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Characterization of the *Spodoptera littoralis* nucleopolyhedrovirus type B *lef-3* gene

by

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B.Sc. Agric., University of British Columbia, 1986

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ABSTRACT:

We constructed a cDNA library with mRNA isolated from Sf9 cells infected with *Spodoptera littoralis* nucleopolyhedrovirus type B (SpliNPV-B) and identified the *lef-3* gene from this library. Northern blot analysis showed that SpliNPV-B *lef-3* mRNA was expressed as a 1.6 Kb transcript at 5 hours post-infection (p.i.), reached high levels at 24 hours p.i., and remained highly expressed at 56 hours p.i.. Transcriptional mapping showed that *lef-3* transcription started from two initiation sites (the distal and the proximal transcription initiation sites) located approximately 9 nucleotides apart. The sequences that modulate *lef-3* expression were investigated by transient expression assays using a reporter gene under transcriptional control of the *lef-3* promoter. Deletion analysis of the 5'-flanking region demonstrated that sequences up to 584 bases 5' of the distal transcription initiation site affected the level of reporter activity, indicating that this region contains transcription regulators. A region that was sufficient to direct basal level of promoter activity, the minimal promoter, was identified. This region encompasses the two transcription initiation sites, two TATA boxes, and a GATA motif. Mutations in the GATA motif resulted in substantial decrease in the level of reporter activity, suggesting that the GATA motif is an important element in the regulation of *lef-3* gene expression. The sequence of a 2.6-kb region (mu 42.8-46.8) encompassing the *lef-3* gene and flanking sequences was determined. Alignment of the predicted amino acid sequence of the LEF-3 polypeptide of SpliNPV-B with the putative sequences of AcMNPV and OpMNPV LEF-3 revealed low levels of sequence conservation (26% and 21% amino acid sequence identity, respectively). This low level of sequence conservation corroborates the view that, within the genus *Nucleopolyhedrovirus*, SpliNPV-B is distantly related to AcMNPV and OpMNPV.

Examiners:

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LIST OF ABBREVIATIONS

AcMNPV	*Autographa californica* nucleopolyhedrovirus
bp	base pairs
BV	budded virus
CPE	cytopathological effects
m.o.i.	multiplicity of infection
nt	nucleotides
OpMNPV	*Orgyia pseudotsugata* nucleopolyhedrovirus
ORF	open reading frame
ODV	occlusion derived virus
PFU	plaque forming units
p.i.	post infection
PIBs	polyhedra inclusion bodies
REN	restriction endonuclease
SpliNPV-B	*Spodoptera littoralis* nucleopolyhedrovirus type B
SSB	single-stranded DNA binding
ULS	untranslated leader sequence

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INTRODUCTION

Baculoviruses form a large group of invertebrate viruses infective primarily to insects of the Order Lepidoptera. They are widespread in the environment and infect more than 600 species of insects and at least two species of crustaceans (Blissard and Rohrmann, 1990; Adams and McClintock, 1991). Diseases caused by baculoviruses, specially the jaundice of silkworm, have attracted attention for hundreds of years. In more recent times, interest in baculoviruses has focused on their ability to reduce insect populations which has led to their use as a method of pest control. With the advent of modern biotechnology, a new role has been conferred upon baculoviruses: they have become an important tool for the expression of foreign proteins whose genes are inserted in the viral genome. In addition, recombinant DNA technology has allowed the development of genetically modified viruses that may become more effective pest control agents than their wild-type counterparts.

The molecular biology of baculoviruses has been extensively investigated in the past 20 years. Most of molecular studies have been done with the *Autographa californica* nucleopolyhedrovirus (AcMNPV), which is the type species of baculoviruses. The entire genome of AcMNPV has been sequenced and many genes have been characterized. However, there are major questions that remain unanswered. For example, the process of viral DNA replication and the mechanisms that determine host range specificity are poorly understood. Furthermore, there has been relatively little molecular investigation of other baculovirus species. The work presented in this thesis relates to the *Spodoptera littoralis* nucleopolyhedrovirus type B (SpliNPV-B), a baculovirus that infects the Egyptian cotton worm, *Spodoptera littoralis* (Lepidoptera: Noctuidae).

In the first chapter of this thesis, I will review some important aspects of baculovirology including an historical background of baculovirology (Section 1.2), baculovirus taxonomy (Section 1.3), the molecular biology of baculoviruses (Section 1.4), host range and safety issues (Section 1.5), practical applications of baculoviruses (Section 1.6), and baculovirus phylogeny and evolution (Section 1.6), and a summary of what is known about the SpliNPV-B (Section 1.7). Finally, I will give an outline of the thesis (Section 1.6). The results of my research are presented in a paper format (Chapters 2, 3, and 4). Chapter 5 is a general conclusion.

Chapter I: Literature Review

HISTORICAL BACKGROUND

The first European report of an insect disease caused by baculovirus infection may be in a poem written in 1527 by Marco Girolano Vida (Benz, 1986), the bishop of Alba, Italy. In this poem, he appears to describe silkworm jaundice, a baculovirus disease that causes the silkworm to swell and rupture releasing a purulent liquid. Later, in 1679, Maria Merian mention the name jaundice (Gelbsucht) in her book about the metamorphosis of caterpillars (Benz, 1986). In this book, she expressed their current belief that jaundice was induced by thunderstorms (Benz, 1986).

Baculovirus occlusion bodies were first reported in the middle of last century by two Italian scientists, Maestri and Cornalia, who noticed that crystal-like granules were found in several tissues of jaundiced silkworms (Benz, 1986). In 1874, these granules were named polyhedral granules by J. Bolle (Benz, 1986), the director of the Austrian Imperial Sericultural Experiment Station in Gorz. Bolle reported that the polyhedral granules (also referred to as polyhedra) were insoluble in glycerol, ethanol, and other organic solvents, but were soluble in alkaline or strong acid solutions (Benz, 1986). In 1906, Bolle recognized the polyhedral bodies as the causal agent of jaundice (Benz, 1986). Shortly after, Whal inoculated nun moth larvae, *Lymantria monacha*, with polyhedral granules and noticed the development of the "wilt disease", another baculovirus infection (Whal, 1909). He observed that polyhedral granules developed in several tissues of the infected larvae and that these granules had distinct shape compared to those collected from jaundiced silkworm (Whal, 1909).

The hypothesis that polyhedral granules caused insect diseases was challenged by Prowazek, who pointed out that after removal of granules the filtered blood of jaundiced silkworm still remained infectious (von Prowazek, 1907). In 1913, a landmark in baculovirology was reached when Glaser and Chapman concluded that the "wilt disease" was caused by a filterable virus capable of passing through a diatomaceous Berkefeld Grade "N" filter (Glaser and Chapman, 1913). However, Glaser and Chapman failed to realize the relationship of the polyhedral granule to the filterable virus and concluded that the polyhedral granules were degenerative products of the disease (Glaser and Chapman, 1916). Another important achievement in the study of baculoviruses was the development of cell culture procedures that could sustain growth of silkworm ovary cells for 2 to 3 weeks (Trager, 1935). In 1930s, Trager infected cultured cells with

baculoviruses and observed virus multiplication and formation of polyhedral granules in cells that were actively dividing (Trager, 1935).

During the 1940s, Bergold conducted experiments with the polyhedra protein and observed that when the polyhedra were dissolved in alkali the virus remained infective. His observation led him to believe that the polyhedra protein was the virus (Benz, 1986). Later Bergold published the first electron micrographs of the polyhedral virus of *Bombyx mori* and *Lymantria dispar* which revealed the rod shape of the virions (Bergold,, 1947). Hughes analyzed ultrathin sections of tissues from infected silkworm by electron microscopy and observed that rod-shaped structures occurred initially in a central chromatic mass and that later bundles of rods became surrounded by a membrane (Hughes, 1953). He concluded that polyhedra were formed by the deposition of a material that surrounds these bundles (Hughes, 1953). The above sketch of the history of baculovirology is a simplification of the major milestones of the field and outlines how the basis of what is presently known about baculoviruses was formed. For a comprehensive review see (Benz, 1986).

A great deal of progress in understanding the biology of baculoviruses has been made over the past two decades using modem tools of molecular biology. The restriction endonuclease pattern of NPV isolates were first analyzed in 1978 (Smith and Summers, 1978), the nucleotide sequence of the AcMNPV polyhedrin gene was determined in 1983 (Hooft van Iddeekinge et al., 1983), and the complete sequence of the AcMNPV genome was published in 1994 (Ayres et al., 1994). Several studies performed in the late 70s and early 80s demonstrated that baculovirus gene expression is regulated in a cascade mode (Friesen and Miller, 1986; Blissard and Rohrmann, 1990). The AcMNPV genes that provide the minimal requirement for transient DNA replication were determined in 1994 (Kool et al., 1994). Baculovirus expression vectors for the expression of foreign proteins in insect cells were first developed in 1983 (Smith et al., 1983; Smith et al., 1983) and this system has been widely used (Maeda, 1994). During the late 80s and the 90s, genetically modified baculoviruses have been developed with the aim of increasing the virus efficiency as a pesticide (Wood and Granados, 1991; Bonning and Hammock, 1996). The first commercial recombinant baculovirus product has recently been released by the American Cyanamid Company, Princeton, NJ, USA.

TAXONOMY

Viruses of the family *Baculoviridae* infect invertebrates and have enveloped rod-shaped virions containing a circular supercoiled double-stranded DNA molecule ranging in size from 90 to 230 Kbs (Blissard and Rohrmann, 1990). The family *Baculoviridae* used to be divided into two subfamilies: the *Eubaculovirinae* and the *Nudibaculovirinae* (Francki et al., 1991). Members of the *Eubaculovirinae* subfamily were characterized by an occlusion body, a proteinaceous matrix that protects the virus once it is released in the environment. The *Eubaculovirinae* subfamily was composed of two genera, the nuclear polyhedrosis virus (NPV) and the granulosis virus (GVs). GVs and NPVs differ in size, number of virions per occlusion body, and in the constitution of their occlusion body. GVs are smaller than NPVs (0.25-0.5μm in diameter compared to 1-15μm for NPVs) and contain a single virion per occlusion body whereas NPVs usually contain several virions per occlusion body (Adams and McClintock, 1991). The occlusion body of the GVs is composed mainly of a protein called granulin which is closely related to the major protein of the NPV occlusion body, the polyhedrin (Rohrmann, 1992). The NPVs were further classified according to the number of virions per envelope as either single nuclear polyhedrosis virus (SNPV), containing only one virion per envelope, or multiple nuclear polyhedrosis virus (MNPV), containing several virions per envelope. Members of the *Nudibaculovirinae* were nonoccluded viruses such as the baculovirus that infects the palm rhinoceros beetle, *Orcytes rhinoceros* (Adams and McClintock, 1991).

Recently, the VIth International Committee on Taxonomy of Viruses has proposed a new classification system for baculoviruses (Volkman et al., 1995). The family *Baculoviridae* is now divided into two genera: Nucleopolyhedrovirus (NPVs) and Granulovirus (GVs) (Volkman et al., 1995). Under the new system nonoccluded viruses do not belong to the *Baculoviridae*. Baculoviruses continue to be named after the host insect from which they were first isolated followed by the genus to which they belong. The VIth International Committee on Taxonomy of Viruses has, however, proposed a new abbreviation system which takes the first two letters (instead of one letter) from the host's genus and species name and abolishes (for most viruses) the designation describing single (S) or multiple (M) nucleocapsids per envelope (Volkman et al., 1995). The abbreviation for a few of the best investigated baculovirus species such as

AcMNPV and OpMNPV has not been altered. In this thesis I will use the classification and abbreviation systems proposed by the VIth International Committee on Taxonomy of Viruses.

MOLECULAR BIOLOGY

Life cycle

NPVs are widespread in the environment and are found in the soil and on plant surfaces. The life cycle of NPVs is characterized by two phases in which two distinctive virions are produced; the occlusion derived virion (ODV) and the budded virus (BV). The infection cycle typically starts when susceptible insect larvae ingest food contaminated with occlusion bodies (Blissard and Rohrmann, 1990). The alkaline condition of the insect midgut and the action of alkaline proteinases break down the polyhedra releasing the ODVs. The free virions pass through the peritrophic membrane, contact microvilli or lateral surfaces of the columnar epithelial cells, and enter these cells by a process of receptor-mediated membrane fusion (Adams and McClintock, 1991; Horton and Burrand, 1993). The naked nucleocapsids (without the envelope) are transported to the nucleus where the viral genomes are released from the capsid (Blissard and Rohrmann, 1990). Expression of viral genes and replication of the viral genome occurs in the nucleus (Blissard and Rohrmann, 1990). The nucleus of the host cell becomes enlarged and an electron-dense structure called virogenic stroma is formed (Vialard et al., 1995). Nucleocapsids are assembled around and within the virogenic stroma, migrate to the cytoplasm, and bud through the cytoplasmic membrane, hence the name budded virions (BV) (Blissard and Rohrmann, 1990). AcMNPV BV progeny are typically observed at 12 hours post-infection (Blissard and Rohrmann, 1990).

Studies with AcMNPV indicate that BV are released into the larval extracellular compartment and spread the infection to various insect tissues through the tracheal system (Engelhard et al., 1994). Once BVs reach these tissues, they enter the cells primarily by absorptive endocytosis and produce viral progeny of two types. Some nucleocapsids migrate to the cytoplasm, bud through the cytoplasmic membrane, and thus give origin to other BVs (Blissard and Rohrmann, 1990). Other nucleocapsids are enveloped *de novo* in the nucleus, embedded within the occlusion body, and thus give rise to ODVs (Blissard and Rohrmann, 1990). Polyhedra are usually first observed at approximately 24 hours post-infection (Blissard and Rohrmann, 1990) and accumulate during the infection which often last from 5 to 7 days. Upon death, the larvae liquefy resulting in the release of large quantities of polyhedra in the environment (up to 25% of the dry weight of the cadavers consist of polyhedra). The polyhedra occlusion

body protects the viruses from the action of UV light and desiccation and allows them to remain infective for long periods of time.

Virus structure

The major structural characteristic of NPVs is the occlusion body which is an unique feature of invertebrate viruses, and is found not only in baculoviruses but also in cytoplasmic polyhedrosis viruses (Family; *Reoviridae*) and in entomopox viruses (Family: *Poxviridae*) (Rohrmann, 1992). The occlusion body of the NPVs is composed primarily of a single polypeptide of about 29kDa called polyhedrin. Polyhedrin is the most conserved baculovirus protein that has been characterized with over 80% amino acid sequence identity among polyhedrins of baculoviruses isolated from lepidopteran species (Rohrmann, 1986). Polyhedrin is a highly expressed protein which forms a multimeric lattice around many virions. The occlusion body of NPVs, or the polyhedra, can have a variety of shapes such as cuboidal, tetrahedral, dodecahedral, or irregular (Adams and McClintock, 1991). The polyhedra is surrounded by a carbohydrate rich structure, called polyhedron membrane or PE, that seems to increases the stability of the polyhedra (Vialard et al., 1995). Although the polyhedra is critical for virus infection in the environment, it is not essential for viral replication in cell culture. For this reason, the polyhedrin locus is often used for insertion of foreign genes in the construction of baculovirus expression vectors.

Another hyperexpressed AcMNPV protein, p10, appears to form fibrillar structures in the nucleus and cytoplasm of infected cells but its function has not been well defined (Rohrmann, 1992). Mutants that lack the p10 protein have a defective or absent PE indicating that p10 plays a role in PE formation (Rohrmann, 1992). Some p10 defective AcMNPV mutants are unable to release the polyhedra from infected cells suggesting that p10 protein is involved in cell lysis (Van Oers et al., 1994).

NPVs are characterized by the production of two virion phenotypes: ODV and BV. ODVs differ from BVs in several aspects including number of virions per envelope, tissue specificity, and mode of entry into cells (Blissard and Rohrmann, 1990). Both forms of virions have an envelope which surrounds a bacilliform nucleocapsid measuring approximately 40 to 60 nm X 250 to 300 nm (Adams and McClintock, 1991). ODVs are enveloped *de novo* in the nucleus and may have from 1 to 29 nucleocapsids tightly fitted in an envelope that seems to be specialized for interaction with columnar epithelial cells of the insect midgut (Blissard and Rohrmann, 1990). In contrast, BVs acquire their membranes when the nucleocapsid buds through the

cytoplasmic membrane and usually have a single nucleocapsid loosely fitted in an envelope that seems to be specialized for interaction with cells and tissues within the insect haemocoel (Blissard and Rohrmann, 1990).

The core of the baculovirus nucleocapsid is a complex formed by *p6.9*, a protamine-like protein, and viral DNA (Wilson et al.,1987). *p6.9* is a small, arginine rich, very basic protein which associates with the viral DNA (Wilson et al., 1987). The basic character of this protein may neutralize the negative charge of the DNA molecule and thus promote the packaging of the DNA in a condensed form (Wilson et al., 1987). Conversely, the phosphorylation of the *p6.9* protein following viral entry into cells may trigger the unpacking of viral DNA (Rohrmann, 1992).

The NPV capsid is a rod-shaped structure that is assembled in the nucleus of infected cells (Blissard and Rohrmann, 1990). The fact that empty capsids are often observed in nuclei of infected cells suggests that the capsid and the nucleoprotein complex (viral DNA and *p6.9* protein) are formed independently (Vialard et al., 1995). The major capsid protein, P39, is distributed over the surface of the capsid and is found in capsids of both ODVs and BVs (Blissard and Rohrmann, 1990; Rohrmann, 1992).

Nucleocapsids that migrate from the nucleus to the cytoplasm acquire a temporary envelope as they pass through the nuclear membrane (Vialard et al., 1995). This membrane, which is rich in a viral encoded protein named P16, is lost when the virus buds through the plasma membrane (Vialard et al., 1995). As the nucleocapsids bud through the cytoplasmic membrane they acquire a membrane that contains GP64 (or GP67), a viral encoded glycoprotein (Blissard and Rohrmann, 1990). GP64 is found throughout the BV envelope but seems to be more concentrated in the terminal region forming peplomers (Volkman et al., 1984). Peplomers are glycoprotein protrusions observed at one end of the virion and are thought to facilitate viral absorption to cell membranes (Adams and McClintock, 1991). The importance of GP64 for the infection process has been demonstrated by studies in which monoclonal antibodies against GP64 neutralized the infectivity of BV (Volkman and Goldsmith, 1985). Recent analysis has demonstrated that GP64 mediates acid-triggered membrane fusion and suggests that GP64 is responsible for fusion of the virion envelope with the endosome membrane during BV entry into the host cell by endocytosis (Blissard and Wenz, 1992; Rohrmann, 1992).

Nucleocapsids that are enveloped *de novo* in the nucleus are packaged into a bilayer membrane. Although no ODV specific membrane protein has been identified to date, indirect evidence suggests that the AcMNPV p74 gene product is associated with the ODV membrane (Rohrmann, 1992). Mutations that inactivate the AcMNPV *p74* gene do not affect viral growth in cell culture, but renders the virus noninfectious to insect larvae (Kuzio et al., 1989).

Gene expression and replication

Baculovirus gene expression is temporally regulated in a cascade mode with three major phases of gene expression: early, late, and very late. The early phase of infection used to be divided into immediate early and delayed-early phase based on the supposed dependence of viral-encoded factors for transcription of the delayed-early genes (Blissard and Rohrmann, 1990). However, this distinction is not entirely accurate. It has been shown that promoters of delayed-early genes are active in transient expression assays in the absence of viral proteins (Theilmann and Stewart, 1991) and that extracts from uninfected *Spodoptera frugiperda* cultured cells can activate delayed-early promoters (Blissard and Rohrmann, 1991; Glocker et al., 1992).

The early phase of infection starts soon after the release of viral DNA into the host nucleus and early genes appear to be transcribed by host RNA polymerase II. The strongest indication of the involvement of RNA polymerase II in the early phase is the fact that early transcription in inhibited by α-amanitin, an inhibitor of eukaryotic RNA polymerase II (Huh and Weaver, 1990; Glocker et al., 1992). In addition, early gene promoters resemble those of Class II genes, the eukaryotic genes transcribed by RNA polymerase II (Blissard and Rohrmann, 1990). A typical Class II gene promoter has two functional regions: i)proximal elements in the sequence immediately upstream the transcription initiation site; and ii) distal regulatory elements located further upstream and which confer cell and stage-specific control (Singer and Berg, 1991).

Baculovirus early genes have a similar organization with the basal promoter often encompassing a TATA box and a CAGT motif at the site of transcription initiation (Theilmann and Stewart, 1991; Blissard and Rohrmann, 1991; Blissard and Rohrmann, 1990). Blissard et al. (1992) investigated the roles of the TATA box and of the CAGT motif in a 43 nucleotide synthetic promoter. A series of mutations were incorporated to this synthetic promoter and the effect of these mutations on transcription activity and on the location of the transcription initiation site was analyzed (Blissard et al., 1992). Their results indicate that the TATA box determines the transcription start site and that the CAGT motif plays a major role in controlling the rate of transcription initiation (Blissard et al., 1992). The promoter elements of the AcMNPV *39k* gene have also been investigated (Guarino and Smith, 1992). This baculovirus promoter has the unique feature of two adjacent TATA boxes, referred to as 'distal' and 'proximal' TATA boxes,

directing transcription initiation at the distal and proximal transcription initiation sites (Guarino and Smith, 1992). The level of activity and the position of transcription initiation were analyzed following mutations in the early promoter elements of the *39k* gene. The results obtained suggest that 39k transcription is controlled by two distinct mechanisms. The proximal TATA box function in cooperation with the CAGT initiator sequence, whereas the distal TATA box controls transcription independently of the sequences surrounding the distal transcription initiation site (Guarino and Smith, 1992). Recently, insect proteins that bind to baculovirus early promoter sequences have been identified. For instance, Krappa and coworkers demonstrated that an *Spodoptera frugiperda* protein binds to the GATA motif found in the AcMNPV *pe-38* gene promoter (Krappa et al., 1992). Similarly, Kogan and Blissard showed that the GATA and CACGTG elements found in the promoter of the OpMNPV *gp64* gene are recognized by *Spodoptera frugiperda* proteins. Mutations in either of these elements resulted in loss of specific binding and reduction in transient level of expression from the *gp64* promoter (Kogan and Blissard, 1994)

The expression of early genes is also influenced by enhancers. Enhancers are DNA sequences that, when located in the same DNA molecule (cis-configuration), can increase expression of some eukaryotic genes in a manner that is not totally dependent on their orientation and distance in relation to the gene promoter. Five regions of the genome of AcMNPV that contain homologous repeated DNA sequences (hr1 to hr5) display enhancer-like activity in transient expression assays (Guarino et al., 1986). In addition, the enhancer activity of hr5 has also been demonstrated when it is inserted in the genome of AcMNPV (Rodems and Friesen, 1993). Analysis of the AcMNPV hr regions has shown that they consist of repeated sequences rich in EcoRI sites and that a single EcoRI minifragment stimulates transcription as efficiently as regions containing multiple repeats (Guarino et al., 1986). Repeated regions found in the genomes of other NPVs do not seem to be closely related to the AcMNPV hr sequences (Blissard and Rohrmann, 1990). Nevertheless, one of the hrs found in the genome of OpMNPV has some similarity to AcMNPV hrs and also displays enhancer activity (Theilmann and Stewart, 1992).

Virus encoded transcription regulators seem to play a critical role in the early stages of AcMNPV infection. For instance, the product of the *ie-1* gene is involved in the

regulation of AcMNPV early and late genes and also affects the expression of host genes (Guarino and Summers, 1986; Kovacs et al., 1991; Lu et al., 1996). The AcMNPV IE-1 protein was first identified as a factor required for the expression of 39k, another AcMNPV early gene (Guarino and Summers, 1986), and its transactivating effect seems to be greatly increased when the target promoters are linked to AcMNPV hr5 (Guarino and Summers, 1986). The presence of the IE-1 protein seems to induce the formation of protein complexes that bind to direct repeats found in the hr5 (Guarino and Dong, 1991). This observation indicates that IE-1 either binds directly to enhancer sequences or mediates binding of a host factor (Guarino and Dong, 1991). The fact that the AcMNPV hr sequences function as enhancers in the absence of IE-1 demonstrates that this viral early protein is not essential for enhancer mediated activation of baculovirus genes (Nissen and Friesen, 1989; Carson et al., 1991).

Another early gene coding a transcription regulator, the AcMNPV *ie-2* gene (formerly called *ie-n*), was identified as a factor that increases the expression of the *39k* gene in the presence of IE-1 (Carson et al., 1988). Later investigation demonstrated that the *ie-2* gene product stimulated its own expression as well as the expression of *ie-1* gene (Carson et al., 1991). Transcripts of *ie-2* are abundant in the early stages of infection but almost undetectable during the late phase (Carson et al., 1991). Carson et al. (1991) demonstrated that transient transcription of *ie-2* gene is increased by the presence of a viral enhancer (hr1). When the *ie-n* reporter gene construct was linked in *cis* to hr-1, expression of reporter gene was augmented by IE-2 but was reduced by IE-1 (Carson et al., 1991). Several other baculovirus early genes whose products influence transcription from early and late genes have been identified.

Viral DNA replication marks the switch from the early to the late phase of infection. Several observations suggest that the RNA polymerase that transcribes late genes is either of viral origin or virus-induced. First, late transcription activity is resistant to both α-amanitin (100 μg/ml), an inhibitor of RNA polymerase II, and to tagetitoxin (4,000 U/ml), an inhibitor of RNA polymerase III (Steinberg et al., 1990; Glocker et al., 1993). Second, only extracts obtained from virus infected cells, but not from noninfected cells, can activate late promoters *in vitro* (Beniya et al., 1996). Third, it has been demonstrated that RNA polymerase extracted from infected cells has a subunit structure that differs from the subunit structure of the three host polymerases

(Yang et al., 1991). Finally, two AcMNPV early genes, the *lef-8* and the *lef-9*, encode putative polypeptides that have conserved RNA polymerase motifs (Lu and Miller, 1994; Passarelli et al., 1994).

Transcription of baculovirus late genes is controlled by short promoters similar to those of bacteriophage T7 and mitochondrial genes (Blissard and Rohrmann, 1990). All late genes identified to date have the conserved A/G/T/TAAG motif which seems to function as both promoter and mRNA start site (Blissard and Rohrmann, 1990). Analysis of the AcMNPV *vp39* gene promoter showed that an 18 bp region surrounding the TAAG motif is critical to transcription activity (Morris and Miller, 1994). The fact that mutations or deletion of sequences flanking this 18 bp segment had minor effects on transcription activity suggests that neither the untranslated leader sequences nor sequences located further upstream from the 18 bp segment are critical for *vp39* promoter activity (Morris and Miller, 1994).

Both late and very late genes are transcribed following viral DNA replication. Later in infection, however, expression of late genes declines whereas very late genes, which are initially expressed at low levels, become highly expressed (Blissard and Rohrmann, 1990; Morris and Miller, 1994). Two very late genes have been identified to date: the *polyhedrin* gene and the *p10* gene. The *polyhedrin* is the major protein of the occlusion body crystalline matrix and also the most conserved baculovirus protein (Blissard and Rohrmann, 1990). The *p10* gene encodes a protein which assembles into fibrillar structures in the nucleus and whose function is still uncertain (Van Oers et al., 1994). Neither of these genes are essential for virus growth and their loci have been used in the construction of vectors for expression of exogenous proteins.

Analysis of the polyhedrin promoter of several NPVs revealed that a 12 nucleotide consensus (AATAAGTATTTT) exists around the transcription initiation site (Blissard and Rohrmann, 1990). This sequence encompasses the core promoter element, TAAGTATT, which seems to be critical for high levels of transcription activity (Ooi et al., 1989). In addition, the AcMNPV polyhedrin gene has an untranslated leader sequence (ULS) that is important for efficient transcription activity (Ooi et al., 1989). It has been suggested that the polyhedrin promoter is a weak late promoter that is strongly activated during the very late phase because of interactions with specific activators (Morris and Miller, 1994). This hypothesis is supported by fractionation experiments

with nuclear extracts of AcMNPV infected *Spodoptera frugiperda* cells (Xu et al., 1995). It has been observed that although two nuclear extraction fractions eluted by phosphocellulose chromatography activated late and very late promoters, only one of these fractions resulted in higher activity for very late promoters compared to late promoters (Xu et al., 1995). The hypothesis that specific factors interact with promoter elements of very late genes is also supported by the identification of a gene whose product enhances the expression of very late promoters in transient assays (Todd et al., 1996). The very late expression factor-1 gene, *vlf-1*, was initially identified in temperature-sensitive AcMNPV mutants that did not produce PIBs at the restrictive temperature (Lee and Miller, 1979) and may be responsible for the strong expression of very late promoters (Todd et al., 1996).

AcMNPV early genes whose products support the transition from the early to the late phase have been identified by a transient expression assay (Todd et al., 1995). In this assay, clones of a genomic library representing the entire genome of AcMNPV are cotransfected with a plasmid carrying a reporter gene under the control of a late and a very late phase gene promoter. Removal of a clone with a baculovirus DNA fragment carrying a gene that is important for late and very late gene expression reduces or eliminates expression of the reporter gene. Once a clone that contributes to late gene expression is found, the gene(s) responsible for this effect can be identified. A total of eighteen AcMNPV genes that influence late and very late gene expression have been identified by this procedure: the late expression factor genes 1 to 11 (*lef-1* to *lef-11*), *ie-1*, *ie-2*, *dnapol*, *p143* (the *helicase* gene), *39K*, *p47*, and *p35* (Todd et al., 1995). The level of reporter gene expression obtained when clones carrying the above eighteen genes are cotransfected into insect cells in culture is the same as the response obtained when an overlapping genomic library containing the whole genome of AcMNPV is cotransfected with reporter plasmids (Todd et al., 1995).

The above transient expression assay does not determine whether the late expression factors are directly involved with late gene expression or with other events which indirectly interfere with late and very late gene expression. Studies with aphidicolin, an inhibitor of both the host α-DNA polymerase and of the virus induced DNA polymerase, demonstrated that late gene expression is not observed when DNA replication is blocked (Huh and Weaver, 1990; Blissard and Rohrmann, 1990; Beniya

et al., 1996). Therefore, the transient expression assay does not distinguish between factors involved in replication and factors involved in transcription.

Recently, Merrington et al. (1996) reported a new approach to identify baculovirus genes involved in late gene expression. In this procedure, mutations in the genome of AcUW1.lacZ, an AcMNPV recombinant which has the *lacZ* gene under transcriptional control of the p10 promoter, were produced by propagating the virus in the presence of 5'-bromodeoxyuridine (BrdU). Mutants deficient in very late gene expression could be detected by plaque assay because they did not express the *lacZ* gene and produced white plaques when grown in the presence of the chromogenic substrate 5-bromo-4-choro-3-indolyl -ß-D-galactoside (Xgal). The mutant VLD1 was selected and the mutation that impaired late gene expression was mapped in the *lef-2* gene (Merrington et al., 1996). This procedure has the advantage of distinguishing between factors that affect late gene expression and factors that interfere with viral DNA replication.

A transient assay has also been used in the identification of baculovirus early genes that are essential for viral DNA replication. This assay is similar to the above described assay that was used to identify the late expression factors. Clones encompassing the whole genome of the viral genome are cotransfected with a plasmid carrying an AcMNPV origin of replication. DNA replication of the plasmid is not observed when certain regions of the AcMNPV genome are not cotransfected. Therefore, genes that are critical for AcMNPV replication were identified after a particular region of the genome was found to be required for replication of the plasmid with the AcMNPV origin of replication. Using this procedure, Kool et al. (1994) ranked the following six early genes as essential for viral DNA replication: *p143* (the helicase gene), *dnapol, ie-1, lef-1, lef-2,* and *lef-3*. In addition, these researchers also concluded that the product of the *p35*, the *ie-2*, and the *pe-38* genes stimulate DNA replication from a plasmid that has the AcMNPV homologous region 2 (hr2) as origin of replication (Kool et al., 1994).

AcMNPV early genes that are essential for DNA replication from a plasmid carrying the homologous region 5 (hr5) as origin of replication were also identified by Lu and Miller (1995). The genes identified by these researchers were the same as those identified by Kool and coworkers (1994) except that the *p35* gene instead of the *dnapol* gene was considered essential for replication (Lu and Miller, 1995). Lu and Miller

(1995) results also suggest that the *ie-2*, *lef-7*, and *dnapol* gene products stimulate DNA replication. Differences in the results of the two experiments may be related to the fact that replication activity was evaluated at later periods in the experiments done by Lu and Miller and that different homologous regions were used.

The functional role of the above genes in AcMNPV replication has not been determined. There is evidence that *ie-1*, *ie-2*, and *pe-38* gene products are transcription regulators and these genes, therefore, could affect replication by assuring proper expression of other baculovirus genes (Guarino and Summers, 1987; Carson et al., 1991; Lu and Carstens, 1993). The *p35* gene product has been well characterized and shown to block apoptosis in vertebrate and invertebrate cells (Clem et al., 1991; Sugimoto et al., 1994). Consequently the *p35* gene product may increase replication of viral DNA by preventing cells from dying (Kool et al., 1994). The *p143* and the *dnapol* genes encode polypeptides with sequence motifs shared by helicases and DNA polymerases respectively (Tomalski et al., 1988; Lu and Carstens, 1991). The involvement of the *p143* gene in replication is also supported by experiments which showed that a DNA replication defective, temperature-sensitive AcMNPV mutant had mutations in the *p143* gene (Lu and Carstens, 1991). The *lef-3* gene product displays single stranded-DNA binding (SSB) activity (Hang et al., 1995) and a nucleotide sequence motif found in SSB proteins (Ahrens et al., 1995). The putative LEF-7 polypeptide displays amino acid sequence motifs similar to motifs found in the sequence of herpes simplex virus type 1 UL29 gene product and LEF-7 also has two single stranded-DNA binding motifs (Lu and Miller, 1995). Little information in known about the function of the *lef-1* and *lef-2* gene products (Kool et al., 1995). A recent experiment, however, has shown that point mutations in the *lef-2* locus resulted in an AcMNPV mutant that had very late gene expression blocked but that did not have its DNA replication impaired (Merrington et al., 1996). As this result disagrees with what has been observed in transient complementation assays, the authors suggest that *lef-2* gene product may have dual function. LEF-2 may be involved in both late gene expression and viral DNA replication and the mutated region of the gene might correspond to a site in the protein that is involved only in very late gene expression (Merrington et al., 1996).

HOST-RANGE AND SAFETY

A remarkable attribute of baculovirus is their host specificity. The majority of baculoviruses have been isolated from lepidopteran species (Blissard and Rohrmann, 1990) and most evidence indicates that baculoviruses will only infect members of the same Order (McIntosh and Grasela, 1994). The only exception in the literature of a baculovirus that cross infect orders are two reports describing the infection of an orthopteran and an isopteran species by SpliNPV, the baculovirus isolated from the Egyptian cotton worm, *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Bensimon et al., 1987; Fazairy and Hassan, 1988). There are no reports, however, which confirm these highly unusual results. Most studies of baculovirus host range have indicated that NPVs infect only insect members of the same genus, and often of the same family (Groner, 1986). No baculovirus infection has ever been reported in vertebrates and only a few orders of invertebrates seem to be susceptible to baculovirus infection (Groner, 1986).

Baculoviruses with multiple capsids per envelope (MNPVs) appear to have the broadest host range (McIntosh and Grasela, 1994). For instance, AcMNPV infects over 30 insect species from 12 Lepidoptera families and also infects cultured cells of *Anthonomus grandis*, a coleopteran species (McIntosh et al., 1992; McIntosh and Grasela, 1994). Another MNPV with broad host range has been isolated from a celery looper, *Anagrapha falcifera* (Hostetter and Willians, 1991). The AfMNPV infects members of 10 different families (Hostetter and Willians, 1991). A comprehensive host range assessment of MbMNPV, the nucleopolyhedrovirus of cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae) has been conducted (Doyle et al., 1990). In these experiments, a total of 66 lepidopteran species, three hymenopteran species, three coleopteran species, and one neuropteran species were exposed to MbMNPV (Doyle et al., 1990). Viral infection was observed in larvae of 33 lepidopteran species, most of them members of the Noctuidae (Doyle et al., 1990). None of the nonlepidopteran species tested were susceptible to the virus (Doyle et al., 1990).

Although cell culture infections have often been used to investigate baculovirus virulence, growth of baculoviruses in a particular cell line is not always an indication that the virus is infective to the insect *in vivo* (McIntosh and Grasela, 1994). For instance, AcMNPV is not infective to gypsy moth larvae, *Lymantria dispar*, but *L. dispar* cell lines that are both permissive and nonpermissive to AcMNPV have been developed

(Goodwin et al., 1978). The fact that cell lines derived from *L. dispar* can be either permissive or nonpermissive to AcMNPV illustrates that cell lines derived from the same host often have different levels of susceptibility to viral infection. Still, *in vitro* infection has been an invaluable tool to gain insight into mechanisms involved in the determination of baculovirus host specificity.

Carbonell et al. (1985) used a recombinant AcMNPV to investigate gene expression in dipteran and mammalian cells. The recombinant AcMNPV used in this study had reporter genes under transcriptional control of the Rous Sarcoma Virus (RSV) long terminal repeat (LTR), a promoter that is highly active in mammalian cells, and under control of the polyhedrin gene promoter (Carbonell et al., 1985). Their results indicated that the recombinant AcMNPV was able to enter into dipteran and mammalian cell lines and that the RSV LTR was mildly active in both cell lines (Carbonell et al., 1985). Although low levels of AcMNPV DNA replication occurred in *Drosophila* cells, expression from the polyhedrin gene promoter was observed in neither the dipteran nor the mammalian cells (Carbonell et al., 1985).

The hypothesis that late and very late genes would not be expressed in nonpermissive cells was disproved by later experiments that used more sensitive techniques. In these experiments, AcMNPV recombinants with reporter genes under control of the *Drosophila melanogaster* heat shock protein 70 (hsp70) gene promoter, and baculovirus early, late, and very late gene promoters were used to investigate gene expression in permissive and nonpermissive cell lines (Morris and Miller, 1992). Unexpectedly, infection with these recombinants revealed that gene promoters from the three viral infection phases were active in nonpermissive cell lines (Morris and Miller, 1992). The site of late and very late transcription initiation were analyzed in viral infected BmN-4, Ld652Y, and Hz1b3 cells, three nonpermissive lepidopterous cell lines, and in Dm cells, a cell line derived from *Drosophila melanogaster.* Primer extension analysis revealed that transcription initiated at the proper TAAG initiation site in these cell lines (Morris and Miller, 1992). Blocking of DNA replication by aphydicolin prevented the expression of late genes in all cell lines except in Dm cell (Morris and Miller, 1992). As the hsp70 promoter was strongly active in the dipteran cell line (Morris and Miller, 1992), the study also indicates that AcMNPV can efficiently enter the nucleus of the nonpermissive *Drosophila* cell line.

Several studies have indicated that although AcMNPV can enter into cultured mammalian cells the virus does not persists in these cells (Volkman and Goldsmith, 1983; Tija et al., 1983; Carbonell et al., 1985; Hartig et al., 1991). For instance, Hartig et al. (1991) investigated the infection of AcMNPV in cultured primate cells and showed that unlike HSV-1 and Reo-3, AcMNPV was not able to persist in the primate cell lines tested. Dot blot analysis showed that AcMNPV was able to enter the primate cells but that no virus DNA could be detected in any cell line tested 7 days after infection (Hartig et al., 1991). In addition, these researchers demonstrated that exposed cells did not display cytopathological effects nor produced AcMNPV progeny (Hartig et al., 1991).

Ld652Y cells, a cell line derived from *Lymantria dispar*, are described as semi-permissive for AcMNPV replication (McClintock et al., 1986). Infection of Ld652Y cells with AcMNPV causes cessation of cell growth and cell clumping at 20 hours p.i., decrease in host DNA synthesis equivalent to that observed during normal baculovirus replication, (McClintock et al., 1986; Guzo et al., 1991) and complete inhibition of protein synthesis of both viral and host origin between 20 and 24 hours post infection (Guzo et al., 1992). Although high levels of AcMNPV RNAs (Guzo et al., 1992) and transcripts of the early, late, and very late phase of infection are produced in Ld652Y cells, AcMNPV transcripts are poorly translated in these cells (Morris and Miller, 1992). AcMNPV DNA replicates in Ld652Y cells but neither polyhedral inclusion bodies (PIBs) nor infective BV are produced (McClintock et al., 1986; Morris and Miller, 1993).

Recently, Thiem et al. (1996) identified a gene from *Lymantria dispar* nucleopolyhedrovirus (LdMNPV) that allows productive infection of AcMNPV in Ld652Y cells. This gene, which they called *hrf-1* (host-range factor-1), encodes a putative polypeptide with a molecular mass of 25.7 kDa rich in glutamic acid and valine residues (Thiem et al., 1996). When Ld652Y cells were infected with vAcLdPS, a recombinant AcMNPV that has *hrf-1* inserted in its genome, PIB production was observed (Thiem et al., 1996). Western blot analysis demonstrated that AcMNPV proteins that were not expressed in AcMNPV infected Ld652Y cells, were expressed following infection with vAcLdPS (Thiem et al., 1996). Although the function of *hrf-1* in host range determination has not been determined, Thiem and coworkers hypothesize two possible roles for *hrf-1*. The cessation of protein synthesis observed in Ld652Y cells following

AcMNPV infection may be the result of cellular defence mechanisms against virus infection. Therefore, the role of *hrf-1* might be to block this mechanism of host defence (Thiem et al., 1996). On the other hand, it is also possible that baculoviruses have a mechanism that selectively hinders host protein synthesis during infection in permissive cells. In that case, *hrf-1* might be a factor that helps maintain viral protein synthesis in Ld652Y cells (Thiem et al., 1996).

The involvement of the NPV *helicase* gene in host range determination was demonstrated after the construction of AcMNPV recombinants that were able to grow in BmN cells, a cell line that does not support AcMNPV growth (Maeda et al., 1993; Croizier et al., 1994). The AcMNPV recombinants that were able to grow in BmN cells had their helicase gene modified due to homologous recombination with the helicase gene of the BmNPV, a baculovirus species that is closely related to AcMNPV (Maeda et al., 1993; Croizier et al., 1994). DNA sequence analysis demonstrated that the mutated helicase genes differed from the wild-type AcMNPV helicase gene only by few amino acid substitutions (Maeda et al., 1993; Croizier et al., 1994). Coinfection of wild-type AcMNPV and BmNPV prevents BmNPV growth in BmN cells and indicated that the AcMNPV helicase gene has an inhibitory effect on BmNPV growth in BmN cells (Kamita and Maeda, 1993). Although the roles of DNA and RNA helicases are associated with the unwinding of DNA and RNA duplexes, other activities have been attributed to helicases (Maeda et al., 1993). In fact, several other viral proteins that exhibit helicase activity have also been shown to induce cytotoxicity (Maeda et al., 1993).

The AcMNPV *p35* gene, whose protein product suppresses virus induced apoptosis in SF21 cells, also seems to be implicated in host range determination (Clem et al., 1991). The involvement of *p35* in host range has been inferred from analysis of AcMNPV *p35* deletion mutants in SF21 cells, a cell line derived from *Spodoptera frugiperda*, and TN368 cells, a cell line derived from *Trichoplusia ni.* Levels of viral DNA replication, as well as late and very late gene expression were greatly reduced in SF21 cells infected with AcMNPV *p35* deletion mutants. However, when TN368 cells were infected, levels of replication, and both late and very late gene expression remained unaltered (Hershberger et al., 1992). The effects observed with the AcMNPV *p35* mutants in SF21 cells are compatible with the processes associated with apoptosis, such as degradation of intracellular DNA and premature cell lysis (Hershberger et al.,

1992). It is likely that in TN368 cells, another AcMNPV gene prevents virus induced apoptosis. *in vivo* experiments demonstrated that while the *p35* deletion mutant exhibited greatly reduced infectivity to *Spodoptera frugiperda* larvae, infectivity to *Trichoplusia ni* larvae was not altered (Clem and Miller, 1993). The *p35* gene, therefore, may also to play a role in AcMNPV host range determination in nature.

The cell specific activity of the AcMNPV *p35* gene has also been demonstrated by transient replication assays (Lu and Miller, 1995). Cotransfection of cultured cells with clones encompassing regions of the AcMNPV genome have demonstrated that the *p35* gene is essential for viral DNA replication in SF21 cells but not in TN368 cells (Lu and Miller, 1995). Similarly, it was demonstrated by transient replication assays that the AcMNPV *ie-2*, *dnapol*, and *lef-7*, which are required for AcMNPV DNA replication in SF21 cells, are not critical for viral DNA replication in TN368 cells (Lu and Miller, 1995). On the other hand, a gene that corresponds to the AcMNPV ORF 70 turned out to be required for AcMNPV late gene expression and DNA replication in TN368 cells but not in SF21 cells (Lu and Miller, 1995). This gene has been named *hcf-1*(host cell-specific factor-1) (Lu and Miller, 1995).

After analyzing the expression of AcMNPV early, late and very late promoters in nonpermissive cell lines, Morris and Miller (1992) suggested that prevention of productive infection is unique for each cell line. The identification of genes that are required for proper infection in specific cell lines corroborates the view of diverse mechanisms of host range determination in cultured cells. Although direct conclusions relating to viral infectivity of insect larvae can not be drawn exclusively from studies of cultured cells, these studies contribute to the knowledge of factors that influence productive infection and virulence. The observations that AcMNPV *p35* mutants have their infectivity impaired in *Spodoptera frugiperda* larvae and that AcMNPV *helicase* gene mutants grow in *Bombyx mori* larvae indicate that the identification of genes that affect host range in cultured cells may pave the way for better understanding of *in vivo* infection process.

PRACTICAL APPLICATIONS OF BACULOVIRUSES

Use as biological control

One of the main attributes that makes baculoviruses attractive as pest control agents is their host-specificity. Because baculoviruses do not infect vertebrates, and thus are safe to humans and other animals, their use poses neither risks of contamination to those working in pest control nor risks of toxic residues accumulating in food and water supplies. Moreover, as baculoviruses in general infect only a few related species of insects, they can be used without disturbing potentially beneficial insects. The ability to infect and kill only related species makes baculoviruses particularly suitable for integrated pest management (IPM) programs. The goal of IPM is to control pests that exceed economic damage threshold without affecting beneficial organisms that can continue to check other potential pests. As a consequence, secondary outbreaks of pest insects observed when broad-spectrum pesticides are used can be avoided. Another positive aspect of baculoviruses in pest control is that they can multiply in their hosts and keep the pests at low level for years making additional control unnecessary.

The first recorded use of baculovirus in pest control seems to have occurred before the second World War when NPVs were accidentally introduced in eastern Canada with parasitoids brought from Scandinavia to control the European spruce sawfly, *Gilpinia hercyniae* (Cunningham, 1988). The first two recorded deliberate applications of baculoviruses in pest control took place in California in 1949 to control the alfalfa caterpillar, *Colias philodice*, and in Ontario in 1950 to control the european pine sawfly, *Neodiprion sertifer* (Cunningham, 1988). Several experimental field trials using baculovirus as pesticides were done in the 1950's and 1960's (Wood and Granados, 1991)

The first baculoviruses insecticide registered in the United States was an NPV effective against *Heliothis* species on cotton, sorghum, soybeans, tobacco, and tomato. The commercialization of this product in the 1970s was a response to increased resistance of cotton pests to chemical pesticides available at the time. Although highly effective in the control of pests, the baculovirus preparation had low economic return for the manufacturers and could not compete commercially when pyrethroids became available.

There are several other examples of successful uses of baculoviruses in the

control of agriculture and forestry pests. For instance, baculoviruses have been successfully used to control the codling moth, *Cydia pomonella*, in apple and pears orchards. In Brazil, thousands of hectares of soybean fields have been treated with AgNPV to control the soybean looper, *Anticarsia gemmatalis*. In forestry, the NPV of *Neodiprion sertifer*, the European pine sawfly, is the most widely used baculovirus and the Douglas-fir tussock moth, *Orgyia pseudotsugata* has been successfully controlled in the USA and in Canada with applications of OpMNPV.

In spite of these successful examples, baculovirus application remains negligible compared to the use of chemical pesticides. One of the main obstacles to wider use of baculovirus is their remarkable level of host-specificity. While highly desirable from an ecological stand point, a pest control agent that is very selective is not very practical. For the user, specificity is a problem when more than one insect pest is present. After application of a baculovirus preparation, the appearance of another pest means that a new pest control method has to be used. For the pesticide producer, a viral insecticide that can be used against a single or a few species offers low returns compared to an agent that can be used against many pest species. Another difficulty is that baculoviruses often have to be applied in the early stages of larval development to be effective and thus correct application requires close monitoring of the development of pests in the field. The time required to kill the insects may be relatively long and, as the insect continues to feed after it becomes infected, and damage above economic threshold may occur. In summary, the combination of additional work, higher risks, and requirement of new set of skills for the farmers and low economic returns to the pesticide producer have been major barriers for widespread use of baculoviruses in modern agriculture.

Recombinant DNA technology and increased knowledge of baculovirus biology offers the opportunity for development of genetically modified baculoviruses that may be commercially more attractive than their wild-type counterpart. Several genes have been inserted in the genome of baculoviruses in order to increase their virulence. For instance, when a diuretic hormone isolated from the tobacco hornworm, *Manduca sexta*, was inserted in the genome of BmNPV the recombinant virus killed infected silkworm larvae 20% faster than the wild-type virus (Maeda, 1995). Reduction in the time required to kill insects was also observed with an AcMNPV recombinant which expresses an insect-specific neurotoxin (AaIT) gene isolated from the North African scorpion,

Androctonus australis Hector (Steward et al., 1991). Similarly, the ∂-endotoxin from the Gram-positive bacterium *Bacillus thuringiensis* has been inserted in the genome of AcMNPV (Merryweather et al., 1990). Although biologically active protein was produced by the recombinant virus, increased virulence was not observed (Merryweather et al., 1990). Another approach for the development of genetically modified baculovirus with increased virulence is the deletion of the ecdysteroid UDP-glucosyl transferase (*egt*) gene. The protein encoded by the *egt* gene interferes with hormonal regulation of host larval development resulting in blockage of molting (O'Reilly and Miller, 1989). As insects stop feeding for molting, the baculovirus *egt* seems to prolong the length of time the insect feeds. *Spodoptera frugiperda* larvae infected with an AcMNPV *egt* deletion mutant were killed faster and consumed less food in comparison to larvae infected with wild-type viruses (O'Reilly and Miller, 1991). The recombinant baculovirus that is presently commercialized by Cyanamid is an AcMNPV *egt* deletion mutant which expresses AaIT.

Recombinant viruses are commercially attractive and thus justify investments for their development as products. On the other hand, genetic alterations that increase host range and virulence could also make baculovirus less attractive from an environmental stand point. The environmental impact of recombinant baculoviruses with increased virulence or broad host range needs to be carefully examined.

Use as vector for expression of foreign proteins

Advances in biotechnology have allowed the production of foreign proteins in a number of organisms. This technology was first applied in prokaryotes, taking advantage of plasmids and bacteriophages as vectors, and was later developed for eukaryotic systems as well. In the later system, animal viruses such as polyoma virus, bovine papilloma virus, adenovirus, herpesviruses, vaccinia virus, and retroviruses have been used as vectors. Similarly, a system that uses baculoviruses for the expression of foreign proteins in insect cells has become widely used.

There are several characteristics that make baculoviruses excellent vectors for the expression of foreign proteins. Their safety to vertebrates is an important factor as recombinant baculovirus are unlikely to be pathogenic to vertebrate cells. The fact that baculoviruses have nonessential genes that are strongly expressed is another important factor as these genes can be substituted by genes encoding foreign proteins. In addition, their double-stranded genome is relatively easy to manipulate and their rod-shaped nucleocapsid can accommodate viral genomes with DNA inserts of up to 10 Kb. Finally, genetic markers can be used to select for recombinants and cell lines that support virus growth do not require special conditions and are easily available (Maeda, 1994).

A baculovirus transfer vector is a plasmid that carries a foreign gene flanked by sequences of a baculovirus nonessential gene. When intact viral genomic DNA and DNA from the plasmid transfer vector are cotransfected into insect cells, the homologous DNA in the plasmid and in the viral genome recombine by homologous recombination. As a consequence of this recombination event, the foreign gene that was flanked by the baculovirus DNA in the transfer vector is inserted into the viral genome. As the recombination rate between the transfer vector and the baculovirus DNA is usually very low, selection of the recombinant virus is a critical step. When the polyhedrin locus is used, the recombinant virus can be identified by plaque purification as occlusion-negative plaques. This procedure may be very difficult and several innovations have improved the selection process and the frequency of recombination. For instance, frequency of recombination can be improved simply by linearizing the baculovirus DNA before cotransfection with the transfer vector (Kitts et al., 1990). Kitts et al. (1990) reported that up to 30% of progeny viruses were recombinants when linearized AcMNPV DNA was used. More dramatic results have been reported with an AcMNPV derivative

which had unique restriction sites inserted flanking an essential gene located in the proximity of the polyhedrin gene (Kitts and Possee, 1993). Cotransfection of viral DNA that had the essential gene deleted with a vector which replaced the missing gene yielded almost 100% recombination efficiency (Kitts and Possee, 1993).

PHYLOGENY AND EVOLUTION

An hypothesis for the evolutionary history of baculoviruses based on their association with invertebrates has been proposed by Rohrmann (1986). The variety of phenotypes observed in baculoviruses (GV, SNPV, and MNPV) is an indication of their ancient relationship with invertebrates, which may date as far back as the appearance of Crustacea in the late pre-Cambrian. The fact that baculoviruses have been identified in both crustaceans and insects might be an indication that an insect-crustacea progenitor was already infected by baculoviruses. Another possibility is that a insect baculovirus cross-infected a Crustacea. In that case, the relationship between baculoviruses and Crustacea might be more recent than the appearance of the Crustacea. In any case, the current molecular data on the crustacean baculovirus is weak and further work is necessary to better characterize these viruses and their relationship to insect baculoviruses. Because MNPV appears to be restricted to the Lepidoptera, while SNPV has been observed in Crustacea, Diptera, Hymenoptera, and Lepidoptera, the progenitor baculovirus is believed to be an SNPV. Furthermore, baculovirus host range also suggests that when the hemimetabolous and holometabolous insects lines diverged, the virus was excluded from the hemimetabolous and may have maintained relationship only with certain groups within the holometabolous.

With the increased availability of genetic data, a much more complete analysis of the genetic relatedness among virus species in now possible. In baculoviruses, sequence information of the *polyhedrin* gene, the most conserved baculovirus gene, has been used to infer their phylogeny. Sequence data of the polyhedrin gene of 18 baculoviruses was used to construct a baculovirus phylogenetic tree (Zanotto et al., 1993). This analysis suggests that the NeseNPV, which infects the Hymenopteran suborder Symphyta (Sawflies), diverged from the Lepidopteran NPVs before the separation of NPVs from GVs (Zanotto et al., 1993). In this tree, the GVs and the NPVs form separate groups and the NPVs are clustered into at least two groups with distinct evolutionary rates. Group I NPVs include *Autographa californica* MNPV, *Bombyx mori* NPV, *Orgyia pseudotsugata* MNPV, *Agrotis segetum* NPV, *Galleria mellonela* MNPV, and *Anticarsia gemmatalis* MNPV. Group II NPVs include *Mamestra brassica* MNPV, *Panolis flamea* MNPV, *Spodoptera exigua* MNPV, *Spodoptera frugiperda* MNPV, *Orgyia pseudotsugata* SNPV, *Panolis flammea* MNPV, and *Lymantria dispar* MNPV. The fact that both NPV groups have

SNPVs and MNPVs is an indication that this morphological feature, single and multiple nucleocapsids per envelope, is not of evolutionary importance. Finally, the phylogenetic tree obtained suggests that there is no strict correlation between baculoviruses phylogeny and insect host phylogeny within the Lepidoptera (Zanotto et al., 1993). Another baculovirus phylogenetic analysis has been recently presented (Barret et al., 1995). In this study, most parsimonious analysis of the sequence data from the *egt* gene and protein of seven NPVs were used to construct a phylogenetic tree (Barret et al., 1995). Using the *egt* gene sequence of *Lymantria dispar* MNPV as an outgroup, this analysis yielded a phylogenetic tree similar to that obtained for the *polyhedrin* gene (Barret et al., 1995).

SpliNPV-B

S. littoralis (Lepidoptera: Noctuidae), the Egyptian cotton worm, is an important pest in Africa, Asia, and Mediterranean regions. In nature, larvae of *S. littoralis* are often infected with two distinct types of baculoviruses: SpliNPV-A and SpliNPV-B. Each of these NPVs is characterized by unique restriction endonuclease (REN) profiles with minor pattern variation within each group. Viruses from each type also differ in plaque morphology and growth characteristics (Cherry and Summers, 1985; Kislev and Edelman, 1982).

The first report that two distinct groups of NPVs infected *S. littoralis* was presented by Kislev and Edelman (Kislev and Edelman, 1982). These researchers collected larvae from 5 different locations in Israel and identified two different virus types with unique REN profiles. The isolates were called SpliNPV-D and SpliNPV-T. Later, Cherry and Summers (1985) analyzed the restriction endonuclease profile of NPVs collected from infected larvae in 21 different regions of Israel. They also observed the same two distinct virus types and named them SpliNPV-A (SpliNPV-T) and SpliNPV-B (SpliNPV-D). In cell culture, SpliNPV-A produces many small polyhedra per cell while SpliNPV-B (SpliNPV-D) produces few, large polyhedra (Cherry and Summers, 1985). Croizier et al. (1986) used REN pattern analysis to characterize 27 clones of NPVs isolated from *Spodoptera littoralis* larvae collected from Morocco, Egypt, and from a laboratory culture in Lyon (France). All variants identified were SpliNPV-B.

Kislev and Edelman (1982) showed by Southern blot hybridization that the genomes of SpliNPV-A and SpliNPV-B are not closely related. Hybridization studies also suggests that nucleotide sequence of SpliNPV-A is more similar to those of *S. frugiperda* NPV and *S. exigua* NPV than to the nucleotide sequence of the genome of *Heliothis armigera* NPV, *Heliothis zea* NPV, and *Autographa californica* NPV (Kislev, 1985). Southern blot analysis was also used to investigate the similarity between the genomes of SpliNPV-B and AcMNPV (Croizier et al., 1989) and demonstrated that, under low stringency conditions, several regions of the genome of AcMNPV hybridized to similar positions in the genome of SpliNPV-B (Croizier et al., 1989). A few regions, however, did not cross-hybridize even under low stringency conditions, and the segment that contains the AcMNPV *p10* gene, EcoRI-Q fragment located at 88.1 to 89.6 map units, hybridized to a fragment spanning from 13.2 to 19.9 map units of the genome of

SpliNPV-B (Croizier et al., 1989). These results indicate that the genomes of these two viruses are not closely related and that they do not exhibit an identical genomic structure in terms of gene location (Croizier et al., 1989).

Wild stock of baculoviruses isolated from diseased *Spodoptera littoralis* larvae and from larvae of the closely related species *S. litura* were analyzed by Maeda et al., 1990). *S. littoralis* larvae were collected in one location in Egypt and *S. litura* larvae were collected from four different locations in Japan. Virus isolates were obtained by plaque purification and classified into four groups (Groups I to IV) based on DNA REN profile and viral growth characteristic in several cell lines. Three isolates originating from wild stock of egyptian *S. littoralis* larvae and three isolates originating from wild stock of japanese *S. litura* were classified into Group II and displayed a REN profile similar to that of SpliNPV-B (Maeda et al., 1990). Several isolates originating from *S. litura* were classified into Group IV and these viruses displayed a REN profile similar to that of SpliNPV-A (Maeda et al., 1990). These results indicate that japanese *S. litura* larvae are infected by variants of both SpliNPV-A and SpliNPV-B. The extent of DNA similarity within and between the four Groups was analyzed by Southern blot hybridizations and confirmed that the genomes of viruses within each group were closely related. In addition, cross-hybridization among viruses of Group II (SpliNPV-B variants) and viruses of Group III demonstrated that their genomes are closely related (Maeda et al., 1990). No detectable similarity was observed between viruses of Group IV (SpliNPV-A) and viruses of the three other Groups.

The sequence of the SpliNPV-B polyhedrin gene was recently reported (Croizier and Croizier, 1994). The SpliNPV-B polyhedrin protein (predicted from the nucleotide sequence) consists of 249 amino acids and thus has one to four additional amino acids at the N-terminal compared to other polyhedrin proteins (Croizier and Croizier, 1994). The transcription initiation consensus of baculovirus late genes (TAAG) was identified 48 nucleotides upstream the start codon (Croizier and Croizier, 1994). Three identical 27 base repeats were identified in the 300 bases that precedes the start of the open reading frame. Within this region, the sequence "AGATAAAA" occurs eight times. The significance of this motif is not known but it contains an hexanucleotide motif that is recognized by an insect GATA-like protein (Krappa et al., 1992; Kogan and Blissard, 1994). The sequence of the *egt* gene of an SpliNPV-B isolate was also recently reported

(Faktor et al., 1995). The amino acid sequence of the putative polypeptide of the SpliNPV-B *egt* gene exhibits 38% identity with the putative egt polypeptide of AcMNPV and 44% identity with the putative egt polypeptide of LdMNPV (Faktor et al., 1995). The physical map of an SpliNPV-B variant isolated from Moroccan larvae (isolate M2) has been constructed using 8 restriction enzymes (Croizier et al., 1989).

In nature, the host range of SpliNPV-B includes at least two species of insects: *S. littoralis* and the closely related species *S. litura* (Maeda et al., 1990). In the laboratory, it has been shown that SpliNPV-B replicates in cell lines established from *S. frugiperda* (SF21) and *S. littoralis* (CLS-79), but does not grow in cell lines derived from *Trichoplusia ni* (TN-368) and *Bombyx mori* (BmN) (Maeda et al., 1990).

Work by Bensimon et al.(1987) suggests that SpliNPV-B infects two species of locust; the African migratory locust, *Locusta migratoria migratorioides*, and the desert locust, *Schistocerca gregaria* (Bensimon et al., 1987). Fazairy and Hassan (1988) investigations indicated that an SpliNPV isolate from Egypt infects the wood-dwelling termites, *Kalotermes flavicollis* (Fazairy and Hassan, 1988). Further investigations confirming SpliNPV infections in insects of the Order Orthoptera (locusts) and Isoptera (termites) have not been reported.

THESIS OUTLINE

In the second chapter of the thesis, the growth characteristics of SpliNPV-B in cultured *Spodoptera frugiperda* cells (Sf9) is presented. The virus titer produced in these cells was determined and the time of initiation of viral DNA replication was estimated.

The identification and characterization of the SpliNPV *lef-3* gene is presented in the third chapter. SpliNPV *lef-3* was identified from a cDNA library constructed with mRNA isolated from SpliNPV-infected Sf9 cells. The expression of the SpliNPV *lef-3* gene was characterized by Northern blot analysis and the site of transcription initiation was identified by ribonuclease protection assay and primer extension analysis. The *lef-3* gene was mapped in the SpliNPV genome and its nucleotide sequence was determined. The nucleotide and predicted amino acid sequences of SpliNPV *lef-3* were compared to the nucleotide and predicted amino acid sequences of the *lef-3* genes from AcMNPV and OpMNPV. An antisense assay was used to evaluate the importance of the SpliNPV *lef-3* gene product to very late gene expression.

In the fourth chapter, we used transient expression assays to investigate sequences that modulate the SpliNPV-B *lef-3* expression. The promoter region required for basal level of *lef-3* expression (the minimal promoter) in Sf9 cells was identified. A GATA motif and two TATA boxes were identified within the minimal promoter. A mutation was incorporated in the GATA motif and the effect of this mutation was evaluated by transient expression assays. The importance of the untranslated leader sequence (ULS) was investigated. Several deletions of the ULS were generated and the effect on transient transcription activity evaluated. Promoter elements of baculovirus were discussed.

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Chapter II: Replication of *Spodoptera littoralis* nucleopolyhedrovirus type-B in Sf9 cells

ABSTRACT:

The replication and growth of *Spodoptera littoralis* nucleopolyhedrovirus type B (SpliNPV-B) was investigated in Sf9 cells, a cell line derived from *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Our results indicate that in Sf9 cells SpliNPV-B replication began between 12 and 16 hours post infection (p.i.) and that the budded virus titer produced was 6.6 X 10^6 plaque forming unit (PFU) per ml. At 5 days p.i. the majority of infected cells displayed cytopathological effects (CPE) typical of virus infection but a low percentage (less than 10%) of the infected cells produced polyhedra.

INTRODUCTION

The family Baculoviridae is divided into two genera, the *Nucleopolyhedrovirus* (NPV) and the *Granulovirus* (GV), and encompass a large number of double-stranded DNA viruses that infect invertebrates. *Spodoptera littoralis* (Lepidoptera: Noctuidae), the Egyptian cotton worm, is an important insect pest in several regions of the world. Two baculovirus species (named SpliNPV type A and SpliNPV type B) have been identified from diseased *S. littoralis* larvae collected in the middle east and in Morocco (Cherry and Summers, 1985; Croizier et al., 1986). These NPVs have unique polyhedra morphology and their DNA produce distinct restriction endonuclease (REN) pattern (Cherry and Summers, 1985). SpliNPV-A and SpliNPV-B have also been isolated from diseased *S. litura* larvae collected in Japan (Maeda et al., 1990). In addition, NPVs that have relatively high levels of DNA similarity to SpliNPV-B but display unique REN profile were isolated from diseased larvae of japanese *S. litura* and were named SpliNPV-C (Maeda et al., 1990).

We have estimated the beginning of viral DNA replication, analyzed the production of budded viruses, and observed the cytopathological effects during the course of infection of SpliNPV-B in Sf9 cells. Our results demonstrate that in Sf9 cells SpliNPV-B replication begins between 12 and 16 hours following inoculation. The budded virus titer obtained was 6.6 X 10^6 PFU per ml. Cytopathological effects (CPE) were observed at 24 hr p.i. and polyhedra inclusion bodies (PIBs) were visible at 48 hr p.i..

MATERIALS AND METHODS

Viruses and cell culture

SpliNPV-B isolate M2 originated from *Spodoptera littoralis* larvae collected in Morocco and was obtained from G. Croizier (Croizier et al., 1989). After receiving polyhedra collected from infected larvae, we extracted the viral DNA and transfected it into Sf9 cells. The transfected cells produced polyhedra and released budded virus into the media. This budded virus was used as inoculum to build a stock of this NPV. Special care was taken to avoid infecting cells at high multiplicity of infection (m.o.i.) which can result in the development of few polyhedra mutants. Sf9 cells were obtained from the American Type Culture Collection. This cell line is derived from ovary tissue of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Cells were maintained in Grace's media with supplements as described by Summers and Smith (1987). Sf9 cells in culture flasks (75 and 175 cm^2) were infected with budded virus of SpliNPV-B at a m.o.i. of about 0.1 PFU per cell in order to build virus stocks. For experiments, cells in 6-well plates were inoculated with budded virus for two hours at an m.o.i. of 10 PFU per cell. After this period the inoculant was removed and fresh media added. In time course analysis, this point was 0 hr p.i.. Virus titer was determined by the end-point-dilution assay in 96-well microplates as described by O'Reilly et al. (1992) using supernatant collected 5 days p.i..

Viral DNA extraction and dot blot analysis

Viral DNA used in these experiments was extracted from budded virus. Approximately 80 ml of media was removed from 175 cm^2 cell culture flasks 5 days p.i. and centrifuged at 1,000 x g for 10 minutes to remove cell debris. The supernatant was transferred to Oakridge tubes and centrifuged at 15,000 x g for 30 minutes at 4 °C. The pellets were resuspended in 400 µl of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) with Proteinase K (0.4 mg/ml) and sodium lauryl sarcosine (0.5%) and incubated overnight at 50 °C. The next day, the solution was extracted with one volume of equilibrated phenol:chloroform, followed by a chloroform extraction. The aqueous phase was transferred to a Centricon-100 tube (Amicon Corporation) and centrifuged in a fixed angle rotor at 2,300 x g until the volume of the solution was reduced to about 50 µl. The DNA was washed twice by adding 1.5 ml of TE to the Centricon tubes followed by centrifugation at 2,300 x g until the volume of the solution was reduced to about 50 µl.

The concentration of DNA was determined by fluorometry (TKO 100 mini-fluorometer, Hoefer Scientific Instruments).

A dot blot hybridization assay was used to estimate the beginning of viral DNA replication. Dot blot analysis was performed with DNA extracted from SpliNPV-infected Sf9 cells at a m.o.i. of 10 PFU per cell. Infected cells were removed from tissue culture flasks at several times p.i., centrifuged, the supernatant was discarded, and the cells lysed in 400 µl of Proteinase K (0.4 mg/ml) and SDS (2%) solution. The DNA was then extracted following the same procedure described above and applied to a nylon membrane (Nytran, Schleicher and Schuell, Inc.) using a manifold dot blotting apparatus (Hybri-dot manifold, Bethesda Research Laboratories). Approximately 5 µg of DNA was diluted in 100 µl of distilled water, heat denatured, and cooled in ice for 5 minutes before it was added to the wells of the dot blot apparatus. Denaturing solution (0.5 M NaCl; 0.5 M NaOH) and neutralizing solution (0.5 M Tris-HCl pH 7.2; 1.5 M NaCl; 1 mM Na2EDTA; pH 7.2) were subsequently added to the membrane following the manufacture's instructions (Schleicher and Schuell, Inc.) . The membrane was dried in a 65°C oven and the DNA fixed by UV crosslinking. Approximately 20 ng of gel purified SpliNPV DNA was radiolabelled with [α-^{32}P] dCTP using a PCR incorporation method (TAG-IT, Bios Laboratory, New Haven, CT, USA) and used as probe in dot blots. Hybridization was performed at 65°C overnight in 5 x SSPE (1.8 M NaCl, 50 mM Sodium phosphate, 5 mM EDTA pH 7.7), 5 x Denharts (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.5% SDS, 50 µg/ml denatured herring sperm DNA, and ^{32}P-labelled probes, following a 4 hour prehybridization without probe. After hybridization, blots were washed twice with 2 x SSPE, 0.1% SDS for 15 minutes at room temperature, and twice with 1 x SSPE, 0.1% SDS at 65°C for 20 minutes. Results were visualized by exposure of Kodak films (X-OMAT AR) to the blots.

RESULTS AND DISCUSSION

The HindIII REN profile of DNA extracted from our SpliNPV-B stock (Fig. 1) appears to be identical to that published by Dr. Croizier for SpliNPV isolate M2 (Croizier et al., 1989). The result of the titer of our stock was 6.6 X 10^6 PFU per ml. This titer is similar to that obtained by Maeda and coworkers (1990) when investigating the growth characteristics of a SpliNPV-B variant in SF21 cells. The budded virus concentration of SpliNPV-B produced in Sf9 cells was approximately 100 fold lower than that of AcMNPV, the type species of the Nucleopolyhedrovirus (our unpublished results).

One important characteristic of NPVs is the existence of three phases of viral gene expression (early, late, and very late phases) in which different sets of viral genes are expressed. Because the shift from the early to the late phase is marked by the onset of viral DNA replication, the time when viral DNA synthesis begins is an important factor when studying the molecular biology of a NPVs. In this study, we have used a dot blot procedure to determine the time at which replicated SpliNPV-B DNA can be detected in Sf9 cells. Our results indicated that replicated SpliNPV-B DNA can be detected by 16 hr p.i. suggesting that replication initiated between 12 and 16 hr p.i. (Fig. 2).

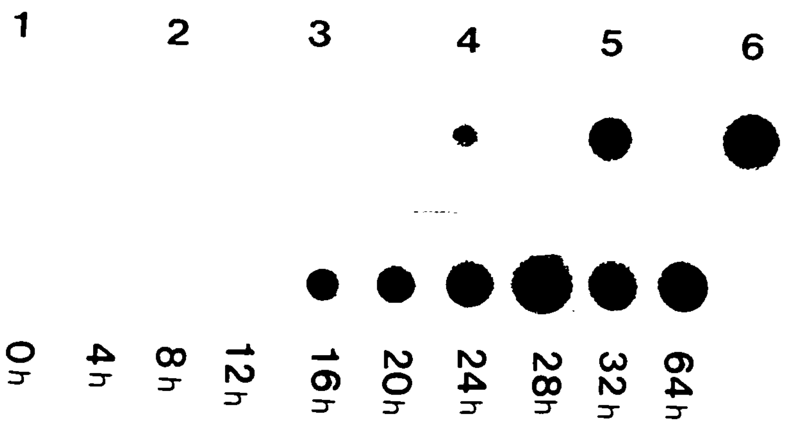
Sf9 cells infected with SpliNPV-B displayed CPE at 24 hr p.i. and PIBs were detected at 48 hr p.i. (Fig 3a, b, and c). At 5 days p.i., only a small proportion of the infected cells (less than 10%) had PIBs. The number of PIBs per cells was usually between 1 to 4 (Fig 3c). Previous investigations have also shown that the percentage of SpliNPV-B-infect cultured cells that contains PIBs is low (Kislev, 1986).

FIGURES

Fig. 1 HindIII REN profile of SpliNPV-B isolate M2. SpliNPV DNA was extracted from budded virus, digested with HindIII, and subjected to agarose gel electrophoresis. Letters on the right indicate the HindIII fragment named according to size. Numbers on the left indicate the size of bands from the Kb ladder.

Hind III
A
B
C-D
E-F
G
H
I
kb
12·2
8·1
6·1
5·1

Fig. 2 Replication of SpliNPV-B DNA. A dot blot assay was used to estimate when replication of SpliNPV-B begins in Sf9 cells. Total DNA was extracted from SpliNPV-infected cells at several times p.i. Bottom row: approximately 5 μg of extracted DNA was used for each time point as indicated under the blots. Top row: increasing amounts of SpliNPV DNA extracted from budded virus was transferred to the membrane as control (position 1 = no DNA; position 2 = 10 ng; position 3 = 50 ng; position 4 = 100 ng; position 5 = 250 ng; position 6 = 500 ng).



1
2
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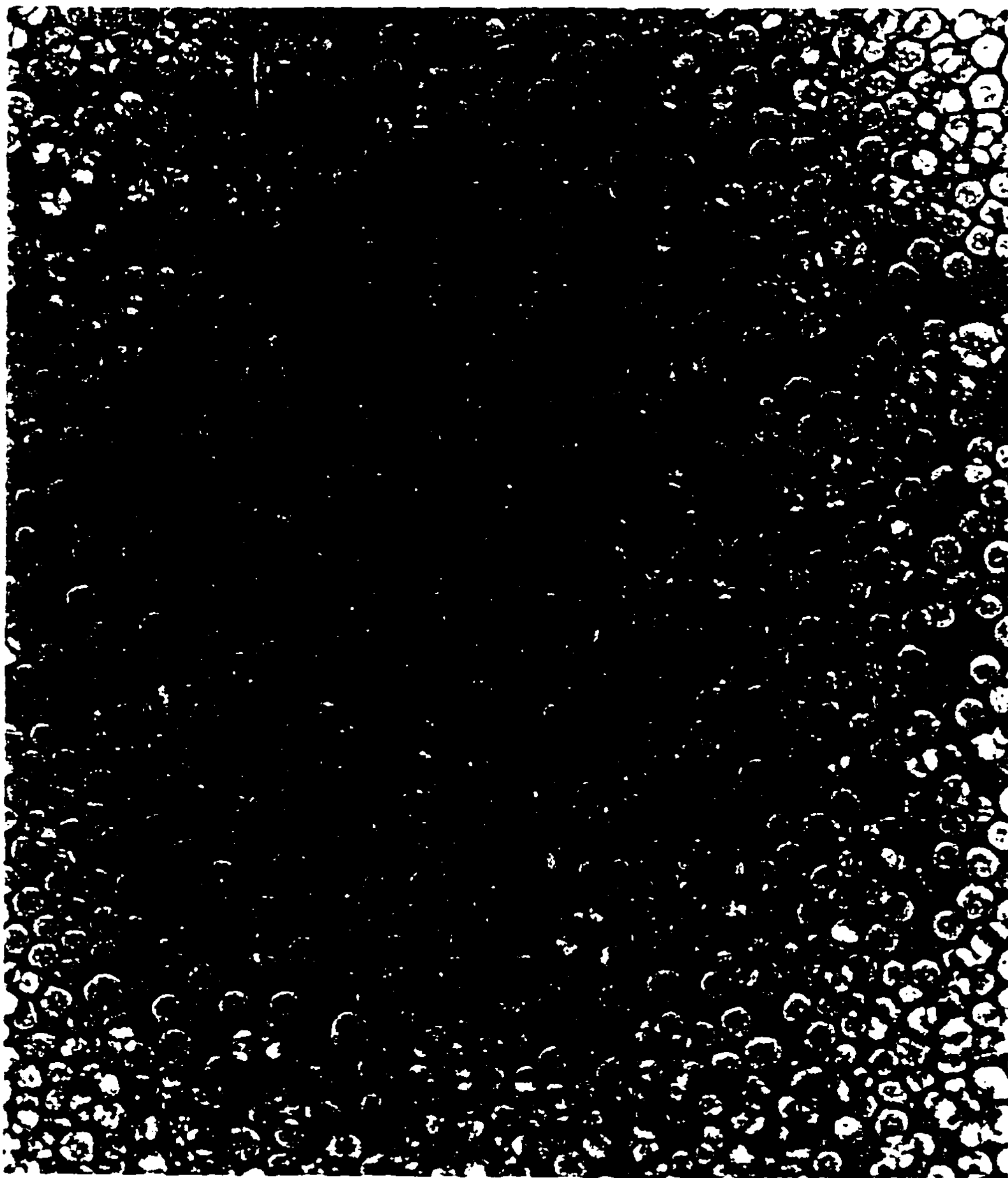
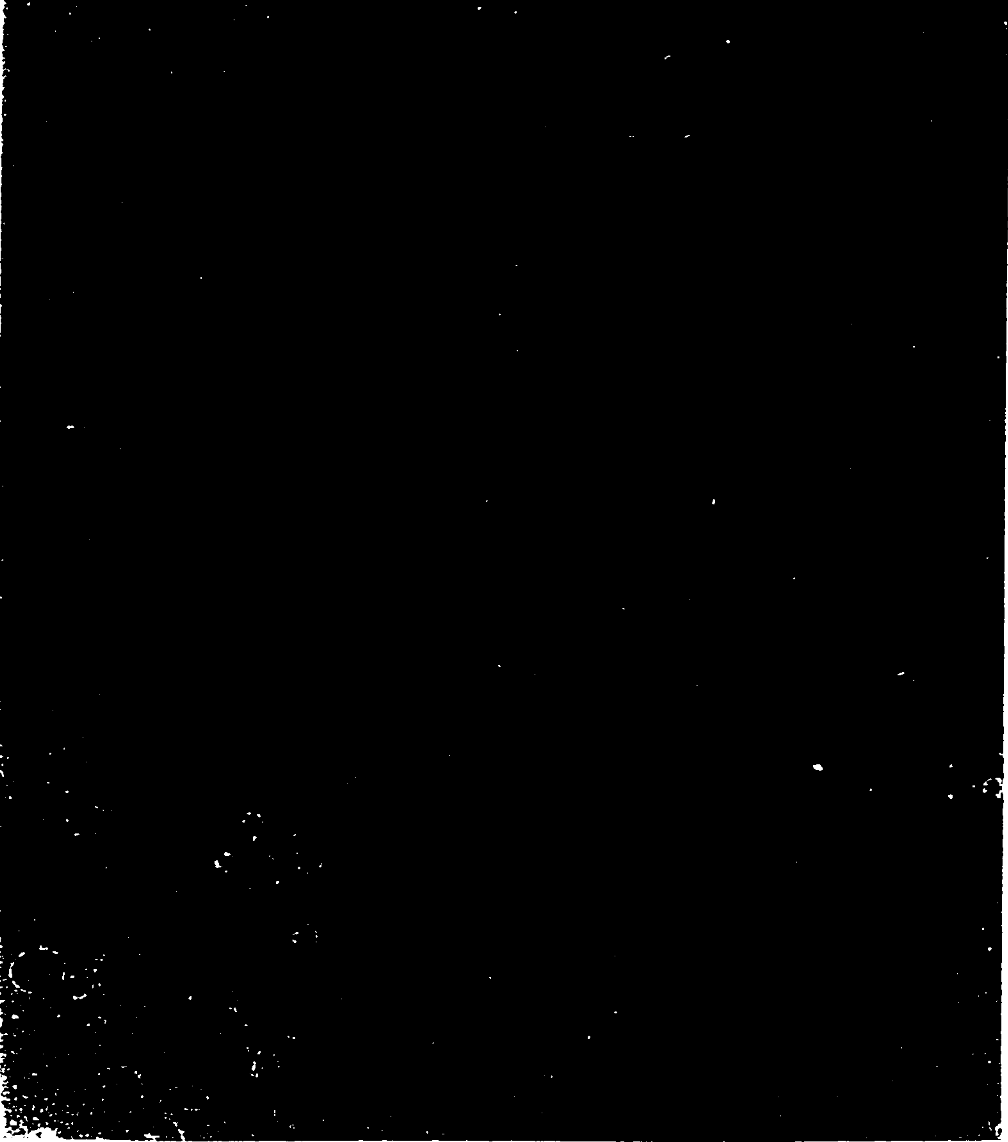
24h
28h
32h
64h

Fig. 3 Sf9 cells infected with SpliNPV-B. (a) uninfected cells; (b) at 24 hours p.i. cells show distinct CPE but no PIBs (arrow indicate cells with CPE); (c) PIBs are observed at 48 hours p.i. (arrow indicate cells with PIBs).

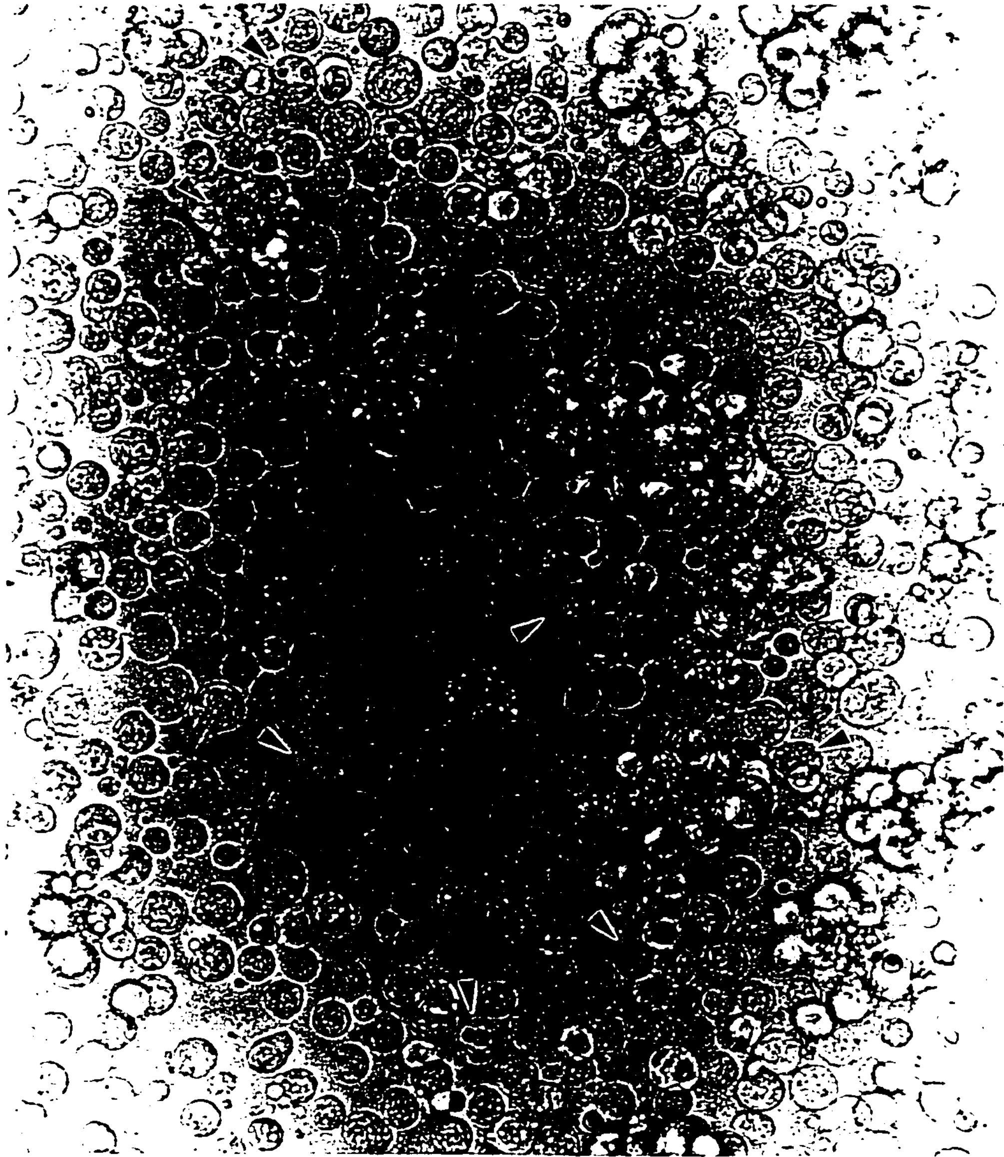
a



b



c



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Chapter III: Identification, sequence, and transcriptional analysis of the Spodoptera littoralis nucleopolyhedrovirus *lef-3* gene

ABSTRACT

We have identified a gene from the *Spodoptera littoralis* nucleopolyhedrovirus type B (SpliNPV-B) with several characteristics that indicates it is homologous to the *lef-3* genes *Autographa californica* nucleopolyhedrovirus (AcMNPV) and *Orgyia pseudotsugata* nucleopolyhedrovirus (OpMNPV). We named this gene SpliNPV-B *lef-3* and mapped its location to 43.6 to 45.5 map units of the SpliNPV-B genome. Nucleotide sequence analysis of this genomic region revealed a major open reading frame encoding a polypeptide of 40.82 Kda. Northern blot analysis showed that SpliNPV-B *lef-3* mRNA was expressed as a 1.6 Kb transcript at 5 hours post infection (p.i.), reached high levels at 24 hours p.i., and remained highly expressed at 56 hours p.i.. We observed that transcription of SpliNPV-B *lef-3* initiated at two distinct sites downstream of two TATA boxes, and that transcription terminated at a polyadenylation site located 9 nucleotides downstream of the putative LEF-3 termination codon.

INTRODUCTION:

Baculoviridae is a large family of double-stranded, circular DNA viruses that infect invertebrates (Blissard and Rohrmann, 1990). The *Spodoptera littoralis* nucleopolyhedrovirus (SpliNPV-B) infects the Egyptian cotton worm, *Spodoptera littoralis*, an insect pest of economic importance in the Mediterranean region, Africa, and Asia (Cherry and Summers, 1985). SpliNPV-B variants have been isolated from diseased *S. littoralis* larvae collected in Israel, Egypt, and Morocco, and also from diseased *S. litura* larvae collected in Japan (Cherry and Summers, 1985; Croizier et al., 1989; Maeda et al., 1990). In this manuscript, we describe the identification and characterization of the SpliNPV-B *late expression factor-3 (lef-3*) gene.

In NPVs, gene regulation occurs in a cascade mode with three major phases of gene expression: early, late, and very late. In the early phase, baculovirus genes are transcribed by host RNA polymerase, and some of these genes have promoters that are strongly stimulated by viral encoded proteins (Carson et al., 1988; Huh and Weaver, 1990; Blissard and Rohrmann, 1990; Glocker et al., 1992). The late phase starts with the onset of viral DNA replication, and late genes are transcribed by an α-amanitin resistant, virus-induced RNA polymerase (Huh and Weaver, 1990; Beniya et al., 1996). Genes of the very late phase are also transcribed after viral DNA replication, but they continue to be highly expressed even at very late times post infection (Blissard and Rohrmann, 1990). The *late expression factor (lef)* genes encode products that stimulate transient expression from late and very late gene promoters (Todd et al., 1995).

Because early genes are critical for viral DNA replication and some early genes seems to play an important role in host range determination, we are interested in characterizing SpliNPV-B early genes. For this purpose, we constructed a cDNA library of mRNA extracted from SpliNPV-infected *Spodoptera frugiperda* cells (Sf9) in the early phase of infection and randomly selected a SpliNPV-specific clone from this library for further analysis. We confirmed the expression of the mRNA from the selected clone and determined the nucleotide sequence of the gene and flanking regions. Analysis of the sequence obtained revealed a major open reading frame (ORF) coding for a putative polypeptide of 349 amino acids with a molecular mass of 40.82 kDa. Search of protein databases using the BLAST network service (Altschul et al., 1990) indicated that

this putative polypeptide had several regions of similarity to the putative polypeptide encoded by the *lef-3* genes of AcMNPV and OpMNPV, but no significant homology to any other sequence in the database. Several other observations led us to conclude that the gene we had isolated was the SpliNPV-B *lef-3* homologue.

Li et al. (1993) identified the AcMNPV *lef-3* gene following the observation that AcMNPV late (capsid protein gene *vp39*) and very late (*polyhedrin* gene) promoters are not active when Sf9 cells cotransfected with an AcMNPV genomic library lacking the fragment spanning from 43 to 48 map units (mu). They identified a gene in this region that restores late and very late promoter activity and this gene was called *lef-3* (Li et al., 1993). Later, Kool et al. (1994) demonstrated by transient complementation assays that the *lef-3* gene product is essential for AcMNPV DNA replication. As late and very late genes are not expressed before viral DNA replication, it is not known whether LEF-3 has any direct function related to the expression of late and very late genes. Results obtained by Hang et al. (1995) suggest that AcMNPV LEF-3 is a single-stranded DNA binding protein (SSB). Recently, Ahrens et al. (1995) have shown by transient complementation assay that the product of the OpMNPV *lef-3* gene is essential for OpMNPV DNA replication.

We mapped the SpliNPV-B *lef-3* gene to position 43.6 to 45.5 mu of the SpliNPV-B genome, a position that is almost identical to the genomic location of the AcMNPV *lef-3* gene (Li et al., 1993). We characterized the expression of SpliNPV-B *lef-3* and used an antisense assay to evaluate whether the SpliNPV-B *lef-3* gene product has a role in late gene expression.

MATERIALS AND METHODS

Viruses and cell culture

SpliNPV-B isolate M2 was obtained from G. Croizier (Croizier et al., 1989) and propagated in *Spodoptera frugiperda* (Sf9) cells maintained in Grace's media with supplements as described by Summers and Smith (1987). Cells were propagated in monolayers in cell culture flasks (25, 75, or 175 cm^2) and were infected with budded virus of SpliNPV-B at a multiplicity of infection (m.o.i.) of 5 PFU per cell. Cells were infected for two hours. After this period, the inoculant was removed and fresh media was added. In time course analysis, this point was 0 hr post infection (p.i.). Mock-infected cells were treated with cell culture media that did not have budded virus. Inhibition of protein synthesis was accomplished by supplementing the media with cyclohexamide (100 µg /ml) from 30 minutes before inoculation until the cells were harvested.

Viral DNA extraction

Viral DNA used in these experiments was extracted from budded virus. Approximately 80 ml of media was removed from 175 cm^2 cell culture flasks 5 days p.i. and centrifuged at 1,000 x g for 10 minutes to remove cell debris. The supernatant was transferred to Oakridge tubes and centrifuged at 15,000 x g for 30 minutes at 4 °C. The pellets were resuspended in 400 µl of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) with Proteinase K (0.4 mg/ml) and sodium lauryl sarcosine (0.5%) and incubated overnight at 50 °C. The next day, the solution was extracted with one volume of equilibrated phenol:chloroform, followed by a chloroform extraction. The aqueous phase was transferred to a Centricon-100 tube (Amicon Corporation) and centrifuged in a fixed angle rotor at 2,300 x g until the volume of the solution was reduced to about 50 µl. The DNA was washed twice by adding 1.5 ml of TE to the Centricon tubes followed by centrifugation at 2,300 x g until the volume of the solution was reduced to about 50 µl. The concentration of DNA was determined by fluorometry (TKO 100 mini-fluorometer, Hoefer Scientific Instruments).

Labelling DNA and hybridization

DNA used as probe in colony hybridization, plaque hybridization, dot blots, Southern blots, and Northern blots were radiolabelled with [α-^{32}P] dCTP using a PCR incorporation method (TAG-IT, Bios Laboratory, New Haven, CT, USA). Hybridizations were performed at 65°C overnight in 5 x SSPE (1X = 0.36 M NaCl, 10 mM Sodium

phosphate, 1 mM EDTA pH 7.7), 5 x Denharts (1X = 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 0.5% SDS, 50 μg/ml denatured herring sperm DNA, and ^{32}P-labelled probes, following a 4 hour prehybridization without probe. After hybridization, blots were washed twice with 2 x SSPE, 0.1% SDS for 15 minutes at room temperature, and twice with 1 x SSPE, 0.1% SDS at 65°C for 20 minutes. Results were visualized by exposure of Kodak films (X-OMAT AR) to the blots.

cDNA library and selection of clones

Poly(A)+ RNA was isolated from SpliNPV-infected Sf9 cells at 8 hrs p.i. using an oligo (dT) column (Invitrogen Corporation, San Diego, CA, USA) and cloned into Uni-Zap XR phage vector according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). SpliNPV-specific cDNA clones were identified by plaque hybridization using SpliNPV-B genomic DNA as probe. In order to begin characterizing SpliNPV, we randomly chose one SpliNPV-derived clone for further investigation. The Uni-Zap XR vector has, within its genome, the sequence of a phagemid, pBluescript SK(-). The phagemid and the cDNA insert were removed from the phage genome by means of an *in vivo* excision procedure (Zap-cDNA synthesis kit manual, Stratagene). The clone we selected for investigation was named pBlue SK Sl-1.

Northern blot analysis

SpliNPV-infected Sf9 cells were harvested at several times p.i. and washed in cold phosphate-buffered saline (PBS). The mRNA was extracted in oligo(dT) cellulose according to the procedures of the Micro-FastTrack Kit (Invitrogen Corporation). Approximately 2 μg of mRNA was separated by electrophoresis in a 0.8% agarose, 0.7% formaldehyde gel (Tsang et al., 1993) and transferred to a Nytran membrane (Schleicher and Schuell, Inc.). RNA extracted from *E.coli* was loaded beside the samples in order to estimate the size of the isolated transcripts. Blots were hybridized at 65°C to radiolabelled SpliNPV-B cDNA gel purified after digestion of pBlue SK Sl-1 with KpnI and NotI (hybridization solution: 5 X SSC (1X = 0.15 M NaCl; 0.015 M NaCitrate, pH 7.0), 5 X Denharts, 0.1% SDS).

Mapping and sequencing the SpliNPV-B cDNA

Once the expression of the selected cDNA was established by Northern Blot, we identified the genomic location of the gene and determined the nucleotide sequence of the gene and flanking regions. For initial localization of the cDNA in the genome of SpliNPV-

B, we performed Southern blot analysis of total SpliNPV-B DNA digested with several restriction enzymes. The blots were then hybridized with labelled cDNA extracted from pBlue SK SI-1. The restriction enzymes BglII, BstEII, EcoRV, and XhoI cleaved the cDNA genomic region into fragments that were relatively small (< 4 Kbp) and thus suitable for cloning into plasmids. Samples of SpliNPV-B DNA were digested with XhoI, EcoRV, BstEII and BglII and cloned into pBluescript KS(+) (Stratagene, USA) using standard cloning procedures (Sambrook et al., 1989). The resultant SpliNPV-B genomic library was screened by colony hybridization using the selected SpliNPV-B cDNA as probe. Four positive clones were selected (pSI-BglII-4.8, pSI-EcoRV-2.4, pSI-EcoRV-20, and pSI-XhoI-35, pBstEII-J). Two of these clones (pSI-BglII-4.8 and pSI-EcoRV-20) were subjected to treatment with exonuclease III and S1 nuclease (Double-stranded Nested Deletion Kit, Pharmacia, Biotech) to generate nested deletion clones. The nested deletion clones were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977). The nucleotide sequence of both DNA strands was determined using pUC forward, M13 reverse, and custom designed primers. Oligonucleotides were end-labelled with [∂-^{32}P] ATP using T4 polynucleotide kinase following standard procedures (Ausubel et al., 1992).

Analysis of sequences and comparison with sequences available in databases

Sequences were assembled and analyzed with the aid of computer programs from the Lasergene Package (DNASTAR, Inc.). The gene sequence and putative ORFs were compared with those of Genbank using the BLAST network service program (Altschul et al., 1990). As the results of BLAST search strongly indicated that the putative product of the major ORF within the SpliNPV-B cDNA was homologous to the polypeptide encoded by the AcMNPV and OpMNPV *lef-3* genes, we started referring to the gene we identified as SpliNPV-B *lef-3*. Nucleotide and amino acid sequence alignments were performed using the Clustal W multiple sequence alignment program (Thompson et al., 1994).

The nucleotide data reported in this work was submitted to the GenBank nucleotide sequence database and was assigned the accession number U77619.

Primer extension analysis

Primer extension analysis was performed using a 21 base oligonucleotide (5'-ATTTCGATTACAACAGACCAA-3') complementary to the untranslated leader sequence

(ULS) of *lef-3* (position -182 to -161 relative to the first AUG codon). Approximately 100 ng of the oligonucleotide was end-labelled with [∂-^{32}P]ATP using T4 polynucleotide kinase according to standard procedures (Ausubel et al., 1992). Unincorporated radioactive nucleotides were removed by three rounds of ethanol precipitation in the presence of 0.4 M ammonium acetate. Incorporation of radiolabel was determined by scintillation counts, and a total of 2 x 10^6 Cerenkov counts of labelled primer was then hybridized to denatured mRNA extracted from SpliNPV-infected Sf9 cells. The labelled primer and 2.0 µg of mRNA were incubated in TE buffer at 37°C for one hour in the presence of RNAguard (Pharmacia). After hybridization, nucleotides, buffer, and reverse transcriptase were added to the reaction. The resulting reaction mixture was the following: 2 mM dNTPs, 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 10 mM dithiothreitol, and 100 units of M-MuLV reverse transcriptase. The reaction mixture was incubated for one hour at 37°C and then treated with phenol-chloroform. The cDNA was precipitated with ethanol, resuspended in 7.5 µl of TE buffer, and subjected to electrophoresis on a 6% polyacrylamide/7.7 M Urea sequencing gel.

Ribonuclease protection assay

Ribonuclease protection assay of the 5'-end of the *lef-3* transcript was performed using the XhoI/BglII fragment (position 1308 to 920 in our sequence) as probe. This fragment was cloned into XhoI and BamHI restriction sites of pBluescript KS(+) and linearized with XhoI. T7 RNA polymerase was used to synthesize a 430 nt RNA probe complementary to the mRNA of the selected cDNA. For 3'-end analysis, plasmid pA3.4 (a plasmid generated for sequencing analysis) was used as template for RNA synthesis. This plasmid was linearized with BstEII and T3 RNA polymerase was used to transcribe a probe of 362 nt complementary to the 3'-end of the *lef-3* transcript. RNA probes were prepared following standard procedures utilizing [α-^{32}P]UTP (Ausubel et al., 1992). Hybridization and RNase digestion were performed with a modified protocol of the Lysate Ribonuclease Protection Kit (Amersham Canada Limited). SpliNPV-infected Sf9 cells were lysed in a guanidine thiocyanate solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl) at a concentration of 1 X 10^7 cells per ml. RNA probe (10^6 Cerenkov counts) diluted in 5 µl of lysis solution was added to 45 µl of cell lysate and allowed to hybridize overnight at 48°C. The next day, the reaction was treated with RNase cocktail (final concentration: 20 units RNase A, 1 unit

RNase T1) for 30 min. at 37°C, followed by a proteinase K treatment (final concentration: 80 µg/ml) for 30 min. at 37°C. The reaction mixture was then extracted with phenol:chloroform and the nucleic acids were ethanol precipitated, resuspended in gel loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF), and analyzed in a 6% polyacrylamide/7.7 M Urea sequencing gel.

Construction of plasmids

Plasmid pAcIE-1, which contains the AcMNPV *ie-1* gene, was obtained from D. Theilmann (Theilmann and Stewart, 1991). Plasmid p39Q-, which contains the promoter region of the AcMNPV *39K* gene, was provided by L. A. Guarino (Guarino and Summers, 1986). Plasmid pSI-ph-luc has the luciferase gene under transcriptional control of the SpliNPV-B polyhedrin gene promoter and was provided by X. Liu (Liu, 1995).

We constructed plasmids that expressed the coding region of *lef-3* in the sense and antisense orientation under the control of the AcMNPV p39 gene promoter (Guarino and Summers, 1986). The plasmid pSI-EcoRV-2.4 was digested with BamHI and BglII yielding a fragment of about 1.2 Kbp encompassing most of the *lef-3* transcript (from position 1310 to position 2496). This fragment was cloned into the BamHI site of the plasmid p39Q- (Guarino and Summers, 1986). Plasmids that had *lef-3* in the sense and in the antisense orientation were selected based on the analysis of fragments generated by diagnostic restriction endonuclease digestions. The selected plasmids were called p39 sense and p39 antisense.

Transfections and luciferase assay

For the antisense experiment, 800 ng of SpliNPV-B DNA and 1 µg of pSI-ph-luc were cotransfected with 4 µg of either p39 sense, p39 antisense, or p39Q-. A total of 1.5 X 10^6 Sf9 cells per well were seeded in a 6 well plate (33 mm) and allowed to attach for 2 hours. We then performed cotransfection using the calcium phosphate coprecipitation procedure (O'Reilly et al., 1992). The Sf9 media was replaced with 1.0 ml of transfection buffer A (Grace's Medium with 10% FBS). Plasmid and viral DNAs were mixed with 1.0 ml of transfection buffer B (125 mM HEPES pH 7.1, 125 mM $CaCl_2$, 140 mM NaCl) and added dropwise to Sf9 cells. Cells were incubated at 28 °C for 4 hours after buffer B was added. The transfection buffer was then removed and replaced with Sf9 media. Cells were incubated for 48 hours at 28 °C.

To perform the luciferase assay, media was removed and the cells were harvested in 1 ml of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4). The cells were counted and pelleted in a microcentrifuge at 4 °C and 4,000 x g for 5 minutes. The pellet was resuspended in 400 µl of cell culture lysis reagent (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100, Promega Corporation), incubated for 15 minutes, and briefly centrifuged to pellet cell debris. A 20 µl aliquot of the cell extract was added to 100 µl of luciferase assay reagent (20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM Coenzyme A, 470 µM luciferin, 530 µM ATP, Promega Corporation) at room temperature. Light emission was measured in a scintillation counter and the amount of luciferase produced was determined from a standard curve generated using purified luciferase (Boehringer Mannheim).

RESULTS

Identification and mapping of an SpliNPV-B cDNA

We used plaque hybridization to identify SpliNPV-B clones from a cDNA library constructed after extracting mRNA from SpliNPV-infected Sf9 cells at 8 hrs p.i (data not shown). We randomly selected one of the clones that had cDNA of viral origin for further investigation. We mapped the cDNA of the selected clone to the BstEII-J fragment (position 43.6 and 45.4 m.u.) of the SpliNPV-B genome (Croizier et al., 1989) (Fig 1a).

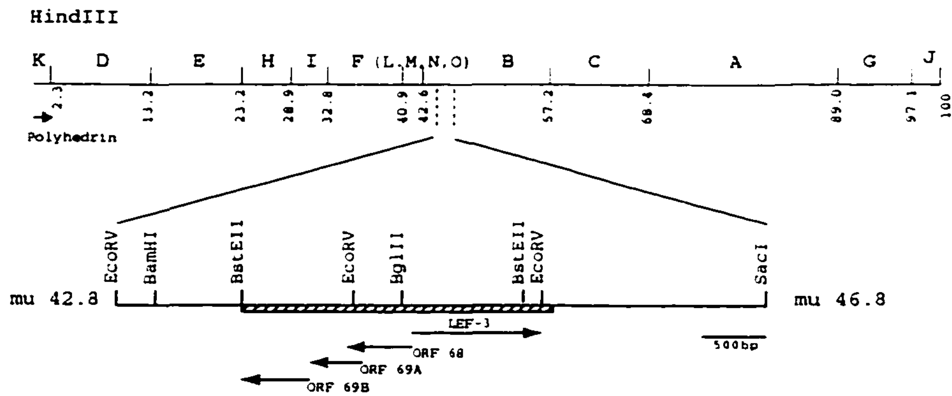
Analysis of the nucleotide sequence of the region spanning from 43.6 to 45.6 mu of the SpliNPV genome.

Both strands of the region spanning from 43.6 to 45.6 mu of the SpliMNPV -B were sequenced (Fig 1b). Computer analysis revealed a major ORF (position 1395 to 2440, Fig. 1b) that lies within the cDNA we selected from the cDNA library. This ORF encodes a putative polypeptide of 349 amino acids corresponding to a molecular mass of 40,820 daltons. Three other smaller ORFs were identified 5' of the major ORF (Fig 1a). Search of available protein sequence databases with the BLAST (Altschul et al., 1990) network service found several regions of homology between the amino acid sequence of the SpliNPV ORFs we identified and the amino acid sequences of ORFs from the genomes of AcMNPV and OpMNPV (Table 1). We, therefore, named the SpliNPV-B ORFs as LEF-3, ORF 68, ORF 69A, and ORF 69B to correspond to their AcMNPV counterparts (Fig. 1a). The major ORF we identified within the cDNA has several regions that displayed homology to the polypeptide encoded by the *lef-3* genes of AcMNPV and OpMNPV (Table 1). Blast search also showed that the ORF we identified 5' of the SpliNPV LEF-3 ORF has regions of homology to the putative product of the AcMNPV and OpMNPV ORF 68 (Table 1). We identified two other ORFs 5' of ORF 68 and named them ORF 69A and ORF 69B. Blast analysis showed that both of these ORFs displayed homology to the AcMNPV ORF 69 but no homology to OpMNPV sequences (Table 1). We used the TFASTA program (Pearson and Lipman, 1988) available at the Genome server at Eerie, France, to align the putative polypeptides encoded by the SpliNPV ORFs with sequences from AcMNPV and OpMNPV identified with Blast analysis. The SpliNPV LEF-3 protein showed 28.5% identity in 235 amino acid overlap with the N-terminal end of AcMNPV LEF-3 protein and 27.2% identity in 250 amino acid overlap with the N- terminal end of OpMNPV

LEF-3 protein. The SpliNPV ORF-68 showed 45.0% identity in 109 amino acid overlap with the N-terminal end of the AcMNPV ORF 68 and 38.5% identity in 122 amino acid overlap with the N-terminal end of OpMNPV ORF 68. The SpliNPV ORF 69A showed 34.7% identity in 101 amino acids overlap with the N-terminal end of AcMNPV ORF 69. The SpliNPV ORF 69B showed 52.8% identity in 72 amino acid overlap of the C-terminal end of AcMNPV ORF 69.

Fig. 1. Location, nucleotide sequence, and predicted amino acid sequence of the SpliNPV-B *lef-3* gene. (a) The SpliNPV-B *HindIII* physical map prepared by Croizier et al. (1989). The location of the *polyhedrin* gene is indicated (Croizier and Croizier, 1994) and an expanded view of the genomic region containing LEF-3, ORF 68, ORF 69A, and ORF 69B is shown below the map. We sequenced the DNA region indicated by the hatched box. (b) Sequence of the SpliNPV *lef-3* gene region from 43.6 mu to 45.6 m.u. The nucleotide sequence of the SpliNPV *lef-3* gene region and the predicted amino acid sequence of the SpliNPV LEF-3, ORF 68, ORF 69A, and ORF 69B are shown. Numbers on the left indicate the nucleotide position in the region sequenced. Numbers on the right indicate the amino acid position in the putative LEF-3 polypeptide. The distal and proximal transcription start sites are indicated with curved arrows above the sequence, the TATA motif is within a box, the polyadenylation signal sequence is double underlined, the GATA motif is indicated by asterisks (*) above the sequence, and restriction endonuclease cleavage sites are underlined (BstEII, Xho, and BglII).

a



b

```
1     GGTGACCATCTGGCGTGCTCGATTCGGATCTCGCTTTTGTCGTATCCGAAAAATTCAATCTTGCAACCGACGCACCGAGTACCGATGGAGCATTCATCCG
      BstEII                                                                                              G
101   AATCTTTTCGAAATCGATTTGGATTTGGCGGCTTTAGATCCTTTTCCAGTATATTTTTCATTATTGTTCAAAATTTTAAATAGCCTCTTCAATGAACTTA
       F  R  K  S  I  S  K  S  K  A  A  K  S  G  K  G  T  Y  K  E  N  N  N  L  I  K  F  L  R  K  L  S  S  I
201   TCTGTTTACGAGCAAATTTGATAGTGTGCAAGTTGACTGAGATCGACTCGAGTTTTTTGTCCAACCTGTTTTTACAAACCAGATACCGTTCAGAGTTGCA
                                                      XhoI
        Q  K  R  A  F  K  I  T  H  L  N  V  S  I  S  E  L  K  K  D  L  R  N  K  C  V  L  Y  R  E  S  N  C
301   TACTCGAGACGATTCCGGTTTGAATACATGGTACTCTTCAAAATTTTTAACAAAGTCCTCGAGCATATTTCTCGTGTCCGACTCGAACGTGTCAAAGATT
        XhoI                                                       XhoI
       V  R  S  S  E  P  K  F  V  H  Y  E  E  F  N  K  V  F  D  E  L  M  N  R  T  D  S  E  F  T  D  F  I
401   TTGACGACGCAATCTCCTCCGACCGACAGGCACGAAAGGATCAGTTGCATTCGCGACGTATCAGCGGCATCGTGAGAGACTCTTGCCTGTTCTCGTCTCC
       K  V  V  C  D  G  G  V  S  L  C  S  L  I  L  Q  M  <-- ORF69B
501   GGTCACGTCGAAACGCCGTCCGCGCACACAAAGTCGCATCGGTTTCCACATTTATTGACAATGTGTCGTACAACAGATTCCGACATTATGTCTCCCGAAC
601   CGTTGTCGTCGACGCCGTCGTCGCCGCCGTCACCACCATAAATTCTCTCGAATCTAGATCCGAATCGTTTGACTATTGACTTGTCGTAGTCTAGATGATT
                                                                                                    I  I
701   GCGCACGTGACACCGAAACCGAGCACGGATTCGCCGTTTACGGCGTTCAAATATCTCACAAACTGTCCCGGTCCGCCGCAAATGTCCAGATATGTTCGAA
       A  C  T  V  G  F  G  L  V  S  E  G  N  V  A  N  L  Y  R  V  F  Q  G  P  G  G  C  I  D  L  Y  T  R  I
801   TGTCGTCGCACACTTTAAACTTGTCGTTGATCTGTTTCAATTTGTAAAAACACCGCTGACCGGACAAGATGGTCGCAGATGATCTTTCCAGCCGCCGTCT
         D  D  C  V  K  F  K  D  N  I  Q  K  L  K  Y  F  C  R  Q  G  S  L  I  T  A  S  S  R  E  L  R  R  R
901   CGCCGACGATATCTCGTAGCTCGAGTAGTCGTCCAGCTTGGATCTCAAAGCGTCGAGTTCGCGTTGATATTTTTCAATCAGTCTGTCGGACGCCGTCGAC
                          XhoI
                                                                                   D  T  D  R  V  G  D  V
       A  S  S  I  E  Y  S  S  Y  D  D  L  K  S  R  L  A  D  L  E  R  Q  Y  K  E  I  L  R  D  S  A  T  S
1001  GCTGTAGCTTTCCGGTCGCCGCCGCCCGTCATGTTCGTATGTGTGGGCAGTAACGGCGGAAATTAATGCGATTAAAAGTGAACAGATAAAAGCGAGGAAG
        S  Y  S  E  P  R  R  R  G  D  H  E  Y  T  H  A  T  V  A  S  I  L  A  I  L  L  S  C  I  F  A  L  F
       A  T  A  K  R  D  G  G  G  T  M  <-- ORF69A
                     -> 5'      -> 5'
1101  ATATAAAGTGGAGTTATATTCAACGGCGCTACGACACGCGACGGTCTGTTCAACGCGACGTTAAAAAACTCACTGTTAATCAACAGATTCCGTTCGTTAA
       I  Y  L  P  T  I  N  L  P  A  V  V  R  S  P  R  N  L  A  V  N  F  F  E  S  N  I  L  L  N  R  E  N  I
1201  TCTCGTACACTATTGGTCTGTTGTAATCGAAATTTTCAAAATCGGCCCTGTTTATTGCCGTCCTGTACGAGACGTAATTTGGACTTGTATGCAATAAATC
         E  Y  V  I  P  R  N  Y  D  F  N  E  F  D  A  R  N  I  A  T  R  Y  S  V  Y  N  P  S  T  H  L  L  D
1301  TATCATCAGATCTTTCCACGCGTGCTCTCTGCTCTCGGGCGACACTTCCACCCGGTCCGAGTTTAATATTCGCCATCGAATTTTAGACATAAATATGAAG
             BglII                                                                      LEF-3 -->  M  K  2
       I  M  L  D  K  W  A  H  E  R  S  E  P  S  V  E  V  R  D  S  N  L  I  R  W  R  I  K  S  M  <-- ORF68
1401  CGTCAATTGAGCAACGGCGACGGCGGCAATCAAGAAAACAACGGCAGCGGCGTGCCTTTGAAGAAACCTGCCGCCGGATCGTATTCTATCAATTTCAAAG
        R  Q  L  S  N  G  D  G  G  N  Q  E  N  N  G  S  G  V  P  L  K  K  P  A  A  G  S  Y  S  I  N  F  K    35
1501  AGACTAAAGGTGAATTGATTGGCAAGAATATGATCAGTCTGAACAATGAACTTTTTTATATAATGAAATTTTTCATTGAAAACGGCAACAAAGACTATTA
       E  T  K  G  E  L  I  G  K  N  M  I  S  L  N  N  E  L  F  Y  I  M  K  F  F  I  E  N  G  N  K  D  Y  Y  69
1601  CGGCAACATGCAACAGTACACCAACATGGAGATTGGCAAGACTTACAAGATAAATTTAAAATATGCCAACCGTAGACTGATCATCGATCGGTACTCTGAG
        G  N  M  Q  Q  Y  T  N  M  E  I  G  K  T  Y  K  I  N  L  K  Y  A  N  R  R  L  I  I  D  R  Y  S  E  102
1701  GACAAAACCGTCGAGTTGCGCGTCGATGTCAAGGATCATTTGTTGTACGAAGACTTTATGGAAGATAATATTGTAACGATCGAGACGCAATTCTTGTGCG
        D  K  T  V  E  L  R  V  D  V  K  D  H  L  L  Y  E  D  F  M  E  D  N  I  V  T  I  E  T  Q  F  L  C  135
1801  GCTTTAGGCCCATTTTTACAAATTACATAAAATTTGTGTTCAACGTAAAGTACAAAAACGAAAGGGATCAGATGTCGATAATGCAAGTCGAATCGATTTG
        G  F  R  P  I  F  T  N  Y  I  K  F  V  F  N  V  K  Y  K  N  E  R  D  Q  M  S  I  M  Q  V  E  S  I  C  169
1901  TGATACATCTCGCGCCATGAACCTGTTTAACGTCGAAACGCATTCGGACCTGTTCAAGCGTATGATGGAGTTGGAGAAGAAATTTTTAAAATTGACCAGA
         D  T  S  R  A  M  N  L  F  N  V  E  T  H  S  D  L  F  K  R  M  M  E  L  E  K  K  F  L  K  L  T  R  202
2001  GTCAAATGTGTCAACAACAAGAACAACTTTAAAAATTTATCTTTTCTGGAAATGAGCAAGATTGAAGTGACCGAAGACTGCGAGACGCCGCCGTTTGATT
         V  K  C  V  N  N  K  N  N  F  K  N  L  S  F  L  E  M  S  K  I  E  V  T  E  D  C  E  T  P  P  F  D  235
2101  GCGACGATTTTGTCGGCAATATAAGTCGAAACAACAAGCAGATATATCACGCCAAAGTGACCAGCTTTAAGGTCAACACGATGTCTAATAACAATATTAA
       C  D  D  F  V  G  N  I  S  R  N  N  K  Q  I  Y  H  A  K  V  T  S  F  K  V  N  T  M  S  N  N  N  I  K  269
2201  AATTGTGTATAGAGTTGAAGACTACGATCAGGACATTAACGGTGTGATATTTTTGAACAATGATCGAAAAGACACAAAGAATAATGAATATGAAAAGGTG
         I  V  Y  R  V  E  D  Y  D  Q  D  I  N  G  V  I  F  L  N  N  D  R  K  D  T  K  N  N  E  Y  E  K  V  302
2301  CTGTTGGATCTCAACCAGACTGCGGACACGATCGGTGACCTGAACGACGTCTACATATACACGTCGAGCGATATAAACGGATATTATACCGTTTTAGGTC
                                       BstEII
       L  L  D  L  N  Q  T  A  D  T  I  G  D  L  N  D  V  Y  I  Y  T  S  S  D  I  N  G  Y  Y  T  V  L  G  335
2401  TAACTTGTTACGATTTGAGTAATTTTGAATATGTTCCCATATGATAATGTATTAATAAAAATACACCAATATTTTTTTTGTTTTTATTGATATCAAAAA
       I  T  C  Y  D  L  S  N  F  E  Y  V  P  I                                                            349
2501  GTCGGACAGGTTCGATTCGAACGTGTCGACCGAGTTGCGTTTAATTTTTTTAATTTAAAAATTTCAAGTCGATCGAAAAGTCGGACAGGTTCGATTCGAA
```

TABLE 1. Blast[a] search results showing regions of highest similarity between the predicted amino acid sequences of SpliNPV ORFs and ORFs from AcMNPV and OpMNPV

Amino acid position in the ORF				
SpliNPV	AcMNPV	OpMNPV	identities [b]	positives [c]
ORF-67[d]	ORF-67	ORF-67		
33-94	32-93	-	35%	59%
106-178	110-182	-	28%	49%
86-166	-	83-163	27%	45%
24-66	-	14-56	39%	53%
ORF 68	ORF 68	ORF 68		
4-85	63-144	-	50%	68%
90-111	150-171	-	36%	77%
4-85	-	5-86	43%	68%
90-112	-	92-114	30%	65%
ORF 69A	ORF 69	-		
60-99	38-77	-	50%	72%
ORF 69 B	ORF 69			
2-95	165-258	-	46%	64%

[a] Database search done with the Blast network service (Altschul et al., 1990) identified several regions in the genome of AcMNPV and OpMNPV which produced high scoring segment pairs with SpliNPV ORFs.

[b] Percentage of identical amino acids in the segment aligned.

[c] Positives refers to the sum of identical and similar amino acids which aligned in the segment. No gaps were created in the alignments

[d] ORF-67 corresponds to the putative coding region of the *lef-3* gene

Northern blot analysis

To investigate the temporal expression of SpliNPV-B *lef-3*, we performed Northern blot analysis with mRNA extracted from SpliNPV-infected Sf9 cells at several times p.i. (Fig. 2a and 2b). This analysis revealed that *lef-3* is expressed as 1.6 kb transcript at 5 hours p.i. The expression of this transcript increases until 24 hours p.i. and it is still expressed at high levels at 56 hours p.i.. Larger overlapping transcripts were observed at 24 hours and 56 hours p.i.. Inhibition of protein expression in infected cells by the addition of cyclohexamide to the media reduced expression of the 1.6 kb transcript and blocked transcription of the larger transcripts (Fig. 2c).

Transcriptional mapping of SpliNPV-B *lef-3*

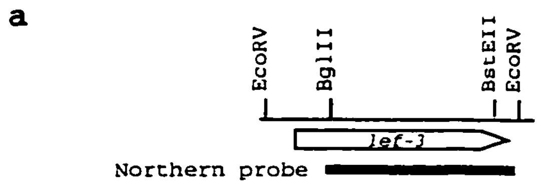
We performed the ribonuclease protection assay to estimate the location of *lef-3* transcription initiation and termination sites (Fig. 3). For 5'-end analysis, a radiolabelled RNA fragment of 432 nucleotides was hybridized to total RNA extracted from SpliNPV-B infected Sf9 cells (Fig. 3a). After RNase digestion, two major protected fragments of about 185 and 199 nucleotides were observed, indicating the existence of two transcription start sites located at approximately 286 and 272 bases upstream from the start codon of the LEF-3 ORF (Fig. 3b). These sites were named the distal and the proximal transcription initiation sites (Fig. 1b). Several larger protected fragments were detected at 12 and 24 hr p.i. suggesting that other transcription initiation sites located further upstream were also active. For 3'-end analysis, a 362 nucleotide RNA probe was hybridized to total RNA extracted from SpliNPV-infected Sf9 cells at 12 and 24 hr p.i. (Fig. 3a). After RNase digestion, a protected fragment of 137 nucleotide was observed (Fig. 3c) suggesting that transcription terminates at approximately position 2471. This places the 3' termini 25 nucleotides downstream the translation stop site and 11 nucleotides downstream the 3' processing signal (AATAAA). We also attempted to discover which transcription initiation sites were active when other viral encoded products were produced. For that purpose we performed ribonuclease protection assays with RNA harvested from SpliNPV-infected cells that had been treated with cyclohexamide. Two protected fragments of 199 nucleotides and 185 nucleotides were observed at 24 hr p.i. indicating that host factors alone were sufficient for transcription initiation at these sites (Fig 3d). Larger protected fragments were not observed under these conditions (Fig. 3d).

Primer extension analysis was performed to map accurately the 5' end of the *lef-3* transcripts using a 21-nt oligonucleotide complementary to sequences 161 bases upstream the LEF-3 ORF. A 119-nt extension product was observed as a weak band at 12 hr p.i. and as a strong band at 24 hr p.i. placing the distal start site at the T located at position 1114 (Fig. 4). As SpliNPV-B replication initiates between 12 and 16 hr p.i. (Chapter II of this thesis), the position 1114 seems to be an early transcription start site. Several other extension products were observed at 24 hr p.i. Three extension products of approximately 104-nt, 105-nt, and 106-nt indicate that transcription also started around the C nucleotides located at position 1129, the proximal transcription start site. Larger extension products observed at 24 hr p.i. agree with the results obtained with Northern blot analysis and ribonuclease protection assay which indicated the presence of transcription start sites located further upstream the distal and proximal transcription start sites.

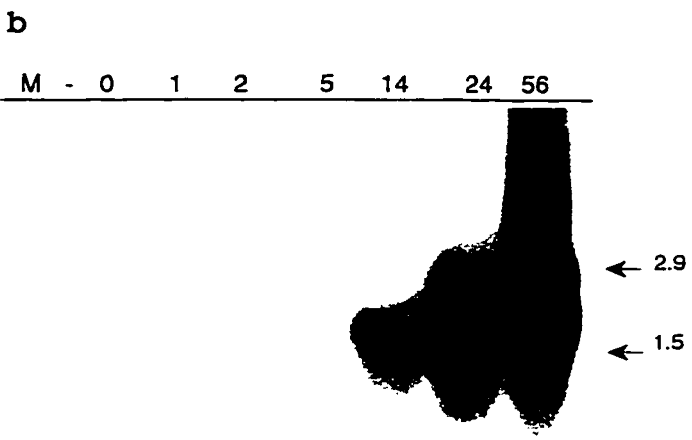
The above data indicate that *lef-3* encodes a major transcript of about 1.36 kb plus a poly(A) tail. This result is in agreement with the estimated 1.6 kb band detected in Northern blot analysis. Although larger overlapping transcripts were observed in Northern blot analysis at 24 hours and at 56 hours p.i., we did not attempt to characterize these transcripts.

Fig. 2. Northern blot analysis of SpliNPV-B *lef-3* transcripts. (a) Schematic representation showing the location of the DNA probe. (b) Northern blot of mRNA isolated from SpliNPV-infected cells (m.o.i.=10) or mock-infected cells (M). Cells were harvested at 0, 1, 2, 5, 14, 24, and 56 hours p.i. as indicated above the lanes. The numbers to the right of the blot refer to the size in kilobases of RNA size markers. (c) Northern blot analysis of mRNA isolated from cyclohexamide treated Sf9 cells. Mock-infected (M) and SpliNPV-infected cells (m.o.i. = 10) were treated with cyclohexamide. Cells were harvested at 5, 14, and 24 hours p.i. as indicated above the lanes. Lane C was loaded with mRNA extracted at 24 hr p.i. from SpliNPV-infected Sf9 cells not treated with cyclohexamide. The numbers to the right of the blot refer to the size in kilobases of RNA size markers.

a



b



c

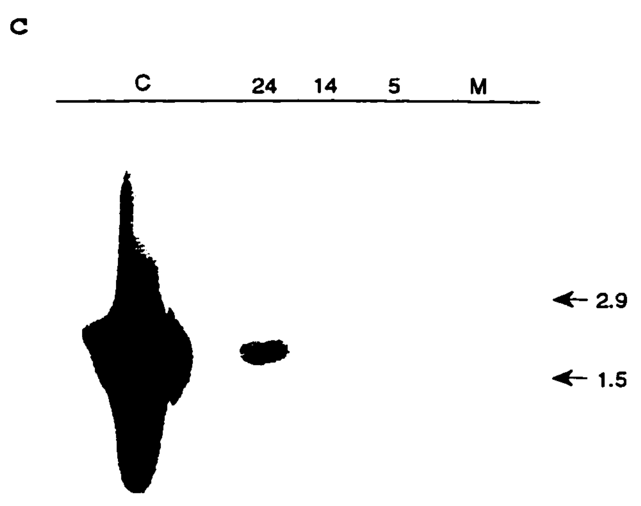


Fig. 3. Transcriptional mapping of *lef-3*. (a) Diagram showing the location of the DNA region used to generate RNA probes for ribonuclease protection assay. The arrow in the diagram represent the *lef-3* transcript (b) Mapping of transcription initiation sites of the SpliNPV-B *lef-3* mRNA. Autoradiogram showing the results of ribonuclease protection assay of mRNA extracted from mock-infected (M) and SpliNPV-infected Sf9 cells harvested at 12 and 24 hours p.i. as indicated above the lanes. The numbers on the left indicate the size of the protected fragments (199 and 185) and the size of the RNA probe (432). The size of the protected fragments was determined by comparison to the *lef-3* sequence shown on the right. (c) Mapping of the 3' end of the SpliNPV-B transcripts. Autoradiogram showing the results of ribonuclease protection assay of mRNA extracted from mock-infected (M) and SpliNPV-infected Sf9 cells. Cells were harvested at 12 and 24 hours p.i. as indicated above the lanes. Molecular weight markers were loaded on the first lane (L). The numbers to the left of the blot refer to the size in kilobases of DNA size markers. The number on the right indicate the estimated size of the protected fragment. (d) Mapping of *lef-3* transcription initiation in cells treated with cyclohexamide. Autoradiogram showing the results of ribonuclease protection assay of mRNA extracted from mock infected (M) and SpliNPV-infected Sf9 cells treated with cyclohexamide (C) and not treated with cyclohexamide (N). Cells were harvested at 24 hours p.i.. Numbers on the right indicate the estimated size of the protected fragments.

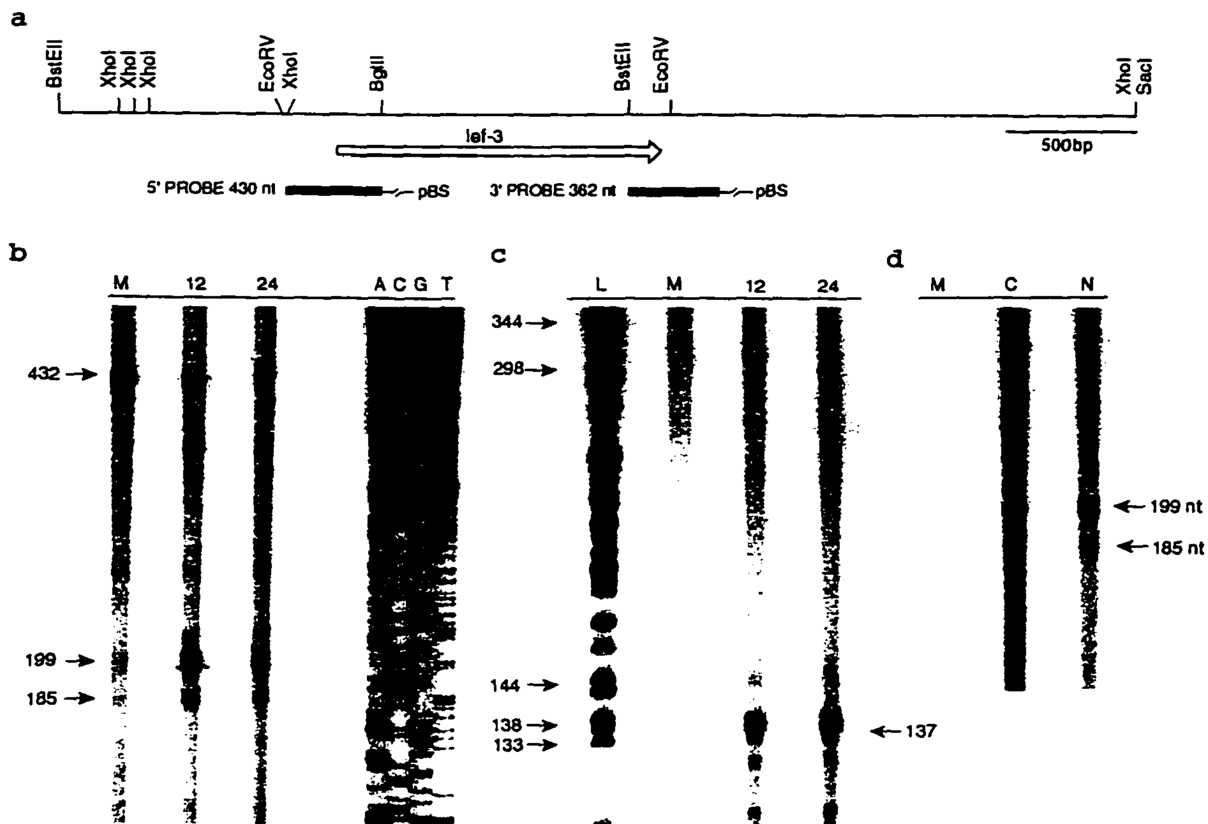
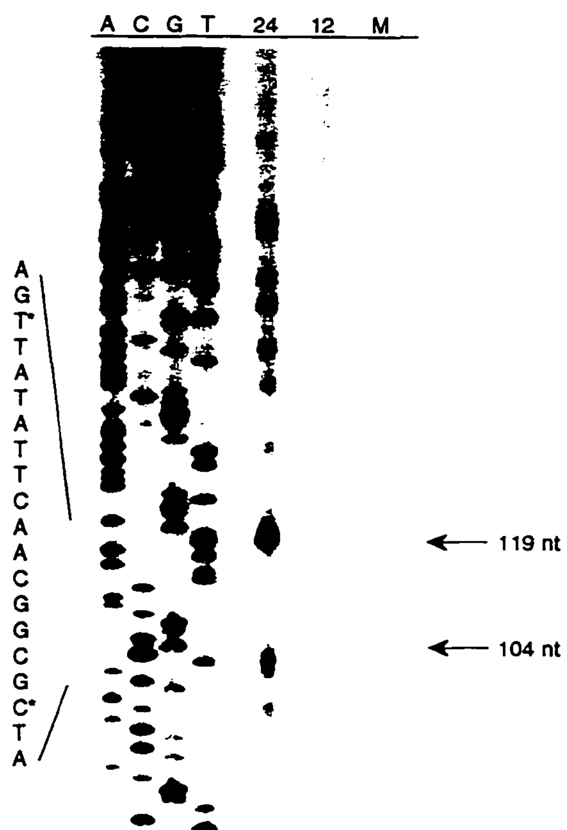

a
BstEII
XhoI
XhoI
XhoI
EcoRV
XhoI
BglI
BstEII
EcoRV
XhoI
SacI
lef-3
500bp
5' PROBE 430 nt
pBS
3' PROBE 362 nt
pBS
b
M
12
24
A C G T
432
199
185
c
L
M
12
24
344
298
144
138
133
137
d
M
C
N
199 nt
185 nt

Fig. 4. Primer extension analysis of SpliNPV-B *lef-3* transcripts. Autoradiogram showing the extension products obtained after hybridization of end-labelled oligonucleotide to mRNA extracted from mock-infected (M) and SpliNPV-infected Sf9 cells. Cells were harvested at 12 and 24 hours p.i. as indicated above the lanes. Sequencing reaction obtained with the same oligonucleotide used for primer extension analysis is shown. The distal and proximal transcription start sites are indicated with asterisks (*) in the sequence shown on the left of the picture. The numbers on the right indicate the estimated size of two extension products.



A C G T 24 12 M
A
G
T*
T
A
T
A
T
T
C
A
A
C
G
G
C
G
C*
T
A
119 nt
104 nt

Nucleotide and amino acid sequence comparison

Alignment of the SpliNPV-B LEF-3 protein and leader sequence with the LEF-3 protein and leader sequence of AcMNPV and OpMNPV is shown on figures 5a and 5b. Table 2 shows the percentage nucleotide identity between the ORFs and the ULSs of the three *lef-3* genes and the percentage identify between the entire length of the three putative LEF-3 polypeptides.

Effect of expressing the anti-sense *lef-3* transcript

To investigate the influence of SpliNPV *lef-3* on transcription from a very late promoter, plasmids expressing the *lef-3* transcript in the sense and antisense orientation were cotransfected with SpliNPV-B DNA and pSI-ph-luc. Transient expression from the polyhedrin promoter was evaluated as the amount of luciferase produced at 48 hours post transfection. Levels of luciferase produced in the presence of the antisense RNA were significantly lower ($\chi 2$, $p<0.01$) than levels produced in the presence of the sense RNA (Fig. 6).

Fig. 5. Alignment of the putative polypeptides and of the ULSs of the AcMNPV, SpliNPV, and OpMNPV *lef-3* genes. (a) Alignment of the predicted amino acid sequence of AcMNPV, SpliNPV, and OpMNPV LEF-3. The conserved SSB motif identified by Ahrens and coworkers (Ahrens et al., 1995) in the amino terminal regions of AcMNPV and OpMNPV LEF-3 is indicated with asterisks (*) below the sequences; (b) Alignment of the ULSs of AcMNPV, SpliNPV, and OpMNPV *lef-3* genes. Alignments were made with the Clustal W multiple alignment program (Thompson et al., 1994). Number on the top of the alignment indicate position within the alignment. Numbers on the left indicate position of the last amino acid or nucleotide in each line. Conserved amino acids and nucleotides are outlined within boxes.

a

```
          1             15 16            30 31            45 46            60 61            75 76            90
1 AcMNPV  -MATKRSLS-GESSG E-------RLIKRMA MASSPKKIRENMKRI SGKLMSKMTLSLDN- -EYHYTERIMSDNKI QEYYGDSCSFKLMDE    79
2 SlMNPV  ---MKRDLSNGDGGN QENNGSGVRLMKPAA G-----SYSINFKET KGELIGKNMISLNNE LFYIMKFFLENQN-- KDYYGNMCQYTNMEI    80
3 OpMNPV  MMAAKREH--ADDGA E-------PARKR-- -------VKENMKRV TGKLLSKMTISLENH LMYFFTERLINENKT EAYYGNLCCFKDLVE    72
                                                          *    *            *                *     **

          91           105 106          120 121          135 136          150 151          165 166          180
1 AcMNPV  GKCYDISLNYVKTKF SQMIQINEYKECEME IETATPMSDYLTNKH FENEDGVNIIVKYKF IYKKINSGLYKMVFE VMYKNLNDDPDVVQV   169
2 SlMNPV  GMTYKINLKYAN--- -RRLIIDRYSE-DKT VELRVDVKDHLLYED FMEDNIMTIETQFLQ CFRRIFTNYIKEVFN VKYKNERCDMSIMQV   165
3 OpMNPV  QECMDVSLNFVKTKY NERKEINEMSKCDAA IDENVAVKLCLTRAD EENEEIMNVLAKLKQ VEKRLGANNYKMVED INMQDAGGAVFVCDV   162
                    *    *

          181          195 196          210 211          225 226          240 241          255 256          270
1 AcMNPV  ECSVNAKTLINLFKN NIKGSDDINEVFKYL KDNENQIFTIYSIKC QQIFNGSNVYMNWNV VNSTFIELCEAKESE AYSNLQNCTNAKINI   259
2 SlMNPV  ESICDTSRAMNLENV ETHS-----DLFMRM MELEKKFLKLTRVKC VNN---KNNFKNLSF LEMSKIEVTEDCETP ----PFDCDDFVGNI   243
3 OpMNPV  ECFANLKVLASAAKA PVKSPDNFNSLMDFY YKNTNTLFYVHCVRC CHTSKGQNEFLNWTA GPSTSLETPSNTDNE DYINLVHS-HSTNNI   251

          271          285 286          300 301          315 316          330 331          345 346          360
1 AcMNPV  SRSNKHVASYNVNVL KSELEENDMGDNKFI VQFKSDELNIADSDD CSTSSDLGKWNKSVF YVNTN----KKTEAD SLQKLCADFNDISML   345
2 SlMNPV  SPNNKQIYHAKVTSF KVNTMSNNN--IKIV YRVEDYDCDING--- ------------VIF LNNDR----KDTKNN EYEKVLLDLNQIADT   312
3 OpMNPV  SPANKHLKSMQLSLE KAEQKINDNGKHSFS VQFKT--LDSMD-DD -----DDTKWHKCVY YVDSNGNKEDPNDTN AVCKLAMDFDQLATC   333

          361          375 376          390 391
1 AcMNPV  LEDNLIKVIIYVTVE NGENHNMNVLGDLKY IEDENEYKFL-    385
2 SlMNPV  IGD-INDVMIYFSSD IN--GYYTMLGLTCY DLSNFEYVPI-    349
3 OpMNPV  LADKITKATIFVTAD NADASTMNLLGLLKH DDEEDEYQFL-    373
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b

```
          1             15 16            30 31            45 46            60 61            75 76            90
1 AcMNPV  ------------GAT CGTTAAATCGGACGG GTCTGTTCAGAGCCG CTCTTATTCTCTCGT TGTACATTGTTAAAG TTTTTGTTTTTAAAT    78
2 SlMNPV  TTATATTCAACGGCG CTACGACACGCGACG GTCTGTTCAACGGCA CGTTTAAAAACTCAC TGTTAATCAACAGAT TCCGTTCCTTAATCT    90
3 OpMNPV  --TTGATTGTGCGGT CGCTGCGACGGACGG GGCTGTGAAGCGCCG CTCTCATTCTCTCGT TCAAAACAATCAGCT CTTTGCGCTTCATCT    88

          91           105 106          120 121          135 136          150 151          165 166          180
1 AcMNPV  TGTACACAATCGGCG TGTTGTAGTCGAAAT TTTCAAAATCGGCTT TTTGAAACATTGTTC TGAACGTGTTGTCGA GCGGCGTGTTGCTGG   168
2 SlMNPV  CGTACACTATTGGTC TGTTGTAATCAAAAT TTTCAAAATCGGCCC TGTTTAATTGCGTCC TGTAGGAGACGTAAT TTGGACTTGTATGCA   180
3 OpMNPV  TGTACACGATTGGCG TGTTGTAGTCAAAGT TTTCAAGATCGGCCC GCTGGAGCATTGTCG GAAAAGTGTAGTCAA GCGGCGTGTCGCGGG   178

          181          195 196          210 211          225 226          240 241          255 256          270
1 AcMNPV  CCACGTTTATAATCA ACTCCCTCCACGCTA ACGAACGGTGCTCTG GCGACACTTCGATTT CGTCGCCATTCAGTA TTTGCCATCGGATAG   258
2 SlMNPV  ATAAATCTATCATCA GATCTTTCCACGCGT GCTCTCTGCTCTCGG GCGACACTTCGACCC CGTCCGAGTTTAATA TTCGCCATCGAAT--   268
3 OpMNPV  CCGCGTTAGCGATCA GTTCTCGCCAAGCCA GCGCCGGGTGATCGG GCGCTACCTCGACCT CGTCGGAACTCAGCA CCTGCCAACGCACCG   268

          271          285 286
1 AcMNPV  ATTCCCACATATCGA CAACAGCAAT    283
2 SlMNPV  -TTTAGACATAAAT- ----------    281
3 OpMNPV  TGTCCCAC------- ----------    276
```

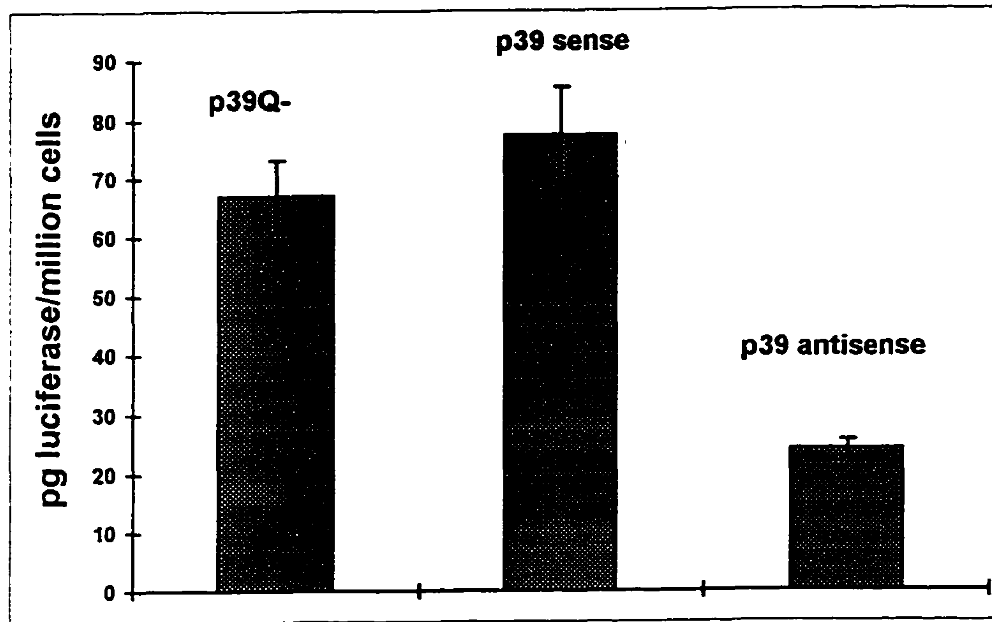
TABLE 2: Percentage identity between nucleotide and amino acid sequences from the *lef-3* genes and putative gene products.

NPV compared	Nucleotide sequence[a]		Amino acid sequence[b]
	ORF	ULS	
SpliNPV and AcMNPV	29%	44%	26%
SpliNPV and OpMNPV	26%	39%	21%
AcMNPV and OpMNPV	41%	59%	37%

[a] The nucleotide sequences of the open reading frame (ORF) and of the untranslated leader sequence (ULS) were aligned with Megalign program (DNASTAR, Inc.)using the Clustal method (Higgins and Sharp, 1989).

[B] The amino acid sequences of the putative LEF-3 proteins were aligned using Clustal W multiple alignment program (Thompson et al., 1994) available at the Clustal W server at the Human Genome Center, Baylor College of Medicine.

Fig. 6. Antisense analysis. A total of 4 µg of p39 sense, p39 antisense or p39Q- were cotransfected with 800 ng of SpliNPV-B DNA and 1 µg of pSI-ph-luc. Cells were harvested at 48 hours post-transfection. Luciferase activity was measure in a scintillation counter and the amount of luciferase estimated by comparison to an standard curve. Activity was linear in the range measured. Results are the means of six independent transfections and error bars represent the standard error.


pg luciferase/million cells
90
80
70
60
50
40
30
20
10
0
p39Q-
p39 sense
p39 antisense

DISCUSSION

We have identified and characterized an SpliNPV-B gene with several characteristics that indicate it is homologous to the AcMNPV and OpMNPV *lef-3* genes. First, the three genes encode a putative polypeptide of approximately the same molecular mass which have a number of conserved amino acids (Fig. 5a). Second, transcriptional analysis indicated that SpliNPV-B *lef-3* had an unusually long ULS, similar in length and nucleotide sequence to the ULSs identified in the *lef-3* genes of AcMNPV and OpMNPV (Fig. 5b). Third, the ORF we identified in the genomic region immediately 5' of the SpliNPV-B *lef-3* gene encodes a putative polypeptide that is similar to the putative polypeptide encoded by AcMNPV and OpMNPV ORF 68, which is located upstream (5') of the AcMNPV and OpMNPV *lef-3* genes. Finally, the SpliNPV-B *lef-3* gene was mapped in a similar genomic position to the *lef-3* genes of AcMNPV and OpMNPV (though the SpliNPV-B *lef-3* gene is in opposite orientation in relation to the polyhedrin gene).

Alignment of the amino acid sequence of the putative SpliNPV-B LEF-3 with the amino acid sequences of AcMNPV and OpMNPV LEF-3 revealed only 26% and 21% sequence identity (Fig. 5). These low levels of sequence conservation are not uncommon between baculovirus genes. For instance, only 26% amino acid sequence identity has been reported for the p10 protein of AcMNPV and SeMNPV (Rohrmann, 1992). The fact that the ULS displayed higher levels of sequence conservation than observed between the nucleotide sequence of the *lef-3* ORF (Table 2) suggests that the ULS have a conserved functional role. Conserved ULSs have also been observed in the polyhedrin gene of several baculoviruses and in the major capsid protein of AcMNPV and OpMNPV (Rohrmann, 1986; Thiem and Miller, 1989). The role of the AcMNPV polyhedrin ULS in transcription regulation was shown by mutation analysis which demonstrated that this region contains promoter elements (Ooi et al., 1989). Because ORF 68 is located in the opposite strand of the ULS of the three *lef-3* genes, it is also possible that the sequence conservation observed in this region is related to sequence conservation between the putative gene encoding ORF 68. Although a gene product has not been identified for ORF 68, analysis of AcMNPV and OpMNPV ORF 68 by the Grail method indicated that ORF 68 has excellent potential to encode polypeptides (Ayres et al., 1994; Ahrens et al., 1995).

In Sf9 cells, replicated SpliNPV-B DNA is detected between 12 and 16 hr p.i.

suggesting that the late phase of infection start between 12 and 16 hr p.i. (Chapter 2 of this thesis). Our results suggests that the SpliNPV *lef-3* gene was moderately transcribed in the early phase of infection and became strongly active in the late phase of infection (Fig. 2b). The transcription pattern we observed for SpliNPV-B *lef-3* differs from that reported for the *lef-3* genes of AcMNPV and OpMNPV. The steady state level of OpMNPV *lef-3* transcripts increases until 12 hours p.i. and then declines after 18 hours p.i. (Ahrens et al., 1995). Transcription of the AcMNPV *lef-3* reaches a peak at 6 hours p.i. and then decreases to low levels at 24 hours (Li et al., 1993). In addition, blocking of protein synthesis by cyclohexamide seems to have opposite effects in the transcriptional regulation of AcMNPV and SpliNPV-B *lef-3* genes. When protein synthesis is blocked by cyclohexamide, steady state level of AcMNPV *lef-3* transcripts increases (Li et al. 1993). In contrast, cyclohexamide greatly reduced the steady state level of SpliNPV-B *lef-3* transcripts (Fig. 2c). It appears that while the AcMNPV *lef-3* gene is down regulated by other viral encoded products, the SpliNPV *lef-3* gene is up regulated by SpliNPV proteins. These results suggests that the expression of the SpliNPV *lef-3* gene is regulated differently during infection.

We identified two transcription initiation sites, the distal and the proximal transcription initiation sites (Fig. 1b). Although primer extension analysis suggests that the distal site was an early transcription initiation site (Fig. 4), ribonuclease protection assay (Fig. 3d) done with mRNA extracted from cells that had been treated with cyclohexamide indicate that both the distal and the proximal transcription initiation sites, but not the sites located further upstream, were active in the absence of viral encoded products. Further analysis is required to define the temporal regulation of the SpliNPV *lef-3* transcription initiation sites. Neither the baculovirus early CAGT motif nor the late promoter ATAAG motifs were found in the transcription initiation region (Fig 1b). On the other hand, a putative TATA box is located immediately upstream the two transcription initiation sites (Fig. 1b). This TATA motif conforms with the consensus TATA sequence (TATAAA)and is also observed in the promoter region of other baculovirus early genes (Theilmann and Steward, 1991; Guarino and Smith, 1992). In addition, the location of the SpliNPV *lef-3* TATA motif in relation to the proximal transcription initiation sites is similar to the location of TATA motifs observed in other baculovirus early gene genes (Theilmann and Steward, 1991; Krappa et al., 1992;

Guarino and Smith, 1992; Blissard et al., 1992). We also identified a GATA motif (AGATAA) located 24 nucleotides upstream the distal transcription initiation site (Fig. 1b). This motif is similar to the GATA motif which is important for transcription regulation of the OpMNPV *gp64* early promoter (Kogan and Blissard, 1994). Our results indicate that the transcription initiation region of SpliNPV *lef-3* does not share common elements with the transcription initiation region of neither AcMNPV nor OpMNPV *lef-3* genes (Li et al., 1993; Ahrens et al., 1995).

Hang et al. (1995) demonstrated that the AcMNPV LEF-3 exhibits SSB activity. In addition, both the AcMNPV and the OpMNPV LEF-3 contain a conserved sequence in their amino-terminal regions that is similar to a motif found in several SSB proteins (Ahrens et al., 1995). The predicted amino acid sequence of the amino-terminal region of SpliNPV-B LEF-3 agrees only partially with the SSB amino acid motif identified in the LEF-3 of AcMNPV and OpMNPV (Fig. 5a). Still, the region that showed highest amino acid homology between SpliNPV-B and AcMNPV LEF-3 (Table 1) is a stretch of 61 amino acids which surrounds the AcMNPV LEF-3 SSB motif (located between amino acids 39 and 93 ; Table 1).

In our antisense experiment, transient expression of the *polyhedrin* promoter was reduced when viral DNA was cotransfected with a plasmid expressing the anti-sense *lef-3* RNA (Fig. 6). This observation suggests that the antisense *lef-3* RNA impaired the expression of the *lef-3* gene product. Reduction in the expression of very late transcription indicates that SpliNPV-B *lef-3* is either involved in viral DNA replication or in very late gene expression suggesting that functional homology with the *lef-3* gene product of AcMNPV and OpMNPV.

Phylogenetic analysis based on the published sequences of the *polyhedrin* gene, the most conserved baculovirus gene, suggests that NPVs cluster into at least two groups with distinct evolutionary rates and that both AcMNPV and OpMNPV belong to the same group (Zanotto et al., 1993). Previous investigations have shown that the genomes of SpliNPV-B and AcMNPV are not closely related (Croizier et al., 1989) and *polyhedrin* phylogenetic analysis which included the SpliNPV-B *polyhedrin* gene suggested that SpliNPV-B is distantly related from both AcMNPV and OpMNPV (our unpublished results). The level of nucleotide sequence divergence between the SpliNPV-B *lef-3* gene and the *lef-3* genes of AcMNPV and OpMNPV, and the unique pattern of SpliNPV-B *lef-3*

expression further support the evidence that SpliNPV-B belongs to a phylogenetic group that is distinct from that of AcMNPV and OpMNPV.

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Chapter IV: Analysis of sequences that regulate the expression of the SpliNPV-B *lef-3* gene

ABSTRACT:

The *lef-3* gene has been identified in the genome of three baculoviruses and it encodes a product that is essential for viral DNA replication. We have identified sequences that regulate the expression of the *Spodoptera littoralis* nucleopolyhedrovirus type B (SpliNPV-B) *lef-3* gene in Sf9 cells. We constructed plasmids with the luciferase gene under transcriptional control of the SpliNPV *lef-3* 5'-flanking region that had undergone deletion mutations and analyzed the transient expression of these constructs. We identified a promoter region that is sufficient to direct basal level of promoter activity and named this region the minimal promoter. This region encompass two transcription initiation sites previously identified by transcriptional mapping, two TATA boxes, and a GATA motif. Mutation in the GATA motif resulted in substantial reduction in reporter gene activity indicating that this motif plays an important role in the regulation of SpliNPV *lef-3* transcription. Our results suggest that the upstream regulatory region of the *lef-3* promoter extends at least 584 bases 5' of the distal transcription initiation site.

INTRODUCTION:

Baculoviruses are occluded viruses that have large double-stranded DNA genomes. Baculoviruses of the genus Nucleopolyhedrovirus are characterized by the occlusion of many virions in a crystalline structure called the polyhedra. The observation that baculovirus genes are temporally regulated in a cascade mode led to the division of gene expression into three phases: the early, the late, and the very late phases (Vialard et al., 1995). Early genes are expressed before viral DNA replication and are transcribed by the host transcription apparatus (Huh and Weaver, 1990; Glocker et al., 1992). The late phase of infection starts with the onset of viral DNA replication and genes of the late phase are transcribed by a viral encoded or viral modified RNA polymerase (Huh and Weaver, 1990; Beniya et al., 1996). Transient replication assays have been used to identify several early genes that are essential for viral DNA replication (Kool et al., 1994; Ahrens and Rohrmann, 1995; Ahrens et al., 1995). These investigations have indicated that the *lef-3* gene of *Autographa californica* nucleopolyhedrovirus (AcMNPV) and *Orgyia pseudotsugata* nucleopolyhedrovirus (OpMNPV) are essential for viral DNA replication (Kool et al., 1994; Ahrens et al., 1995). The fact that the AcMNPV *lef-3* gene product displays single-stranded DNA binding (SSB) activity (Hang et al., 1995) and that the two LEF-3 polypeptides have conserved SSB DNA motif in their putative amino acid sequences (Ahrens et al., 1995) further support the hypothesis of the involvement of LEF-3 in the process of viral DNA replication.

In a previous study, we identified an *lef-3* homologue in the genome of *Spodoptera littoralis* nucleopolyhedrovirus type B (SpliNPV-B) (chapter III of this thesis). We observed that only 26% and 21% amino acid sequence identity exists between the putative LEF-3 polypeptide of SpliNPV-B and those of AcMNPV (Li et al., 1993) and OpMNPV (Ahrens et al., 1995;). We also noted that the pattern of expression of the SpliNPV *lef-3* gene was different from that of AcMNPV and OpMNPV *lef-3* genes (Li et al., 1993; Ahrens et al., 1995; Chapter III of this thesis). In this report we present the results of our investigation on promoter elements that control transcription of the SpliNPV *lef-3* gene.

The promoter elements of baculovirus early genes display an organization that is similar to that of eukaryotic type II genes, which are transcribed by class II RNA

polymerase. Transcription of several baculovirus early genes begins at a conserved CAGT motif which appears to function as an initiator element (Blissard and Rohrmann, 1990; Guarino and Smith, 1992). A TATA box is often present in baculovirus early promoters and studies have indicated that this baculovirus promoter motif has similar function to the eukaryotic TATA box (Blissard and Rohrmann, 1991; Blissard et al., 1992; Guarino and Smith, 1992). In addition, several investigations have demonstrated that baculovirus early promoters have upstream regulatory regions that extend several hundred base pairs 5' of the transcription initiation site. On the other hand, the expression of late and very late genes is controlled by short promoters that resembles those found in mitochondria and bacteriophages (Blissard and Rohrmann, 1990). All baculovirus late and very late promoters have a conserved ATAAG motif which seems to function as both promoter and transcription initiation site.

High steady state levels of the *lef-3* transcripts of AcMNPV, OpMNPV, and SpliNPV are observed in the early phase of infection (Li et al., 1993; Ahrens et al., 1995; Chapter III of this thesis). Levels of AcMNPV and OpMNPV *lef-3* transcripts decline with the onset of the late phase whereas high steady state levels of the SpliNPV *lef-3* transcripts were still observed at 56 hours post infection (p.i.) (Li et al., 1993; Ahrens et al., 1995; Chapter III of this thesis). Transcriptional analysis indicates that the three *lef-3* genes have a long untranslated leader sequence (ULS) and that transcription does not initiate from a CAGT motif (Li et al., 1993; Ahrens et al., 1995; Chapter III of this thesis) . Although the nucleotide sequence of the SpliNPV *lef-3* gene ULS displays considerable homology to sequences of the ULS of the *lef-3* genes of AcMNPV and OpMNPV (44% and 39% respectively) the promoter of the three *lef-3* genes do not seem to share similar elements (Li et al., 1993; Ahrens et al., 1995; Chapter III of this thesis).

We have used a transient expression assay to identify regions important in the regulation of SpliNPV *lef-3* expression in Sf9 cells. We constructed plasmids with the luciferase gene under transcriptional control of the SpliNPV *lef-3* 5'-flanking region that had undergone deletion mutations and analyzed the transient expression of these constructs. We defined a region that was capable of directing basal level of transcription activity, the minimal promoter, and showed that this regions contains the two transcription initiation sites previously identified by transcriptional mapping (Chapter

III of this thesis), two TATA boxes, and a GATA motif. Sequences upstream the minimal promoter increased the levels of reporter activity several folds, suggesting that they contain transcription regulatory sequences. Mutation in the GATA motif resulted in substantial reduction in reporter gene activity indicating that this motif plays an important role in the regulation of SpliNPV *lef-3* transcription.

MATERIALS AND METHODS

Viruses and cell culture

SpliNPV-B isolate M2 was obtained from G. Croizier (Croizier et al., 1989) and propagated in *Spodoptera frugiperda* (Sf9) cells maintained in Grace's media with supplements as described by Summers and Smith (Summers and Smith, 1987). Cells were propagated in monolayers in cell culture flasks (25, 75, or 175 cm^2) and were infected with budded virus of SpliNPV-B at a multiplicity of infection (m.o.i.) of 5 PFU per cell. Mock-infected cells were treated with cell culture media that did not have budded virus. Cells were infected for two hours. After this period, the inoculant was removed and fresh media was added. In time course analysis, this point was 0 hr post infection (p.i.).

Viral DNA extraction

Viral DNA used in these experiments was extracted from budded virus. Approximately 80 ml of media was removed from 175 cm^2 cell culture flasks 5 days p.i. and centrifuged at 1,000 x g for 10 minutes to remove cell debris. The supernatant was transferred to Oakridge tubes and centrifuged at 15,000 x g for 30 minutes at 4 °C. The pellets were resuspended in 400 µl of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) with Proteinase K (0.4 mg/ml) and sodium lauryl sarcosine (0.5%) and incubated overnight at 50 °C. The next day, the solution was extracted with one volume of equilibrated phenol:chloroform, followed by a chloroform extraction. The aqueous phase was transferred to a Centricon-100 tube (Amicon Corporation) and centrifuged in a fixed angle rotor at 2,300 x g until the volume of the solution was reduced to about 50 µl. The DNA was washed twice by adding 1.5 ml of TE to the Centricon tubes followed by centrifugation at 2,300 x g until the volume of the solution was reduced to about 50 µl. The concentration of DNA was determined by fluorometry (TKO 100 mini-fluorometer, Hoefer Scientific Instruments).

Construction of plasmids for analysis of promoter sequences upstream the ULS

Plasmid plef3FR contains the entire coding region of the SpliNPV *lef-3* gene and approximately 2000 nucleotides of sequences upstream the distal transcription start site in a cloned fragment spanning from the BamHI site at 42.9 mu to the EcoRV site located immediately upstream of the 3' end of the *lef-3* gene at 45.6 mu (Fig. 1a and b).

We constructed pBluc by cloning the luciferase gene and the SV 40 polyA signal (SalI/XhoI fragment) of pGL-2 basic (Promega Corporation) into the XhoI site of pBluescript II KS. Diagnostic restriction digestion was used to identify the clone that had the luciferase gene in the desired orientation (the 5'-end of the luciferase gene adjacent to the AccI site of pBluescript II KS).

The ClaI/BglII fragment of plef3FR was removed and cloned into pBluc that had been linearized with AccI and BglII. The resulting plasmid had the luciferase gene under transcriptional control of the *lef-3* promoter region (Fig. 1c). This plasmid was called plef3Luc and it contained 1001 bp upstream the *lef-3* distal transcription start site and 200 bp of ULS. The translation start site used in this construct was the ATG codon of the *luciferase* gene.

To identify the *lef-3* minimal promoter and upstream regulatory sequences, a series of 5' deletion mutants were generated from plasmid plef3Luc. Plasmid plef3Luc was linearized with NotI and SacI and unidirectional deletions were generated using Exonuclease III and S1 nuclease following standard procedures (Henikoff, 1987). We selected seven nested deletion plasmids for transient expression analysis: plef3Luc deletion -584, -468, -359, -183, -45, -11, and +60 (Fig. 1d). Negative and positive numbers indicate the position of the last bp in the deletion subclones in relation to the distal transcription start site of the *lef-3* gene (negative upstream and positive downstream). Plasmid pAcIE-1, which contains the AcMNPV *ie-1* gene, was cotransfected with deletion subclones. This plasmid was obtained from D. Theilmann (Theilmann and Stewart, 1991).

Fig. 1 Construction of plef3Luc and deletion subclones. (a) SpliNPV-B HindIII physical map prepared by Croizier et al. (1989) showing the location and orientation of the *polyhedrin* gene (Croizier and Croizier, 1994) and of the *lef-3* gene. The enlarged section shows the location of the lef-3 gene ORF and upstream region. (b) Plasmid plef3FR contains the entire coding region of the *lef-3* gene and approximately 2000 nucleotides of sequences upstream the ULS. Plasmid plef3Luc has the luciferase gene under transcriptional control of the lef-3 promoter. Plef3Luc was constructed by inserting the ClaI/BglII fragment of plef3FR into pBluc that was linearized with AccI and BglII. The ClaI/BglII fragment contains approximately 200 nucleotides of the *lef-3* ULS and 1000 nt upstream the *lef-3* ULS. The translation start site used in this construct was the ATG codon of the luciferase gene. (d) Subclones of plef3Luc with deletions in the 5' end region of the *lef-3* promoter were generated by unidirectional digestion with Exonuclease III followed by S1 nuclease treatment. The position of the distal transcription initiation site is indicated (+1). Thick bars represent the amount of *lef-3* DNA segment of each subclone. The size of the promoter segments upstream the distal transcription initiation site is indicated by negative numbers. One of the deletion subclones analyzed had the 5' region deleted up to 60 nt downstream the distal transcription start site (+60). The arrow curved arrow indicates the location of the transcription initiation sites (tis).

a

HindIII

K D E H I F (L,M,N,O) B C A G J

2.3 13.2 23.2 28.9 32.8 40.9 42.6 57.2 68.4 89.0 97.1 100

Polyhedrin

mu 42.9 BamHI BstEII ClaI EcoRV BglII LEF-3 BstEII EcoRV mu 45.5

500bp

b

BamHI BstEII ClaI EcoRV tis BglII LEF-3 BstEII EcoRV

plef3FR

ClaI/BglII digestion

ClaI BglII

Clone fragment into the AccI/BglII site of pBluc

c

AccI/ClaI EcoRV tis BglII luciferase SV40 polyA

plef3Luc

d

-584 +1 luciferase SV40 polyA plef3Luc-584

-468 +1 plef3Luc-468

-359 +1 plef3Luc-359

-183 +1 plef3Luc-183

-45 +1 plef3Luc-45

-11 +1 plef3Luc-11

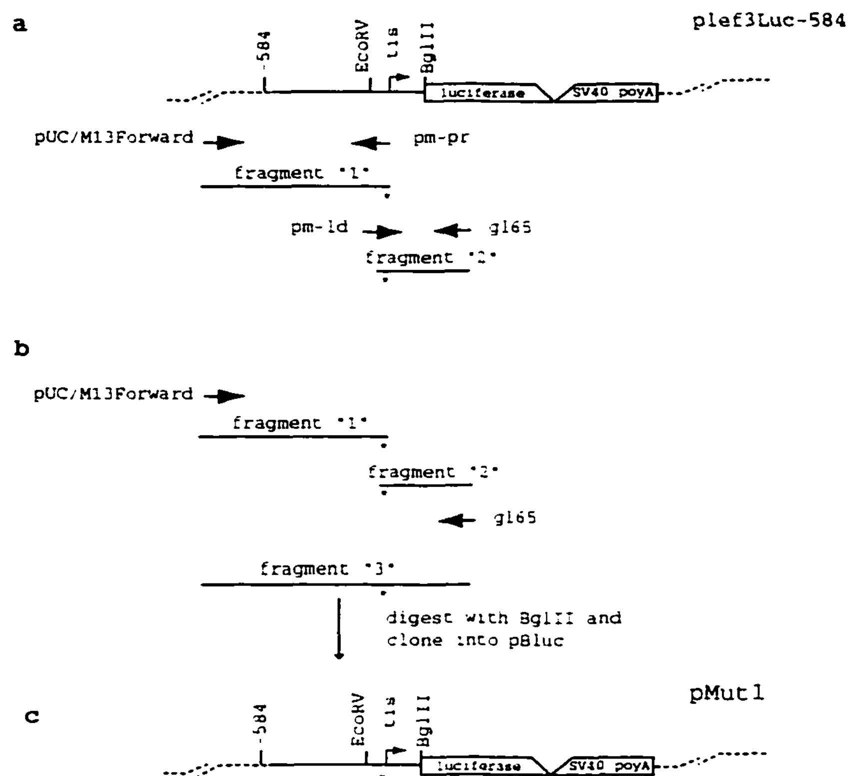
+60 plef3Luc+60

Construction of a plasmid that has mutations in the "GATA" motif

The sequences surrounding the *lef-3* transcription start sites has two TATA box motifs and a GATA motif (Chapter III of this thesis). To verify the importance of the GATA motif for transcription from the *lef-3* promoter we used a PCR based technique to introduce mutations in the GATA motif (Vallejo et al., 1994). Mutation was achieved by amplifying two regions of the *lef-3* promoter with primers that had the desired change in the GATA motif Fig 2a, b, and c). Two mutagenizing primers were used: oligo pm-ld (5'-AGT GAA CAG CTA GAA GCG AGG AAG-3') and oligo pm-pr (5'-CTT CCT CGC TTC TAG CTG TTC ACT-3'). These 24-mers are complementary to each other, extend over the GATA motif, and introduce two nucleotide changes in the GATA motif. The nucleotide changes are underlined. Plasmid plef3Luc-584 was used as template for amplification. pUC/M13 forward primer (5'-CGC CAG GGT TTT CCC AGT CAC GAC-3') is complementary to a nucleotide sequence located immediately upstream the 5'-end of the *lef-3* promoter fragment cloned into plef3Luc-584. Primers pUC/M13 forward and pm-pr were used to create the mutated fragment that extends from the GATA motif until the end of the *lef-3* promoter (Fig 2a). This fragment of 644 bp was called fragment "1" . Primer gl-65 (5'-GGC GTC TTC CAT TTT ACC AAC AG-3') is complementary to a nucleotide sequence located in the 5'-end of the *luciferase* gene. Primers gl-65 and pm-ld were used to create the mutated fragment that extends from the GATA motif until the beginning of the *luciferase* gene (Fig 2a). This fragment of 296 bp was called fragment "2". PCR reactions were done with 5 units of Pfu enzyme (Stratagene) using a buffer supplied by the manufacture (20 mM Tris- HCl pH 8.8; 2 mM $MgSO_4$; 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 0.1% Triton X-100; 100 µg/ml nuclease-free BSA). The PCR cycling parameters were 1 cycle at 94°C for 4 minutes, followed by 30 cycles at 94°C for 30 seconds, 55° for 30 seconds, and 72°C for 1 minute. The reaction volume was 50 µl and approximately 50 ng of primers and 10 ng of plef3Luc-584 template were used in each reaction. Fragments "1" and "2" were gel purified with Wizard columns (Promega) for subsequent use. Because fragments "1" and "2" overlap each other in the region where the mutation was introduced, it was possible to splice them together in a PCR amplification reaction (Fig 2b). In this reaction, fusion of the two fragments was achieved by the extension of overlapping strands and PCR amplification of the extended product (called fragment "3") was achieved by the use of the two outside primers

(pUC/M13 forward and gl-65). Approximately 200 ng of each of the two fragments and 50 ng of each primer (pUC/M13 forward and gl-65) were used in the overlap extension and amplification reaction. A total of 5 units of Pfu enzyme (Stratagene) was used with the buffer supplied by the manufacture in a reaction volume of 50 µl. The PCR cycling parameters were 1 cycle at 94°C for 4 minutes, followed by 30 cycles at 94°C for 30 seconds, 55° for 30 seconds, and 72°C for 1 minute. The two outside primers were added to the reaction only after the initial 5 cycles. A fused fragment that we called "3" was obtained from this reaction. Fragment "3" was purified in a Wizard column (Promega) and digested with BglII enzyme. The digested fragment was cloned into the SmaI/BglII site of pBluc and the resulting plasmid was called pMut1 (Fig. 2c).

Fig. 2. Construction of a subclone that has the GATA motif mutated. (a) plef3Luc-584 was used as a template in a PCR amplification reaction to generate the mutated fragments "1" and "2" as described in Materials and Methods. (b) Fragment "3" was obtained by overlap extension of fragments "1" and "2". (c) Fragment "3" was digested with BglII and cloned into pBluc. The resulting plasmid was called pMut1 and has the AGATAA sequence mutated to AGCTAG. The position of the mutation in the fragments and in pMut1 is indicated by asterisks (*). The position of the primers used in the PCR reaction is indicated by arrows. Four oligos were used as primers: pUC/M13 forward, pm-pr, pm-ld, and gl65. Curved arrows indicate the location of the transcription initiation sites (tis).


a
plef3Luc-584
-584
EcoRV
tis
BglII
luciferase
SV40 poyA
pUC/M13Forward
pm-pr
fragment "1"
pm-ld
g165
fragment "2"
b
pUC/M13Forward
fragment "1"
fragment "2"
g165
fragment "3"
digest with BglII and
clone into pBluc
c
pMut1
-584
EcoRV
tis
BglII
luciferase
SV40 poyA

Construction of plasmids for analysis of the ULS

To analyze the ULS we constructed three plasmids with *lef-3* promoter/luciferase gene fusions. PCR reactions were used to synthesize fragments that were cloned into pBluc (Fig. 3). Approximately 50 ng of genomic SpliNPV-B DNA and 50 ng of primers were used in each reaction in a volume of 50 µl. A total of 5 units of Pfu enzyme (Stratagene) and the buffer supplied by the manufacturer were used for PCR reactions. The PCR cycling parameters were 1 cycle at 94°C for 4 minutes, followed by 30 cycles at 94°C for 30 seconds, 55° for 30 seconds, and 72°C for 1 minute. Oligo up1536 (5'-ACT CTT GCC TGT TCT CGT CTC C-3') was used as forward primer. This oligo is complementary to sequences located 635 bp upstream the distal transcription initiation site. Two oligos were used as reverse primers: oligo Bg1140 (5'-CGA GAT CTC GTG TCG TAG CGC CGT TGA ATA-3') is complementary to nucleotides that are immediately downstream of the two transcription initiation sites and has a BglII overhand in the 5'-end; oligo dl01 (5'-GCG GAT CCA TTT ATG TCT AAA ATT CGA TGG CGA ATA TT-3') is complementary to nucleotides located immediately upstream the *lef-3* translation start site and has a BamHI overhang in the 5'-end. PCR reaction with oligos up1536 and Bg1114 resulted in a fragment of 647 bp that was purified in a Wizard column (Promega), digested with BglII, gel purified, and cloned into the SmaI/BglII site of pBluc. The resultant plasmid was called pdel1. PCR reaction with oligos up1536 and dl01 resulted in a fragment of 902 bp which was purified in Wizard column (Promega), digested with BamHI, gel purified, and cloned into the SmaI/BglII site of pBluc. The resultant plasmid was called pCI. A second PCR reaction was done with oligos up1536 and dl01. The PCR product obtained was digested with BglII and cloned into the SmaI/BglII site of pBluc. The resultant plasmid was called pdel2.

DNA sequencing

The DNA sequence of the *lef-3* gene and promoter regions have been previously determined (Chapter III of this thesis). In this work, we confirmed the *lef-3* sequence in the deletion subclones and in pMut1 by the dideoxynucleotide chain termination method using custom designed primers (Sanger et al., 1977). The sequence of the ULS and of the 5' flanking regions are shown in figure 4.

Fig. 3 Amplification of PCR fragments for construction of subclones with different length of ULS. The PCR amplified fragments "deletion 1" and "entire ULS" were cloned in pBluc as described in Materials and Methods. Fragment "entire ULS" was digested with BglII. One of the resultant fragments had the promoter region of *lef-3* and the ULS up to the BglII position. This fragment was called "deletion 1" and was cloned into pBluc.

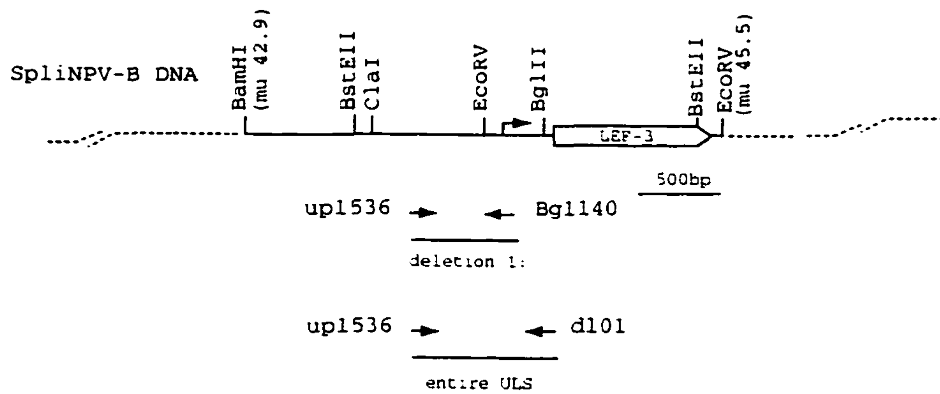

SpliNPV-B DNA
BamHI
(mu 42.9)
BstEII
ClaI
EcoRV
BglII
LEF-3
BstEII
EcoRV
(mu 45.5)
500bp
up1536
Bgl140
deletion 1:
up1536
dl01
entire ULS

Fig. 4. Sequence of the region 5' of the ORF encoding LEF-3. Nucleotide sequence of the *lef-3* ULS and 5' flanking regions is shown. Numbers of the right indicate the nucleotide position in relation to the distal transcription start site. The position of the last bp in the deletion subclones is underlined, the TATA motifs are indicated by boxes, and the distal (5'd) and proximal (5'p) transcription initiation sites are indicated with curved arrows. The BglII site and the site where the LEF3 ORF begins are shown.

```
TTGACGACGCAATCTCCTCCGACCGACAGGCACGAAAGGATCAGTTGCATTCGCGACGTATCAGCGGCATCGTGAGAGACTCTTGCCTGTTCTCGTCTCC  -514
                                                                                 oligo up1536
                               -584
GGTCACGTCGAAACGCCGTCCGCGCACACAAAGTCGCATCGGTTTCCACATTTATTGACAATGTGTCGTACAACAGATTCCGACATTATGTCTCCCGAAC  -514
                                              -468
CGTTGTCGTCGACGCCGTCGTCGCCGCCGTCACCACCATAAATTCTCTCGAATCTAGATCCGAATCGTTTGACTATTGACTTGTCGTAGTCTAGATGATT  -414
                                                          -359
GCGCACGTGACACCGAAACCGAGCACGGATTCGCCGTTTACGGCGTTCAAATATCTCACAAACTGTCCCGGTCCGCCGCAAATGTCCAGATATGTTCGAA  -314

TGTCGTCGCACACTTTAAACTTGTCGTTGATCTGTTTCAATTTGTAAAAACACCGCTGACCGGACAAGATGGTCGCAGATGATCTTTCCAGCCGCCGTCT  -214
                               -183
CGCCGACGATATCTCGTAGCTCGAGTAGTCGTCCAGCTTGGATCTCAAAGCGTCGAGTTCGCGTTGATATTTTTCAATCAGTCTGTCGGACGCCGTCGAC  -114
                                                                     -45               ******
GCTGTAGCTTTCCGGTCGCCGCCGCCCGTCATGTTCGTATGTGTGGGCAGTAACGGCGGAAATTAATGCGATTAAAAGTGAACAGATAAAAGCGAGGAAG  -14
 -11           ⌐→ 5'd        ⌐→ 5'p                                                 oligo pm-dl and pm-pt
                                                                        +60
ATATAAAGTGGAGTTATATTCAACGGCGCTACGACACGCGACGGTCTGTTCAACGCGACGTTAAAAAACTCACTGTTAATCAACAGATTCCGTTCGTTAA  +86
                      oligo bgl148
TCTCGTACACTATTGGTCTGTTGTAATCGAAATTTTCAAAATCGGCCCTGTTTATTGCCGTCCTGTACGAGACGTAATTTGGACTTGTATGCAATAAATC  +186

TATCATCAGATCTTTCCACGCGTGCTCTCTGCTCTCGGGCGACACTTCCACCCGGTCCGAGTTTAATATTCGCCATCGAATTTTAGACATAAATATGAAG  +286
      BglII                                                                   oligo dl81
                                                                                               →
                                                                                           LEF-3 ORF
```

Transient expression assay

To perform transient expression assays, a total of 1.5 X 10^6 cells per well were seeded in a 6 well plate (33 mm) and allowed to attach for 2 hours. We then performed cotransfection using the calcium phosphate coprecipitation procedure (O'Reilly et al., 1992). The Sf9 media was replaced with 1.0 ml of transfection buffer A (Grace's Medium with 10% FBS). Plasmid and viral DNAs were mixed with 1.0 ml of transfection buffer B (125 mM HEPES pH 7.1, 125 mM $CaCl_2$, 140 mM NaCl) and added dropwise to Sf9 cells. A total of 1 µg of plasmid DNA per well was used in transfections. A total of 800 ng of SpliNPV DNA was used in cotransfections. Cells were incubated at 28 °C for 4 hours after buffer B was added. The transfection buffer was then removed and replaced with Sf9 media. For promoter activity analysis and antisense experiments cells were incubated for 48 hours at 28 °C. For time course analysis, cells were incubated from 4 to 48 hours. To perform the luciferase assay, media was removed and the cells were harvested in 1 ml of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4). The cells were counted and pelleted in a microcentrifuge at 4 °C and 4,000 x g for 5 minutes. The pellet was resuspended in 400 µl of cell culture lysis reagent (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100, Promega Corporation), incubated for 15 minutes, and briefly centrifuged to pellet cell debris. A 20 µl aliquot of this cells extract was added to 100 µl of luciferase assay reagent (20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM Coenzyme A, 470 µM luciferin, 530 µM ATP, Promega Corporation) at room temperature. Light emission was measured in a scintillation counter and the amount of luciferase in each assay was determined from a standard curve generated using purified luciferase (Boehringer Mannheim).

RESULTS:

Analysis of the upstream regions of *lef-3* promoter

To identify regulatory elements that control transcription of the SpliNPV-B *lef-3* gene, we constructed plasmids with the luciferase gene under transcriptional control of the SpliNPV *lef-3* 5'-flanking region that had undergone deletion mutations (Fig. 1d and Fig. 4) and analyzed the transient expression of these constructs. Three sets of experiments were done: a) transfection of nested deletion clones by themselves; b) cotransfection of nested deletion clones and SpliNPV-B DNA; and c) cotransfection of nested deletion clones and pAclE-1. Levels of transcription were evaluated based on the amount of luciferase activity produced at 24 hours post-transfection. Transfection of plef3Luc clones deletion -11 and +60 resulted in background levels of luciferase activity (Fig. 5a, b, and c). Transfection of plef3Luc-45 resulted in a substantial increase over background levels and we called the level of activity obtained from this plasmid the "basal activity" of the SpliNPV *lef-3* gene promoter. The region extending up to 45 bp upstream from the distal transcription initiation site will be referred as the minimal promoter (Fig. 4). Transfection of plef3Luc deletion clones -183, -359, -468, and -584 resulted in levels of luciferase activity several fold above the basal level (Fig. 4a, b, and c). Cotransfection of deletion subclones with either SpliNPV-B DNA or plE-1 resulted in greatly increased levels of reporter gene activity for all clones (Fig. 5a, b, and c).

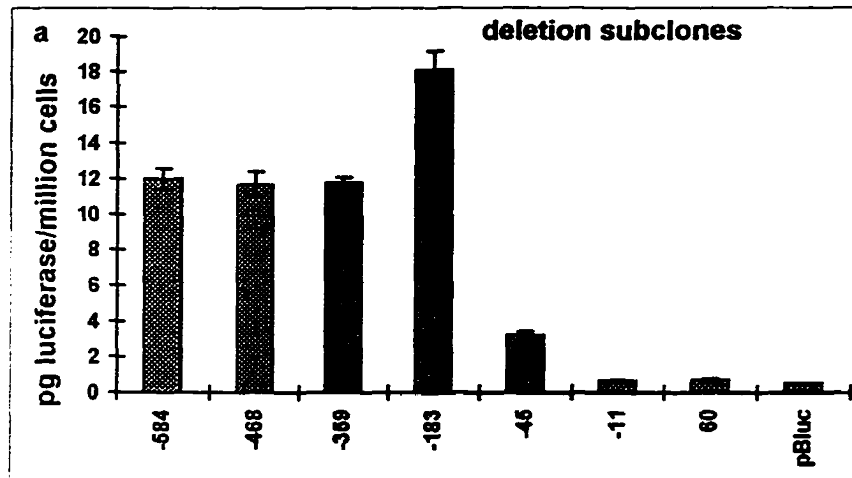
Mutation analysis of the GATA motif

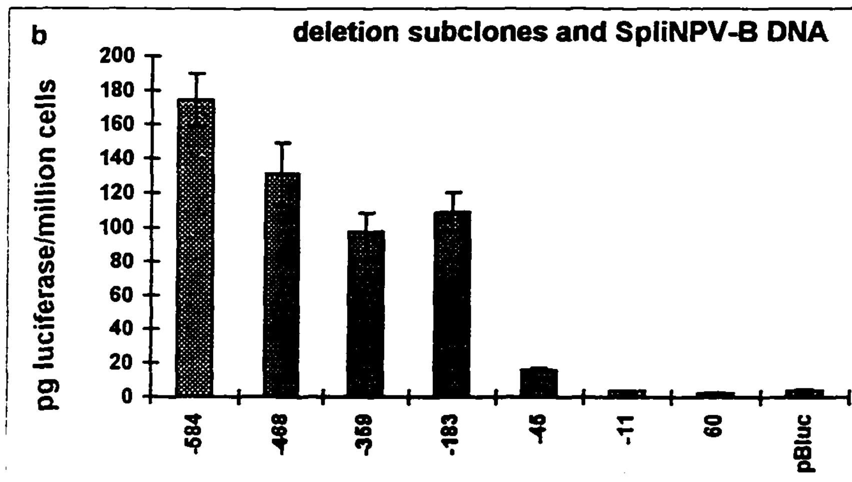
The minimal promoter we identified in the SpliNPV *lef-3* promoter contains a GATA motif. We used a PCR method to construct a subclone of plef3Luc-584 which has the AGATAA sequence mutated to AGCTAG. This subclone was called pMut1. Levels of reporter gene activity for pMut1 was almost four fold lower than that for plef3Luc-584 in the absence of other viral products (Fig. 6a). When cotransfected with SpliNPV-B DNA, there was a 43% reduction in the level of pMut1 compared to that of plef3Luc-584 (Fig. 6b).

Effect of the ULS in transient expression from the *lef-3* promoter

Transient expression of plasmids with different length of the original *lef-3* ULS in the presence and in the absence of viral encoded products is shown in figure 7a and b.

Fig. 5. Analysis of transient expression of deletions subclones. Sf9 cells were transfected with plef3Luc subclones that had portions of the lef-3 promoter region deleted from the 5' end. Cell were harvested at 24 hours post-transfection and luciferase activity was measured in a scintillation counter. The amount of luciferase was estimated by comparison to a standard curve. Activity was linear in the range measured and results are the means of at least three independent transfections. (a) Approximately 1 µg of each deletion subclones were transfected by themselves; (b) cotransfected with 500 ng of pIE-1; (c) or cotransfected with 800 ng of DNA from SpliNPV-B.


a
deletion subclones
pg luciferase/million cells
20
18
16
14
12
10
8
6
4
2
0
-584
-468
-369
-183
-45
-11
60
pBluc


b
deletion subclones and SpliNPV-B DNA
pg luciferase/million cells
200
180
160
140
120
100
80
60
40
20
0
-584
-468
-369
-183
-45
-11
60
pBluc

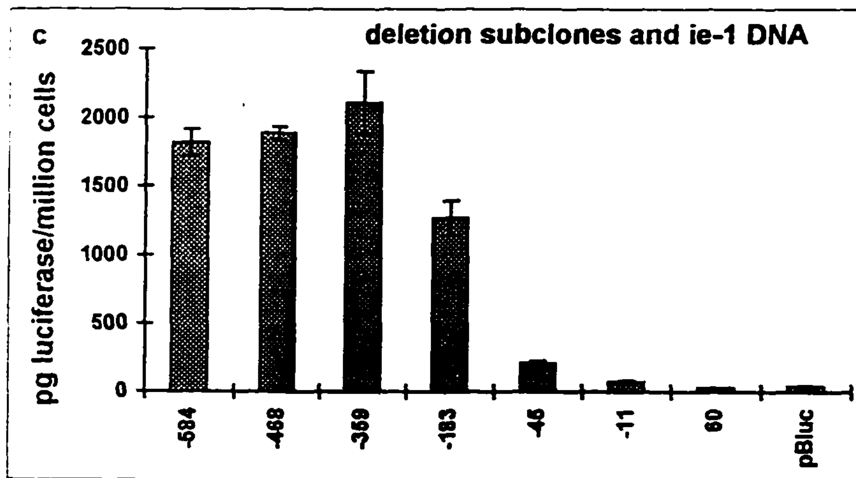
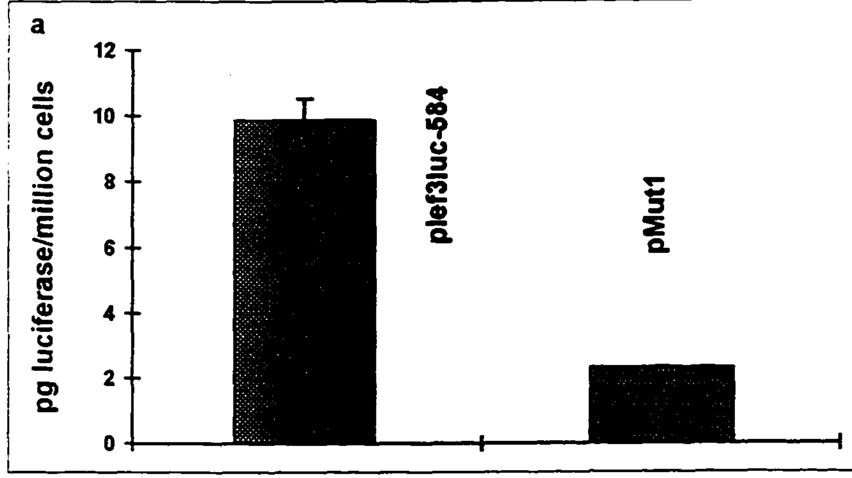

c
deletion subclones and ie-1 DNA
pg luciferase/million cells
2500
2000
1500
1000
500
0
-584
-468
-369
-183
-45
-11
60
pBluc

Fig. 6. Effect of mutation in the GATA motif. pMut1 had the AGATAAAAG motif we identified in the minimal promoter region of *lef-3* mutated to AGCTAGAAT as described in Materials and Methods. (a) Sf9 cells were transfected with 1 μg of pMut1 or plef3Luc-584 by themselves or (b) cotransfected with 800 ng of DNA from SpliNPV-B. Cell were harvested at 24 hours post-transfection and luciferase activity was measured in a scintillation counter. The amount of luciferase was estimated by comparison to a standard curve. Activity was linear in the range measured and results are the means of at least three independent transfections.


a
pg luciferase/million cells
0
2
4
6
8
10
12
plef3luc-584
pMut1

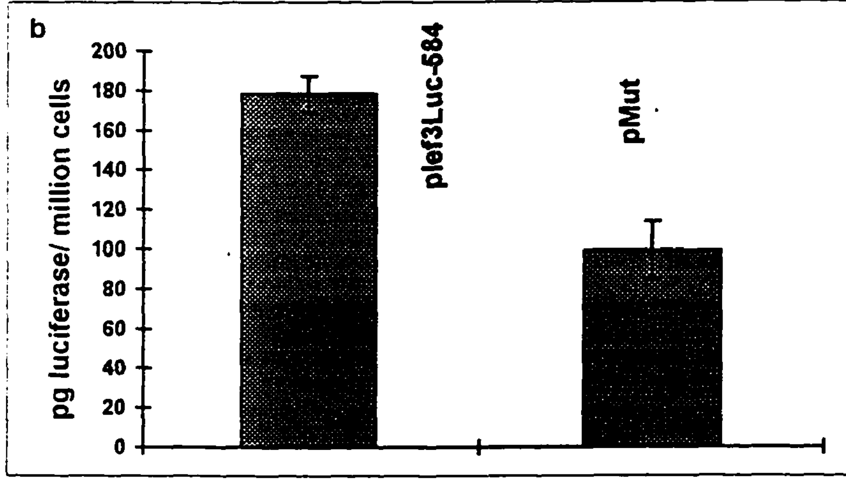
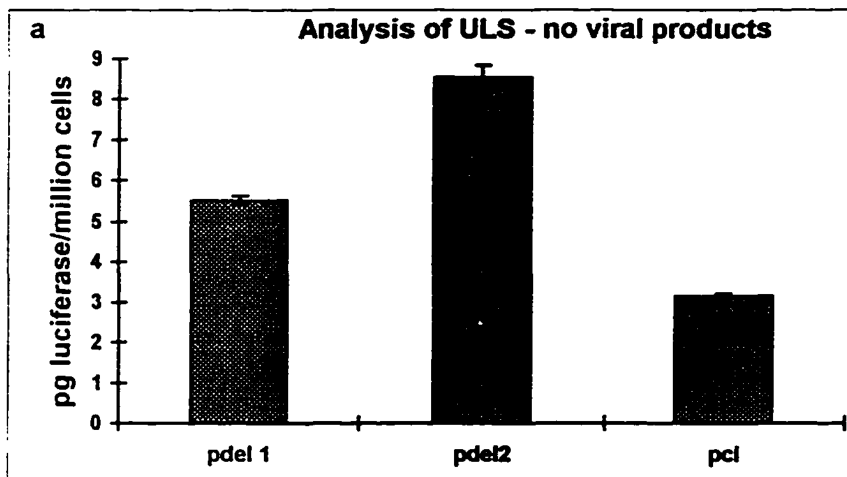
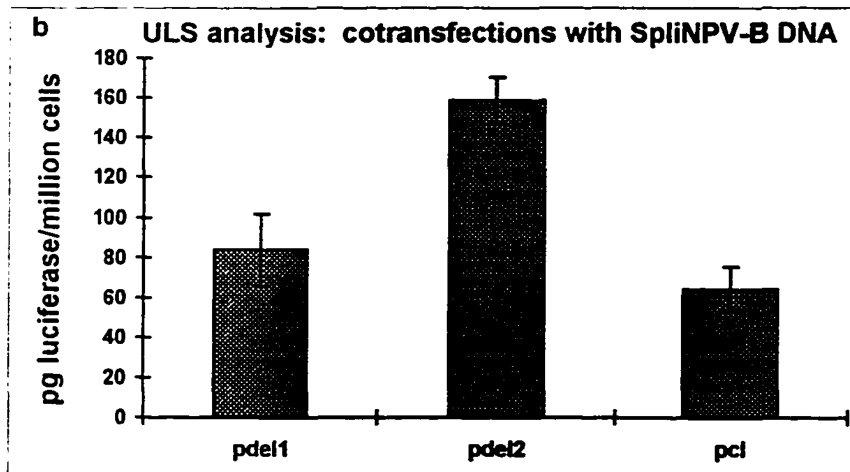

b
pg luciferase/ million cells
0
20
40
60
80
100
120
140
160
180
200
plef3Luc-584
pMut

Fig. 7. Luciferase activity from subclones with different length of *lef-3* ULS. pdel1 has the entire ULS deleted; pdel2 has approximately 200 nt of the ULS; and pcl has the entire ULS preserved. (a) Approximately 1 μg of each of these constructs were either transfected into Sf9 cells by themselves or (b) cotransfected with 800 ng of DNA from SpliNPV-B. Cell were harvested at 24 hours post-transfection and luciferase activity was measured in a scintillation counter. The amount of luciferase was estimated by comparison to a standard curve. Activity was linear in the range measured and results are the means of at least three independent transfections.


a
Analysis of ULS - no viral products
pg luciferase/million cells
9
8
7
6
5
4
3
2
1
0
pdel 1
pdel2
pcl


b
ULS analysis: cotransfections with SpliNPV-B DNA
pg luciferase/million cells
180
160
140
120
100
80
60
40
20
0
pdel1
pdel2
pcl

DISCUSSION

We have characterized the transcriptional activity of the SpliNPV *lef-3* gene (Chapter III) and observed that it is expressed throughout infection. We also showed that transcription of the *lef-3* gene starts at two major sites (distal and proximal initiation sites) located in a region that has two adjacent TATA boxes (Fig. 4). To identify the location of potential *lef-3* transcription regulatory sequences and the minimal promoter, subclones of plef3Luc that had deletions in the 5' sequences upstream the *lef-3* transcription initiation sites were constructed. These subclones were transfected into Sf9 cells or cotransfected with SpliNPV-B DNA or with pIE-1, a plasmid that contains the entire AcMNPV *ie-1* gene, whose product is a strong transactivator of several baculovirus promoters.

Although the levels of expression were greatly reduced in the absence of viral encoded products, the overall trend of expression was similar in the presence or absence of viral products. For instance, basal levels of promoter activity were obtained with subclone plef3Luc-45 in the three sets of experiments (Fig. 5a, b, and c). Therefore, the *lef-3* sequences up to 45 nucleotides 5' of the distal transcription initiation site were defined as the "minimal promoter". Similarly, in the three sets of experiments, a dramatic increase in reporter activity was observed when sequences between -45 bp and -183 bp were part of the subclone (Fig. 5a, b and c), suggesting that positive regulatory elements are located in this region.

In the absence of other viral factors, maximum levels of luciferase activity were produced with the subclone that contained 183 bp upstream the distal transcription initiation site (Fig. 5a). The lower activity observed with the three subclones that contain sequences further upstream (plef3Luc-359; plef3Luc-468; and plef3Luc 584) suggests that negative regulatory elements exist in the sequences between -183 bp and -365 bp. Cotransfection with SpliNPV DNA or with pIE-1 overcame this inhibitory effect (Fig. 5b and c).

A gradual increase in reporter activity was observed when subclones that have sequences from position -359 bp to position -584 bp were cotransfected with SpliNPV-B DNA (Fig. 5b), suggesting that the upstream regulatory region of the *lef-3* promoter extends at least up to 584 bp 5' of the distal transcription initiation site. In contrast, cotransfection with AcMNPV *ie-1* gene produced highest levels of luciferase activity

with plef3Luc-359 (Fig. 5c).

It has been shown that *Spodoptera frugiperda* cells contain a protein which recognizes GATA motifs (Krappa et al., 1992; Kogan and Blissard, 1994). In addition, mutation analysis demonstrated that two GATA motifs identified in the upstream regulatory region of the OpMNPV *gp64* gene are involved in the regulation of *gp64* early transcription (Kogan and Blissard, 1994). Croizier and coworkers observed 8 repeats of the sequence AGATAAAA within a short region upstream the SpliNPV-B *polyhedrin* gene and suggested that this motif may be a regulatory element of a gene located 5' of the *polyhedrin* gene (Croizier and Croizier, 1994). The *lef-3* minimal promoter region we defined contained the distal and proximal transcription initiation sites, two TATA boxes, and a GATA motif (AGATAAAA) identical to the repeated sequences observed 5' of the *polyhedrin* gene. To determine the importance of the *lef-3* GATA motif in transcription regulation, we constructed a plef3Luc-584 subclone, named pMut1, in which the sequences of the GATA motif were modified (Fig. 2a, b, and c). Transfection of pMut1 into Sf9 cells resulted in a four fold decrease in reporter activity, indicating that the GATA motif is recognized by host transcription factors (Fig. 6a). A substantial reduction in reporter activity was also observed when pMut1 was cotransfected with SpliNPV-B DNA, suggesting that the GATA motif is important for the regulation of *lef-3* expression (Fig. 6b).

SpliNPV-B *lef-3* gene has a long ULS similar to that observed in the *lef-3* genes of AcMNPV and OpMNPV. Plasmid constructs with deletions in the ULS were prepared to evaluate the effect of this region in transient transcription activity (Fig. 3). Clone pdel1, which had almost the entire ULS deleted, produced slightly higher reporter activity than pCl, which has the entire leader sequence (Fig. 7a and b). On the other hand, clone pdel2, which had 80 bp of the 3' end of the ULS deleted, produced substantial higher levels of luciferase activity. These results indicate that the ULS influence gene expression in a complex fashion and that it may contain elements that affect mRNA stability and/or promoter activity.

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Chapter IV: Conclusions

Baculoviridae is a large family of occluded viruses that infect invertebrates. This family is divided into two genera, the Nucleopolyhedrovirus (NPVs) and the Granulovirus (GVs), which differ in size, number of virions per occlusion body, and in the composition of their occlusion body. *Spodoptera littoralis* nucleopolyhedrovirus type-B (SpliNPV-B) isolates have been identified from diseased larvae of *S. littoralis* collected in Israel, Egypt, and Morocco and from diseased larvae of *S. litura* collected in Japan.

We observed the growth characteristics of SpliNPV-B in Sf9 cells and noted that although most cells displayed strong cytopathological effects, only a small percentage of cells (less than 10%) produced polyhedra. Low production of polyhedra seems to be a characteristic of SpliNPV-B in cell culture since this was also observed in a cell line derived from *S. littoralis*. Titers of SpliNPV-B budded virus in Sf9 cells were between 10^6 to 10^7 PFU per ml of media. Similar titers were obtained for SpliNPV-B from *S. litura* grown in SF21 cells. The initiation of viral DNA replication was estimated to be between 12 and 16 hours post-infection.

The characterization of the SpliNPV-B *lef-3* gene revealed that it has unique characteristics compared to the *lef-3* genes of AcMNPV and OpMNPV. The number of conserved amino acid sequences in the putative polypeptide encoded by SpliNPV-B *lef-3* is low compared to the predicted amino acid sequence of the AcMNPV and OpMNPV (26% and 21% respectively). The expression pattern of SpliNPV-B *lef-3* gene also differed from that of AcMNPV and OpMNPV *lef-3* genes. Our results showed high steady state levels of SpliNPV *lef-3* transcripts throughout the infection whereas levels of the AcMNPV and OpMNPV *lef-3* transcripts decline sharply in the late phase of infection.

Phylogenetic analysis of the polyhedrin gene, the most conserved baculovirus gene, indicates that within the NPVs, SpliNPV-B is distantly related from AcMNPV and OpMNPV. The level of sequence conservation observed among the *lef-3* genes of SpliNPV-B, AcMNPV, and OpMNPV supports the phylogenetic tree obtained from the polyhedrin gene. The unique pattern of expression observed for the SpliNPV-B *lef-3* gene is another indication that this virus is distantly related from AcMNPV and OpMNPV.

Our results suggest that the function of the three *lef-3* genes is likely to be similar. Transient complementation assay has indicated that the *lef-3* genes of AcMNPV and OpMNPV are essential for viral DNA replication. We used an antisense assay to show

that when the antisense *lef-3* RNA was expressed, transcription from viral very late promoter was reduced. Since very late phase promoters are only expressed after viral DNA replication, the inhibition observed in the antisense assay could be a result of a delay in viral DNA replication. The fact that the amino acid sequences of the three putative LEF-3 polypeptides have a partially conserved single-stranded DNA binding protein (SSB) motif further indicates that the three proteins have the same function.

The transcription of the *lef-3* gene is regulated by sequences spanning up to 584 nucleotides upstream the distal transcription initiation site. The *lef-3* minimal promoter encompass two transcription initiation sites identified by transcriptional mapping, two TATA boxes, and a GATA motif. We demonstrated that the GATA motif found in the promoter of the *lef-3* gene was critical for promoter activity both in the presence and in the absence of other viral encoded products. Identical GATA motifs have been identified in the promoter of the AcMNPV *pe-38, me-53, 35k*, and OpMNPV *ie-1* genes. In addition, the upstream regulatory region of the OpMNPV *gp64* early promoter contains two GATA motifs that are important in the transcriptional regulation of this gene. The two *gp64* GATA motifs differ by one nucleotide to the GATA motif of the *lef-3* promoter.

The evaluation of baculovirus safety and the possibility of widespread use of wild-type or recombinant viruses in the future depends on a deeper and broader understanding of how these viruses interact with their hosts and how they relate to each other. The additional information about SpliNPV-B and the *lef-3* gene contribute to a better appreciation of the diversity of these viruses and of the extend to which nucleotide sequence differences can be found among their genes. The investigation of the *lef-3* promoter has provided further data about a promoter motif that seems to be of critical importance to several baculovirus genes.