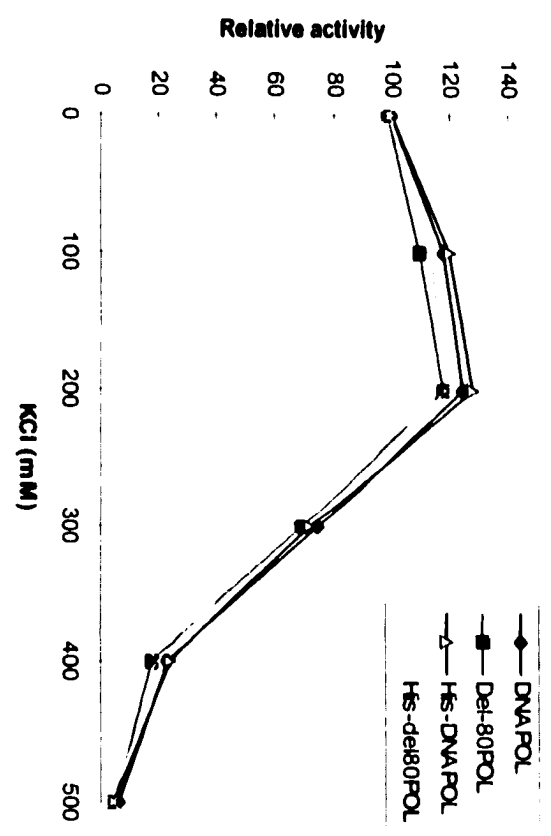
Previous studies determined that viral DNA polymerases are sensitive to aphidicolin, which may compete with dNTPs for binding to DNA polymerase (Hang & Guarino, 1999; Padrali-Noy & Spadari, 1980; Spadari *et al.*, 1982; Sheaff *et al.*, 1991). Similar to AcMNPV DNAPOL, the SpliNPV DNAPOL polymerase activity was inhibited by aphidicolin. Forty percent of the original activity was detected in the presence of 0.25 ug/ml aphidicolin, and 20% was detected in the presence of 0.5 ug/ml aphidicolin (Figure 6.3d). The heat sensitivity was also examined (Figure 6.3e). All enzymes displayed similar heat sensitivities, losing approximately 80% activity after incubation at 50 °C for 9 min (Figure 6.3e).

All enzyme preparations, from both bacterial and baculovirus expression systems, showed the same pattern of polymerase catalytic activity, suggesting that the DNA polymerase activity is intrinsic to the SpliNPV DNAPOL, and that the first 80 amino acids are not essential for *in vitro* DNA polymerase activity.

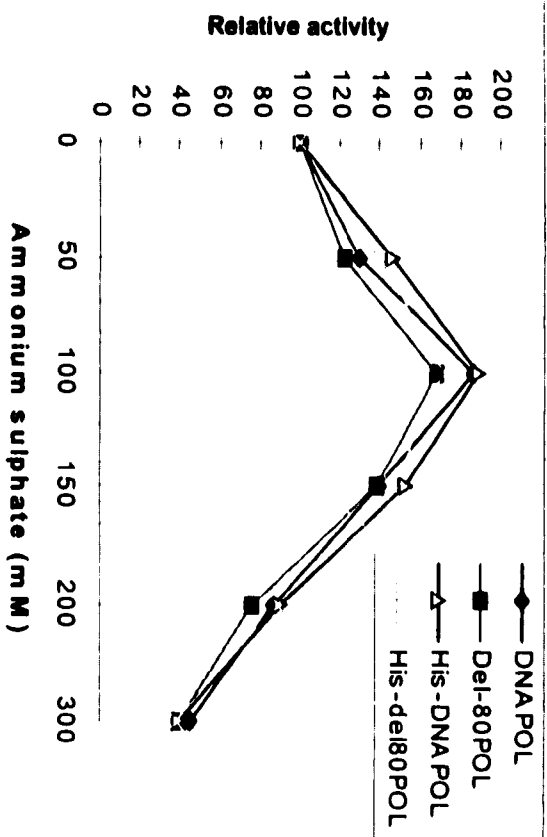
(a)



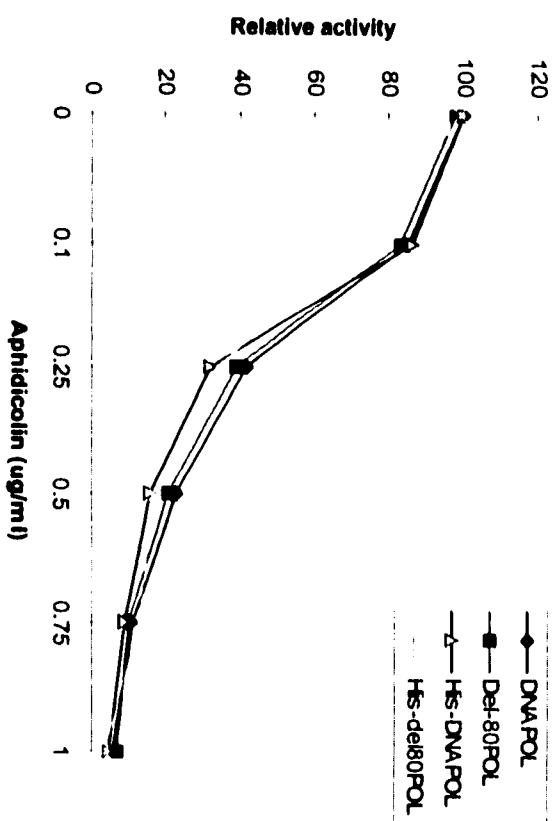
(b)



(c)



(d)



(e)

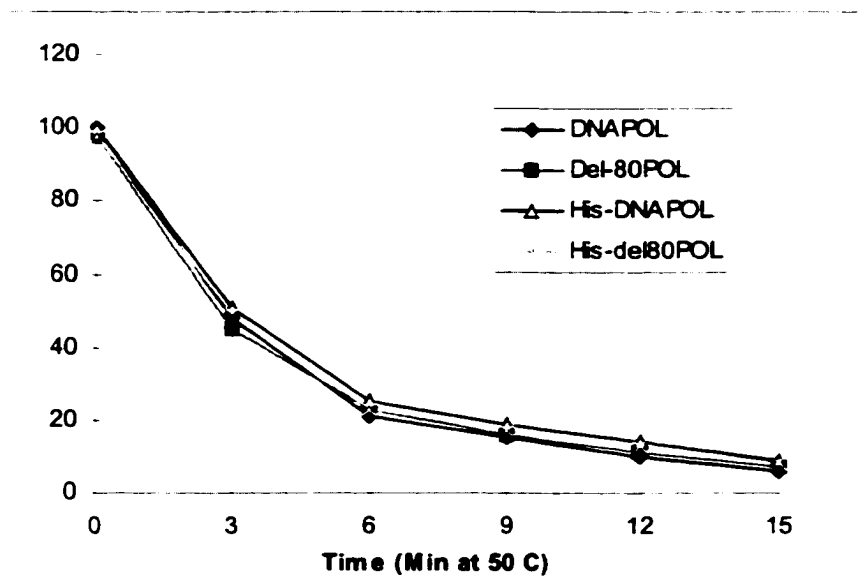


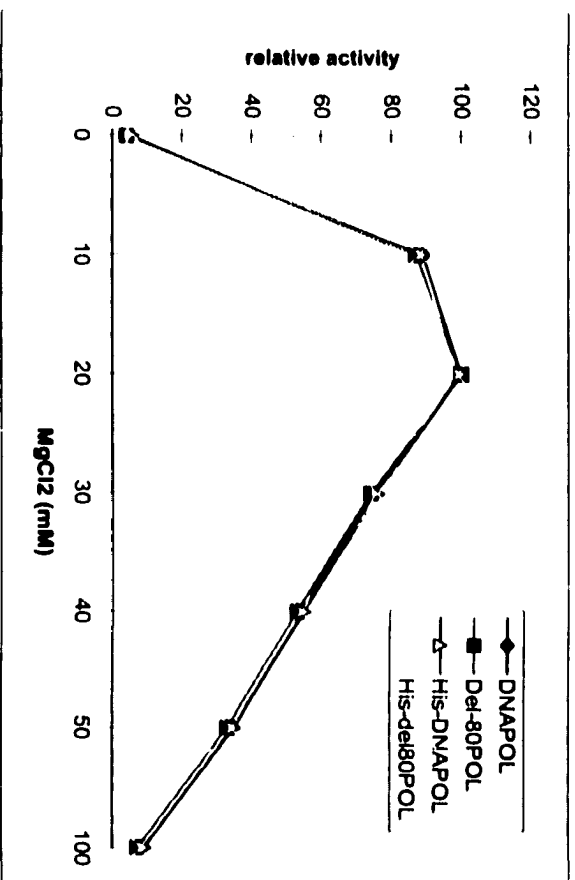
Figure 6. 3. Recombinant SpliNPV DNAPOL DNA polymerase activity. Effects of different components and conditions were assayed using activated calf thymus DNA as a template. The standard assays as described in the text were carried out with 0.2 U of purified DNA polymerase in the presence of various concentrations of **a)** MgCl_2 , **b)** KCl , **c)** $(\text{NH}_4)_2\text{SO}_4$, in the presence of **d)** aphidicolin at different concentrations, or **e)** preincubated at 50 °C for various lengths of time.

6.4.4. 3'-5' exonuclease activity

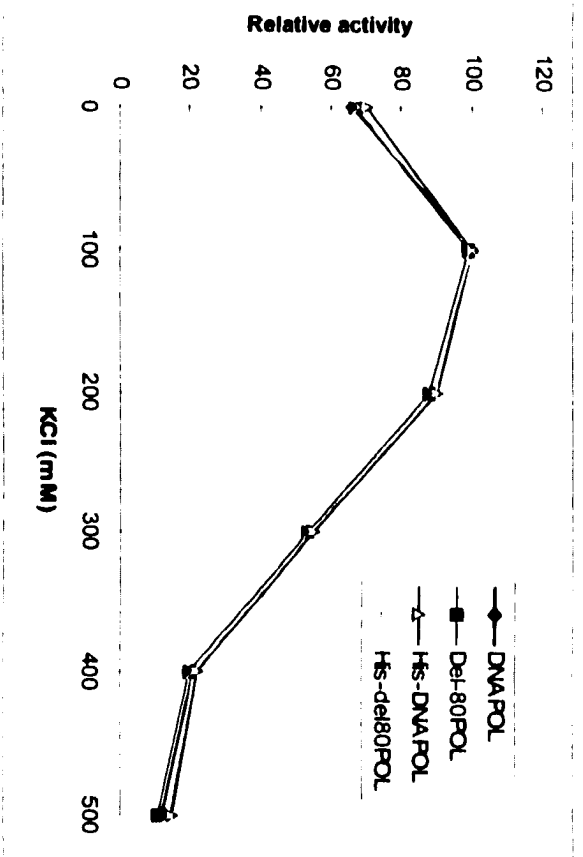
Virus DNA polymerases are known to have a 3'-5' exonuclease activity which is responsible for correction of mismatched dNTPs. Thus, the 3'-5' exonuclease activity of DNA polymerase acts in opposition to the polymerase activity and serves as a proofreader, by removing polymerase errors. Three exonuclease domains (Exo I, Exo II, and Exo III) have been proposed to be responsible for the activity. Highly conserved amino acid sequences encoding these domains were identified within the SpliNPV DNAPOL and in other baculovirus DNA polymerases (Huang & Levin, 2000). The 3'-5' exonuclease activities of the full-length DNAPOL and the $\Delta 80$ mutants from the two expression systems were measured and quantitated. The enzymes exhibited robust exonuclease activity. The 3'-5' exonuclease activity required Mg^{2+} ions (Figure 6.4a). The exonuclease activity was maximal at 20 mM $MgCl_2$ and 100 mM KCl (Figure 6.4a and 6.4b), while the maximal polymerase activity was detected at 15 mM $MgCl_2$ and 200 mM KCl (Figure 6.3b). The inclusion of 200 mM $(NH_4)_2SO_4$ reduced exonuclease activity of all four enzymes by 30% (Figure 6.4c).

Aphidicolin, which was shown to be an inhibitor of the polymerase activity of SpliNPV, also affected the 3'-5' exonuclease activity (Figure 6.4d). Exonuclease activity was reduced to 30% of the original level at 0.25 $\mu g/ml$ aphidicolin, and to approximately 10% at 0.5 $\mu g/ml$ aphidicolin. The results suggest that the same aphidicolin-sensitive enzymatic system is responsible for both the polymerase activity and the exonuclease activity. The exonuclease activities of the SpliNPV full-length DNAPOL and the $\Delta 80$ mutants were inactivated by heat with nearly identical patterns (Figure 6.4e). The enzymes lost 50% of the exonuclease activity after a 3 min preincubation at 50 °C. The exonuclease activity from all preparations demonstrated a similar pattern of enzymatic activity, suggesting that the exonuclease activity is intrinsic to SpliNPV DNAPOL, and that the first 80 amino acids are not required for the activity.

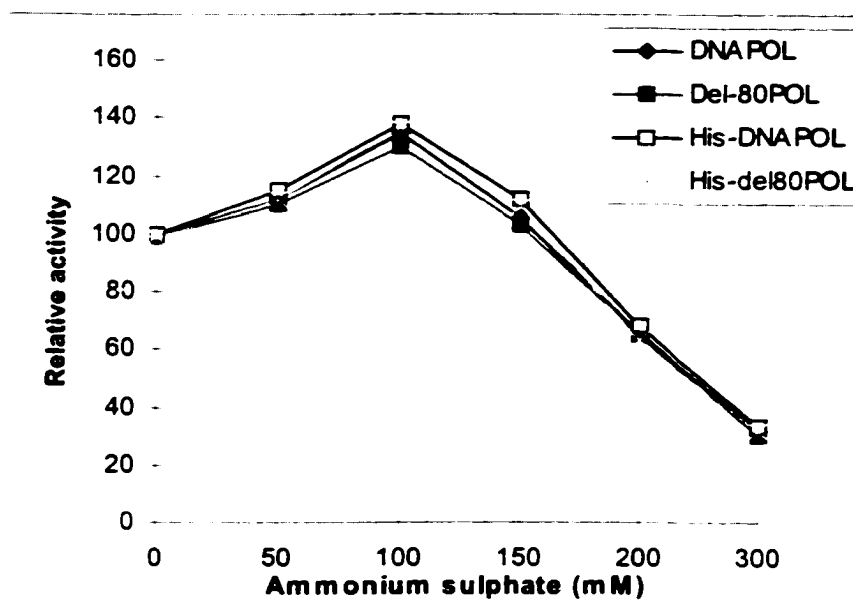
(a)



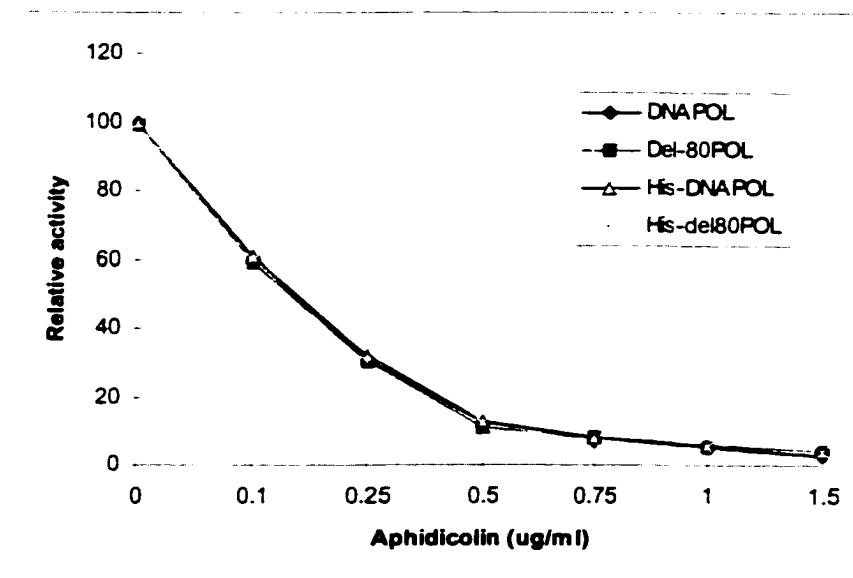
(b)



(c)



(d)



(e)

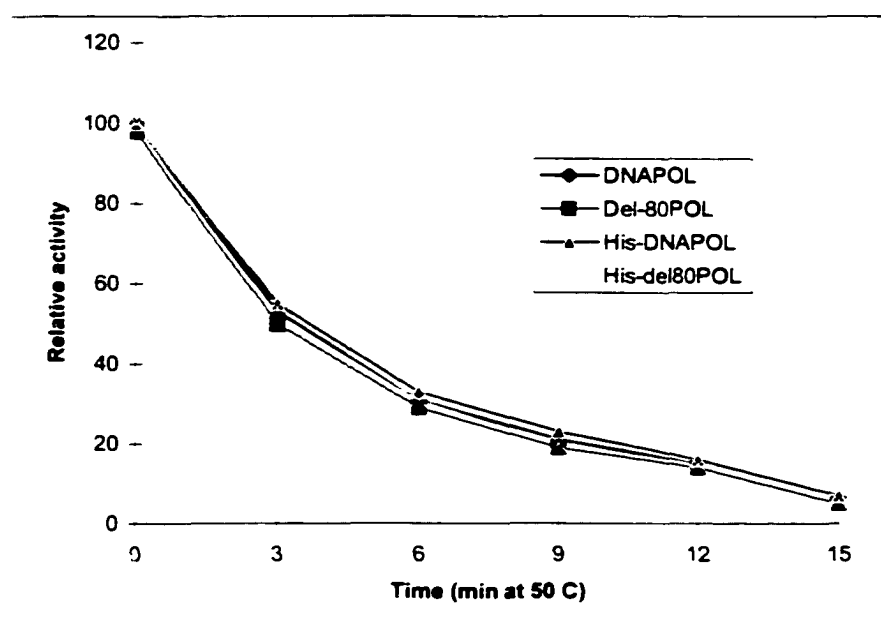


Figure 6.4. Recombinant SpliNPV DNAPOL exonuclease activity. Effects of different components and conditions were assayed using activated calf thymus DNA as a template. The standard assays as described in the text were carried out with 0.2 U of purified DNA polymerase in the presence of various concentrations of **a)** MgCl_2 , **b)** KCl , **c)** $(\text{NH}_4)_2\text{SO}_4$, **d)** in the presence of aphidicolin at different concentrations, or **e)** preincubated at 50°C for various lengths of time.

6.4.5. Replication of singly-primed single-stranded M13 DNA

Having observed the DNAPOL utilizing gapped DNA activated calf thymus DNA as primer-templates, I further investigated the ability of the full-length DNAPOL to replicate single-stranded DNA. The 18-mer oligonucleotide M13 DNA primer was incubated with single-stranded M13 DNA, purified DNA polymerase from the baculovirus expression system, and three deoxyribonucleoside triphosphates. The reaction was completed by the addition of [α - 32 P]dCTP and aliquots were removed at the indicated times. Radiolabelled M13 DNA products were detected after 3 min of incubation. A strong signal was detected by 5 min after addition of dCTP, indicating that SpliNPV DNAPOL synthesized DNA molecules of approximately 7200 nucleotides, corresponding to the entire length of M13 DNA (Figure 6.5). The replication of singly-primed single-stranded M13 DNA assay also revealed that the Δ 80 mutant shared the same characteristic with the full-length SpliNPV DNAPOL, resulting in the replication of M13 DNA (data not shown).

6.5. Discussion

The induction of a virus-specific DNA polymerase activity in NPV-infected cells was demonstrated in the mid 1980s. Since then, Only the AcMNPV and BmNPV DNA polymerases have been characterized (Hang & Guarino, 1999; McDougall & Guarino, 1999). The AcMNPV DNAPOL was purified from AcMNPV-infected Sf9 cells and from the recombinant baculovirus-infected Sf9 cells. Functional analyses revealed that the polymerase and 3'-5' exonuclease activities are intrinsic to the enzyme (Hang & Guarino, 1999; McDough & Guarino, 1999). The studies reported here demonstrate that the SpliNPV DNAPOL could be expressed either in *E. coli* or in Sf9 cells in an active form. The properties of the over-expressed SpliNPV DNAPOLs were compared with a mutant counterpart in which the first 80 amino acid residues were deleted. Assays using activated calf thymus DNA as a template showed that the polymerase and 3'-5' exonuclease activities were also intrinsic to SpliNPV DNAPOL polypeptide. Deletion of the first 80 aa residues did not affect SpliNPV DNAPOL enzymatic activities.

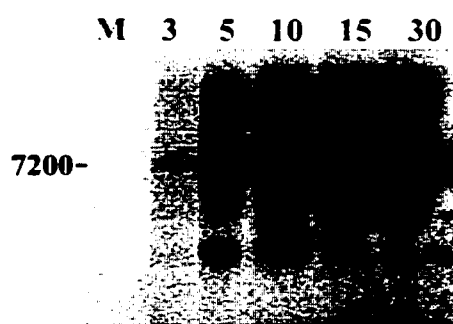


Figure 6.5. Replication of singly-primed M13 DNA by SpliNPV DNAPOL. Purified full-length DNAPOL (200 fmol) was incubated with 20 fmol singly-primed M13 DNA at 37° C. At the indicated time points (minutes), the reactions were terminated by the addition of stop buffer. The reaction products were precipitated, resuspended in sample buffer, and separated on a 1% alkaline agarose gel and visualized by autoradiography. The position of full-length M13 DNA (7200 nt) is indicated on the left.

The maximum polymerase activity of SpliNPV DNAPOL was observed with 15 mM MgCl₂, 200 mM KCl, and 100 mM (NH₄)₂SO₄. The 3'-5' exonuclease activity was maximal at 20 mM MgCl₂ and 100 mM KCl. The enzymes were sensitive to the polymerase inhibitor, aphidicolin, as well as heat treatment. These enzymatic activities of the SpliNPV DNAPOL were very similar to those of AcMNPV and BmNPV (Mikhailov *et al.*, 1986; Hang & Guarino, 1999; McDougal & Guarino, 1999), suggesting functional conservation among NPV DNA polymerases, due to the importance of maintaining viral genetic stability. It has been hypothesized that the earliest enzymatic activity to appear in evolution was that of the polynucleotide polymerases, the ability to replicate the genomes as accurately as possible being a prerequisite for evolution itself (Steitz, 1999).

The analyses of the enzymatic activities of SpliNPV DNA polymerase demonstrated that the enzyme needs cation ions for its DNA polymerase and exonuclease activities. The results are consistent with studies of other family B DNA polymerases, suggesting functional conservation among DNA polymerases. Amino acid sequence comparisons revealed that the SpliNPV DNAPOL shares DNA polymerase domains and exonuclease motifs with other family B DNA polymerases. Crystal structural analyses of family B DNA polymerase suggest that independent of their detailed domain structures, these DNA polymerases appear to share common overall architectural features, which consist of thumb, palm, and finger domains (Brautigam, & Steitz, 1998). Moreover, structural and functional studies supported the hypothesis that the phosphoryl transfer reaction of all polymerases is catalyzed by a two metal ion mechanism (Beese & Steitz, 1991; Derbyshire *et al.*, 1991). The active site features two metal ions that stabilize the resulting penta-coordinated transition state. One of the metal ions activates the primer's 3'-OH for attack on the α -phosphate of the dNTP. Another ion plays a dual role of stabilizing the negative charge that builds up on the leaving oxygen and chelating the β - and γ -phosphates (Brautigam & Steitz, 1998).

While the N-terminal part of DNAPOLs is less conserved among baculoviruses, the studies indicated that the N-terminal truncated form of SpliNPV DNAPOL (Δ 80DNAPol) shared the same characteristics as full-length DNAPOL *in vitro*. Interestingly, this result is in agreement with the study on the mammalian DNA Pol δ

(Schumacher *et al.*, 2000). When DNA synthesis was measured with the RFC-dependent Pol δ assay, the full-length and the Δ NPol δ (missing first 80 aa) were equally active, suggesting that the 80 amino acids at the N-terminus do not function in catalysis *per se* (Schumacher *et al.*, 2000). However, further functional analysis indicated that the holoenzyme containing Δ NPol δ was significantly less efficient and slower than that containing the full-length Pol δ , suggesting that the N-terminal part of Pol δ is involved in the interaction with other proteins, such as SSB and RPC (Schumacher *et al.*, 2000). Further investigation is needed to address the importance of the first 80 aa residues of the SphiNPV DNAPOL during viral DNA replication *in vivo*.

Conclusions

The ability of SpliNPV to successfully infect several lepidopteran insects makes it a suitable candidate for use as a biological insecticide. My initial studies revealed a low level of SpliNPV DNA replication in a cell line (MSE4) derived from a grasshopper, *Melanopus sanguinipes* (Order: *Orthoptera*), which is of interest to the baculovirus host-range research. Further characterization of SpliNPV infection in grasshoppers *in vivo* would provide further insight into the mechanisms of NPV host specificity.

My effort in NPV host-range studies was extended by molecular analysis of SpliNPV in permissive and semi-permissive cell lines that supported virus replication at various levels, and in a non-permissive cell line. Along with the characterization of virus gene expression, and comparison of reporter gene expression under the transcriptional control of either SpliNPV or AcMNPV promoters, the data suggested that the limit to productive infection in semi- or non-permissive cells was at the level of early viral gene transcription. These results could be useful in assessing the safety of baculoviruses as well as in improving the efficacy of baculoviruses as biological control agents.

The identification and characterization of a *cis*-acting factor, the non-*hr ori*, and a *trans*-acting factor, the *dnapol* gene, from SpliNPV further elucidated the unique genetic features of this virus. Functional analyses of the non-*hr ori* suggested that host transcription factors play a key role in the virus DNA replication.

Detailed characterization of the SpliNPV DNA polymerase protein revealed that it possesses biochemical properties similar to those of the AcMNPV and BmNPV DNA polymerases, and suggests that the conserved amino acid sequence structures found in the SpliNPV DNA polymerase convey functional conservation among DNA replication proteins in NPVs. While the N-terminus of the SpliNPV DNAPOL was not essential for enzymatic activities, it may be involved in the protein-protein interaction during virus DNA replication *in vivo*. Further characterization of the DNA polymerase protein and its relation with other essential replication proteins would provide useful information regarding the mechanism of virus DNA replication.

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Appendix. The *Spodoptera littoralis* nucleopolyhedrovirus *dnapol* sequence

```

AF215639 . Spodoptera littora...[gi:7407076]

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VERSION     AF215639.1  GI:7407076
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SOURCE      Spodoptera littoralis nucleopolyhedrovirus.
ORGANISM    Spodoptera littoralis nucleopolyhedrovirus
            Viruses; dsDNA viruses, no RNA stage; Baculoviridae;
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REFERENCE   1  (bases 1 to 6401)
AUTHORS     Huang,J. and Levin,D.B.
TITLE       Identification, Transcription, and Sequence Analysis of the Spodoptera
            littoralis Nucleopolyhedrovirus (SpliNPV) DNA Polymerase Gene
JOURNAL      Unpublished
REFERENCE   2  (bases 1 to 6401)
AUTHORS     Huang,J. and Levin,D.B.
TITLE       Direct Submission
JOURNAL      Submitted (12-DEC-1999) Biology, University of Victoria, Canada,
            P.O.Box 3020 STN CSC, Victoria, B.C. V8W 3N5, Canada
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BASE COUNT 1673 a 1405 c 1551 g 1772 t

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