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Molecular studies on the densovirus of *Mythimna loreyi*, MlDNV, a candidate for biological control

Par

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SUMMARY

Densoviruses of arthropods belong with the parvoviruses of vertebrates to the *Parvoviridae* family. These isometric viruses, with a diameter between 22 to 25 nm, contain genomes of 4-6 kb of linear, single-stranded DNA. The limited number of coding genes implies a high dependence on a coding complement from their hosts and may explain their narrow host range. Densoviruses are potential biological control agents due to their specific and acute effect. Yet, their use in biological control has been delayed for a long time since early unconfirmed reports claimed that these insect viruses infect vertebrate cells in culture. However, the advance in molecular biology research makes it increasingly clear that many differences exist between vertebrate and invertebrate biology. In turn, viruses that are obligatory parasites of invertebrates are adapted to the distinct biology of their hosts, thus fitness of these insect viruses to vertebrates is not anticipated. On the other hand, some vertebrate viruses such as bunyaviruses and flaviruses, that are transmitted to their hosts through an insect vector, can readily replicate and survive for long periods in their intermediate insect hosts without damage. However, these viruses have RNA genomes that carry several viral accessories, which makes the virus less dependent on the cell machinery.

Little is known about the biology of densoviruses, although in recent years several densovirus genomes have been cloned. In the current work, two new densoviruses have been characterized and their expression and tropism mechanisms have been explored. The first objective of this thesis was to study the molecular biology of three densoviruses; *Mythimna loreyi*, *Galleria mellonella* and *Helicoverpa armigera* densoviruses (MlDNV, GmDNV and HaDNV), respectively. These viruses were isolated from lepidopteran pests that are economically important in agriculture. Their genomes were cloned and the complete sequence and genomic organization were determined. They have genomes of about 6 kb and ITRs of around 550 nts which are the longest among all parvoviruses. A high level of sequence identity (90%) was found among them. The cloned genomes were shown to be infectious both *in vitro* and *in vivo* by initiating infections typical to that of the wild-type virus, after transfection. Studying their expression showed that they employ alternative

splicing and leaky scanning mechanisms to produce three nonstructural proteins (NS1-3) from one viral transcript. These viruses use a leaky scanning mechanism to produce a set of four structural proteins (VPs) from one transcript produced from the complementary strand without employing splicing or internal ribosome entry sites.

The second objective was to localize the allotropic determinants that control the tropism of GmDNV and MlDNV. The two viruses share over 90% sequence identity but differ in their host preference. MlDNV is polyspecific, infecting several lepidopteran species, whereas GmDNV is restricted to its host *Galleria mellonella*. The two infectious clones, pMl28 and pGm1 of MlDNV and GmDNV, respectively, were used to swap a number of domains that covered the whole genome to obtain chimeric genomes. We established a cellular system, consisting of two cell types, LD652 that is permissive for both viruses and T-ni03 that is permissive for MlDNV only, as markers to distinguish these phenotypes. The data obtained after transfection of the different viral chimerae in these cells showed that neither the coding sequences nor the viral origin of replication were responsible in controlling the virus tropism. The transfer of the MlDNV VP promoter into GmDNV background extended the tropism of GmDNV to T-ni03 cells. However, the full expression of GmDNV in these cells needed also the trans-activation of the viral NS proteins. These data showed that the *in vitro* tropism of these densoviruses is controlled by the two viral promoters of NS and VP, respectively, in contrast to the allotropic determinants of vertebrate parvoviruses.

The third objective was to study *in vitro* the safety of densoviruses MlDNV and GmDNV as a first step before proceeding towards homologation (regulatory standards for biological control agents). Both infection by virus preparations and transfection by the viral infectious clone were carried out on several mammalian cell types, COS-7, 293 T and PT as well as L929. Using indirect immunofluorescence assays, no virus replication could be detected in mammalian cells. However, in insect cells, virus antigens were readily detected after infection and transfection. Molecular studies were also carried out to define the level at which the virus replication was blocked. These tests were performed specifically with

L929 cells since they were previously reported to be permissive for densovirus infection. Virus transcription in these mammalian cells was not detected even after the transfer of the virus genome into the cells by transfection. Viral DNA could integrate into L cell chromosomes after transfection but not after infection, which indicates a blockage in the virus DNA transfer to the nucleus. However, after integration no viral transcription could be detected. Thus, our results contradict the previous reports about the permissiveness of mammalian cells to densoviruses.

The collective data presented in the current thesis contribute to the understanding of the biology of densoviruses. These viruses use unique expression strategies compared to other members of *Parvoviridae*. They do not infect mammalian cells in culture, and hence, *in vivo* homologation tests that are required before proceeding to field applications are expected to be successful.

Mohamed El-Far

Dr. Peter Tijssen
Research Director

To the memory of my big brother, Aly

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LIST OF ABBREVIATIONS AND SYMBOLS

Å	:	Angstrom
a.a.	:	amino acid
AB	:	antibody
bp	:	base pair
BSA	:	bovine serum albumin
CAT	:	chloramphenicol acetyl transferase
CPE	:	cytopathic effect
cpm	:	counts per minute
DNV	:	densovirus
DPE	:	downstream promoter element
EMSA	:	Electrophoretic Mobility Shift Assay
FACS	:	Fluorescence-Activated Cell Sorter
FBS	:	fetal bovine serum
ffu	:	fluorescent forming unit
GFP	:	green fluorescent protein
GmDNV	:	*Galleria mellonella* densovirus
HaDNV	:	*Helicoverpa armigera* densovirus
hrs	:	hours
IF	:	immunofluorescence
Inr	:	Initiator
ITR	:	inverted terminal repeat
kb	:	kilo bases
kDa	:	kilo Dalton
kV	:	kilo volts

MlDNV	:	*Mythimna loreyi* densovirus
NS	:	non-structural protein
nts	:	nucleotides
OD	:	optical density
ORF	:	open reading frame
PBS	:	phosphate-buffered saline
PLA2	:	phospholipase A2
RCR	:	rolling circle replication
REN	:	restriction endonuclease
rpm	:	rotation per minute
RT	:	room temperature
S	:	sedimentation coefficient
ssDNA	:	single-strand deoxyribonucleic acid
$TCID_{50}$	:	tissue culture infectious dose causing 50% cytopathic effects
U	:	unit
UV	:	ultraviolet
V	:	volt
VP	:	viral protein
VP1up	:	viral protein 1 unique part
w	:	weight

PREFACE

The history of mankind has been marked by two great revolutions. The agricultural revolution, around 10 thousand years ago, enabled man to establish secure and constant colonies as well as a social organization that has been gradually urbanized over time. The second was the industrial revolution, mostly in the 19th century, which resulted from the accumulation of the technological knowledge over the past millennium. These revolutions have permitted an unprecedented growth in the world population. This increase in the population also provided the industry with intellectual and labor forces to serve different aspects of its structure. The industry by itself enabled newer and higher standards for mankind, which with other factors accelerated the population growth as well as increased the average of life span.

The twentieth century, which was remarkable due to technological, medical and socio-political changes, has witnessed unparalleled growth of the world population particularly after World War II. According to the report of the U.S. Bureau of the Census (World Population Profile: 1998), the world population has tripled in the last century. Such an increase in the population size required huge changes in agriculture by the reclamation of new land in order to produce enough food for the populations and their domestic animals. Increasing the cultivated land, especially for monocultures, has affected the structure of insect pest populations. Insect populations increased to high concentrations leading to enormous damage and losses in the yield of agricultural products. Several methods have been adapted to reduce pest populations. Among these methods were the application of chemical pesticide that are effective but environmentally harmful. A persistent and broad-spectrum compound, often termed the "miracle" pesticide, DDT, emerged and was widely used in the world from the early 1940s until the mid 1970s when it was banned from use in the Americas. In just a few decades, the use of DDT and many other registered chemical pesticides against several types of insect pests as well as most important disease-carrying vectors (of malaria, Chagas disease and

leishmaniasis) became a world wide practice. These applications have contaminated the environment and affected the quality of agricultural products. They also affected human and animal health, destabilized the natural balance between insects and their natural enemies by their non-target effects and provoked resistance in insect pests against many chemical treatments.

The United Nations (U.N.) organized on 16 June 1972 a summit in Stockholm on the global environment and development. All nations shared the notion that practices that reduce the harm to the environment should be supported. This was confirmed in 1992 during the Earth Summit in Rio de Janeiro with the U.N. declaration: "Human beings are at the centre of concerns for sustainable development. They are entitled to a healthy and productive life in harmony with nature" (Principle 1. Report of the United Nations conference on environment and development, Rio de Janeiro, June 3-14, 1992).

Alternatives to the use of chemical pesticides in agriculture are essential. In this regard, biological control of insect pests to achieve good protection of agricultural products without harming the environment is promising. It was practised for a long time but has been more emphasized during the last few decades. The term biological control refers to the use of natural enemies such as pathogens (viruses, bacteria, fungi, protozoa and nematodes), parasitoids and predators, to reduce the damage caused by a pest population. It is, in turn, a major component of the so called Integrated Pest Management (IPM). The strategy of IPM represents a multi-tactic approach that considers all factors affecting crop health, including plant nutrition, horticultural practices, and all suitable means of pest suppression to levels below an economic threshold. In many cases, single means are not adequate or do not achieve a healthy and economically viable crop. This may be due to the sheer multitude of factors affecting a biological agent which makes it difficult for one practice or control factor to provide a

unique solution. This, in turn, requires a variety of choices in order to fit with the goal of the site-specific IPM strategy. Therefore, the key for a successful IPM strategy resides in its adaptability, i.e., it should be flexible enough to incorporate, or to alternate, appropriate practices or control factors in a continuous fine-tuning whenever a current one fails to meet the goals.

INTRODUCTION

The number of densovirus isolates from a variety of insect orders and families is increasing rapidly. Several studies have demonstrated that these viruses are ubiquitous pathogens associated with their hosts whether in acute or occult forms to control their populations. Their acute effect, mortality, takes effect a few days after infection depending on the inoculating virus concentration and the insect larval stage. One of the most prominent manifestations of infection of insects is the loss of appetite early after infection, a symptom that is highly desired in biological control programs. The application of these viruses as biological control agents would be of great importance particularly in regions where chemical pesticides are widely employed. In Egypt, the populated and exploited land for agriculture takes up only 5-7% of the total surface (one million km^2) with a population exceeding 70 millions. This restricted land is extensively used three to four times each year in order to produce enough food for the growing population. The monoculture practices consequently led to the concentration of insect pest populations which required the extensive use of broad-spectrum chemical pesticides. The use of viruses as well as other insect pathogens in controlling their host pest populations is critically needed in order to reduce the harmful effect of these chemical pesticides on the environment and water table. In recent years, several densoviruses have been isolated from different locations in Egypt in a collaboration research program by the Faculty of Agriculture of Cairo University, Egypt, the IRD (Institut de la recherche pour le développement, France) and INRS - Institut Armand-Frappier, Canada). Among these viruses, MlDNV and HaDNV, two potential candidates for biological control, are studied in this thesis.

The research on the biology of densoviruses is still in its early stages. However, during the past 10 years, an increasing number of publications described the cloning and determination of the complete genome sequence of members from the different genera of *Densovirinae*. The cloned genomes have the ability to initiate typical infection when

transfected both *in vitro* and *in vivo*. The last characteristic is of special interest since it would facilitate the research on the biology of these viruses *in vitro*. The densoviruses share with the *Parvoviridae* members the property of possessing a mono-partite linear single-stranded DNA genome. The telomeres of this genome carry hairpin structures that are included either in ITRs (inverted terminal repeats) or in unique sequences and which function in DNA replication. Members of the densovirus genus are unique among all parvoviruses by having an ambisense genomic organization in which both viral strands code for viral proteins. The expression strategies of these densoviruses are not yet clear. However, some evidence points to their use of a leaky scanning mechanism to produce a set of N-terminal extended structural proteins (Bergoin and Tijssen, 1998).

Factors that control the virus tropism in densoviruses are not yet studied. On the other hand, the tropism of vertebrate parvoviruses is controlled by a few number of a.a. found usually on or near the three-fold spike on the viral capsid protein. However, a few reports raise the importance of the intracellular environment in determining permissiveness for parvovirus infection.

In the first chapter of this thesis, a comparison has been made with vertebrate parvoviruses with which the densoviruses share some basic characteristics. The second chapter includes the materials and methods that were employed in this research and which were not described in the already published parts of this thesis. The subsequent chapters introduce the results obtained during the course of my research project and which gave rise to three publications. Two of these articles summarize the work that has been done on the molecular biology of the potential biological control agents, GmDNV and MlDNV, in chapters III and IV. A separate part, chapter V, has been dedicated to the cloning, sequencing and determination of the genomic organization of a newly isolated densovirus (HaDNV), a new member of the classical densoviruses. The

subsequent chapter VI details results on densovirus tropism that have been carried out on GmDNV and MlDNV by creating several chimeras. Manuscripts of the work described in chapters V and VI are in preparation. Chapter VII includes the third published article that describes the work that has been done on the safety of densoviruses in terms of infection of mammalian cells *in vitro*. Finally, the last chapter constitutes a general discussion in which the obtained results are compared to recent publications. This chapter emphasizes the impact of these results on densovirus research as well as some perspectives that arise from our findings.

CHAPTER I :
REVIEW OF LITERATURE

VIRUSES AND BIOLOGICAL CONTROL

During the last few decades, entomopathogenic viruses, a group of invertebrate pathogens that is highly divergent and host-specific, have been regarded as an effective and important factor in biological control programs. Viruses that are primarily or exclusively found in insects are currently placed in 12 families and one unclassified group (Miller, 1998). These insect pathogens have been considered as advantageous biological insecticides due to their important regulatory effects on insect populations rather than being eradication tools. They do not pollute the environment, do not persist in the food chain, are specific for their insect targets, and consequently, they do not harm beneficial insects or the ecosystem (Agathos, 1991).

Several successful examples of the use of viruses in insect pest control have been reported, of which the best-known are the baculoviruses (Yearian and Young, 1981; Granados and Federici, 1986). Applications of wild-type baculoviruses have successfully provided pest control for soybeans in Brazil and the pine and spruce forests of North America against the ravages caused by holometabolous larvae (Possee and King, 1994; Moscardi, 1999).

Baculoviruses are known for their slow killing effect. Therefore, success during the application is strongly dependant on the crop to be protected, i.e. whether or not it can tolerate some moderate level of damage (McLeod *et al.*, 1978; Smith *et al.*, 1987). This disadvantage led to the development of genetically engineered baculoviruses (GEBs). These genetically modified viruses are designed to carry genes for exogenous proteins which would speed up the control of insect pests. Yet, their use is still controversial due to safety concerns (Carbonell and Miller, 1987; Maeda, 1989; Wood and Granados, 1991; Huang *et al.*, 1997). Thus, other types of viruses that act rapidly will be of great

importance as viable alternatives to chemical pesticides. As an example, the polydnaviruses, which are introduced into insect larvae through parasitism to temporarily reduce or knock out the insect immunity in favour of a parasitoid, are potential candidates for biological control with insect viruses (Lavine and Beckage, 1995).

An emerging group of insect parvoviruses, termed the densoviruses (DNVs), contains natural pathogens that are highly virulent to their hosts and are very promising as biological agents. In oil palm fields in Colombia and Ivory Coast infested with *Sibine fusca* and *Casphalia extranea*, respectively, application of as few as 10-50 infected larvae/ha was sufficient to achieve over 90% protection (Genty and Mariau, 1975; Belloncik, 1990). Field trials to control different species of medically important mosquitoes with a DNV isolated from the mosquito *Aedes aegypti* showed that the virus had significant efficacy and could successfully be used in the biocontrol of these insects (Buchatsky *et al.*, 1987). Several other densoviruses have great potential as part of the biological control arsenal but are still awaiting a risk assessment (Belloncik, 1990; Bergoin and Tijssen, 1998; Fédière, 2000).

Insect viruses and safety concerns

Since they have very small genomes, viruses as obligate intracellular parasites are well adapted to the biology of their host cells to initiate and to complete their life cycle. Evidence of genetic differences between invertebrate and vertebrate cells, such as promoter elements and transcription initiator sequences required for exact transcription, was previously shown (Courey and Tjian, 1988; Santoro *et al.*, 1988; Kutach and Kadonaga, 2000). However, potential health hazards for such insect pathogens should be addressed before their use in biological control programs. Both *in vitro* and *in vivo* tests

dealing with pathogenicity and toxicity of insect viruses should be carried out on domestic mammals as well as on wildlife animals such as birds and aquatic organisms (Gröner, 1986). There is no solid report about a clear pathogenicity of an insect virus on vertebrates. However, several unconfirmed reports claimed the replication of certain insect viruses in mammalian cells. Kurstak *et al.* (1969 a and b) published the first controversial report on an insect parvovirus, isolated from the greater wax moth *Galleria mellonella* (GmDNV) as a pathogen for mammalians. In their work, they indicated that the virus can infect and transform murine L cells in culture. They used direct immunofluorescence to detect viral antigens in cells infected with purified virus inocula. Several unsuccessful attempts were subsequently made *in vitro* to reproduce such infections either with GmDNV (Kawase *et al.*, 1990) or AalDNV, another type of densovirus isolated from mosquito cells C6/36 derived from *Aedes albopictus* (Jousset *et al.*, 1993). In a similar case, McIntosh and Shamy (1980) reported that the baculovirus, *Autographa californica* multicapsid nucleo-polyhedrovirus (AcMNPV) can fairly infect the Chinese hamster cells (CHO), by showing an increase in virus titers after infection. Conversely, Gröner *et al.* (1984) failed to reproduce the results of McIntosh and Shamy using the same methodology. Thus, they interpreted their observations simply as a matter of insect virus uptake in mammalian cells followed by gradual elution in the cell culture medium, a case that would explain the results of Kurstak and co-workers on the insect parvovirus. Li *et al.* (1997) showed that an entomopoxvirus from *Amsacta moorei* (AmEPV) can readily enter vertebrate cells and moreover can express early genes followed by an abortion in the infectious cycle. It is noteworthy to mention that in certain cases, cells can adsorb and partially engulf viruses, even though they do not support virus multiplication (Allison and Valentine, 1960; Dales, 1973). This may also be the case for the entry of several insect viruses into vertebrate cells (Tjia *et al.*, 1983; Granados, 1987).

Insects arose early in the evolutionary pathway and mechanisms that control their biology should carry striking differences to the later emerging vertebrates. Several studies have been made to explore the transcriptional machinery of insect cells (Hoey *et al.*, 1990; Hoey *et al.*, 1993; Kokubo *et al.*, 1993; Arkhipova, 1995; Burke and Kadonaga, 1996; Burke and Kadonaga, 1997). One of the most studied basal eukaryotic promoter elements is the TATA box, which is usually located at -25/-30 bp from the RNA start site. This sequence is recognized by the TATA binding protein (TBP), which induces DNA bending and participates in the transcription by all three RNA polymerases (Hernandez, 1993). Although, a significant number of TATA-less promoters have been recognized in vertebrates such as the human interferon regulatory factor-1 (IRF-1) gene, the TATA box is the most common core promoter element (Thomas *et al.*, 1997). However, almost one half of studied Drosophila promoters can be classified as TATA-less promoters (Arkhipova, 1995). Burke and Kadonaga (1996) recognized a conserved downstream core promoter element (DPE) within TATA-less promoters in Drosophila, which is required for sequence-specific binding of transcription factor TFIID. The DPE is a distinct 7-nucleotide element that is located at about + 30 (typically, from + 28 to + 34) relative to the transcription start site. This element acts in conjunction with the initiator sequence, Inr (CATG), to provide a binding site for TFIID in the absence of a TATA box. Decreased transcription levels resulting from disrupting the TATA box in TATA-containing promoters could be recovered by the insertion of a DPE at a downstream position (+ 28 to + 34) in the defective promoters (Burke and Kadonaga, 1996). DPE-like motifs were also found in mammalian promoters that are TATA-less. Thomas *et al.* (1997) found that the promoter of the genes encoding human (h) and mouse (m) interferon regulatory factor-1 (Miyamoto *et al.*, 1988; Sims *et al.*, 1993) is TATA-less and contains a sequence that conforms to the 7-nucleotides DPE consensus sequence at the appropriate + 30 downstream position.

Interestingly, by studying 205 Drosophila promoters, Kutach and Kadonaga (2000) showed that DPE with only six nucleotides, is positioned exactly from + 28 to + 33 to the transcription start site. The element is present as often as the TATA box, with the majority of core promoters containing either a TATA box or a DPE (Fig. 1). The DPE, with the consensus A/G/T-C/G-A/T-C/T-A/C/G-C/T, exhibits a strict spacing to the Inr sequence, where introduction or deletion of a single nucleotide between both motifs causes a reduction in transcriptional activity and TFIID binding. The large distribution of these new promoter elements within insect genes signifies an evolutionarily distant machinery for transcriptional regulation to which the invertebrate viruses have to adapt themselves.

Another striking difference resides in the absence of the Sp1 transcription factor from certain insect cells such as Drosophila SL2 cells that are used in the study of this factor (Courey and Tjian, 1988). This would render a GC rich motif in the promoter region nonfunctional except that another homologous Sp1 factor is present in insect cells (Tijssen and Bergoin, 1995). On the other hand, insect genes usually have a CAGT-box at the site of transcription initiation, located 25-30 nts downstream from the TATA box and that fits with the consensus arthropod initiator element (A/C/T)CA(G/T)T (Blissard and Rohrmann, 1989; Cherbas and Cherbas; 1993; Pullen and Friesen, 1995).

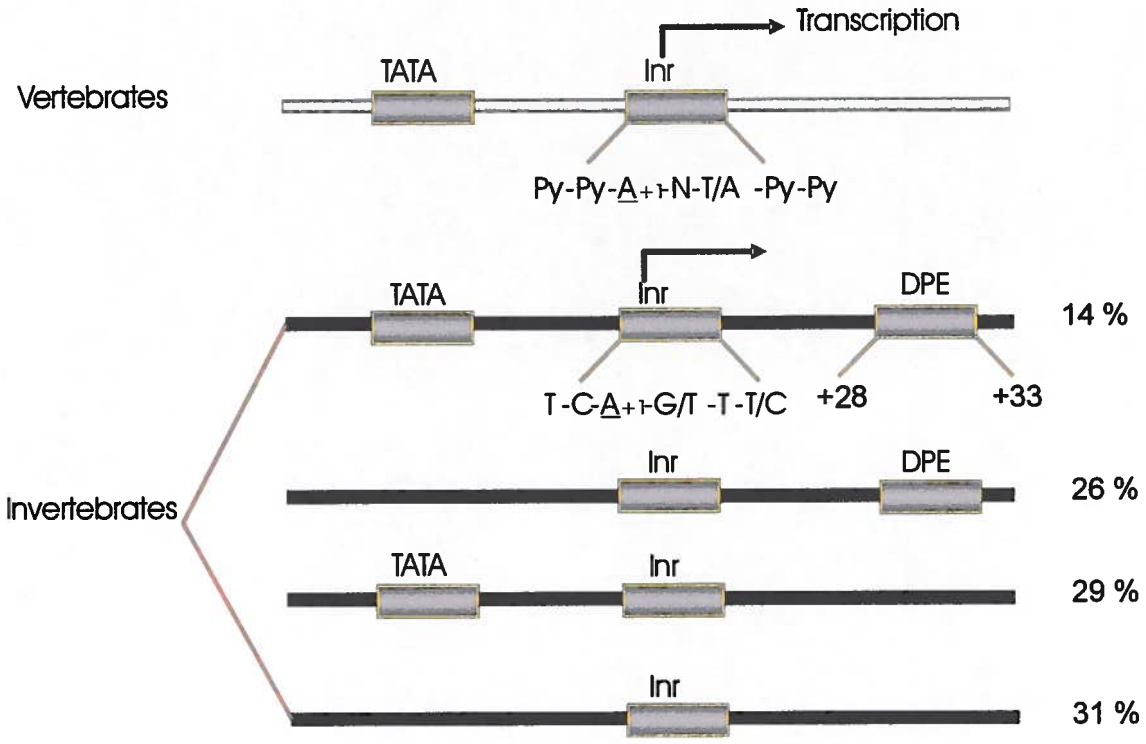


Fig.1. Distribution of the potential DPE in Drosophila promoters (adapted from Kutach and Kadonaga, 2000). Comparison of conserved initiator sequences between vertebrates and invertebrates. Numbering under the DPE is relative to the A_{+1} transcription start site.

Genotype history

Densoviruses constitute a group of pathogens that are highly pathogenic and fatal to their hosts. They were first reported in 1964 when Meynadier and his colleagues observed a high mortality within the insect rearing facility of larvae of the greater wax moth *G. mellonella* in their research unit at St-Christol, Montpellier (France). They observed an outbreak caused by a new virus that was identified as a densonucleosis virus according to its cytopathological symptoms (Meynadier *et al.*, 1964; Vago *et al.*, 1964). Shortly after the isolation of GmDNV, BmDNV-1 was discovered in sericultural farms in Ina City, Japan (Kawase *et al.*, 1976; Watanabe *et al.*, 1976; Kawase *et al.*, 1985). Since then, there are an increasing number of densoviruses isolated from different insect orders especially Lepidoptera comprising economically important butterflies and moths. Due to their virulence and efficiency in stopping insect feeding early after infection, densoviruses are highly attractive for pest control (Belloncik, 1990; Bergoin and Tijssen, 1998).

Taxonomic structure of *Densovirinae*

All small (20-25 nm) icosahedral non-enveloped viruses containing linear, single-stranded DNA genomes, are considered to be parvoviruses (Tijssen and Bergoin, 1995). Densoviruses are thus members of the *Parvoviridae* family that contains two subfamilies; *Densovirinae* with members exclusively infecting invertebrates and *Parvovirinae* with members infecting vertebrates (three genera: *Parvovirus* and *Erythrovirus*, as autonomously replicating viruses, and *Dependovirus* with members that need a helper virus). Today, 40 years after the first report on their occurrence, densoviruses have been found to be

ubiquitously infecting many insect species that belong to different orders: economically important lepidopteran insects, medically important Diptera, as well as Dictyoptera, Orthoptera and Odonata (Table 1) (Reviewed by Bergoin and Tijssen, 1998 and Fédière, 2000, Tijssen *et al.*, 2004). Interestingly, densoviruses were also reported in hosts outside the class Insecta, in Decapoda, (shrimps and crabs), leading to interesting questions about the evolution of this emerging group of viruses (Lightner and Redman,1985; Shike *et al.*, 2000).

Very little is known about the molecular biology of densoviruses since they constitute an emerging group of viruses, and hence, their classification is still far from being settled. Thus far, there are around thirty densoviruses divided into three genera, *Densovirus, Iteravirus* and *Brevidensovirus* (van Regenmortel *et al.*, 2000). As a result of the growing knowledge about these densoviruses, the diversity of their host of isolation, the cloning of their complete genomes and determination of the genomic organization, the first genus can now be divided into three subgroups (Tijssen *et al.*, 2004). Subgroup A contains the classical ambisense densoviruses usually isolated from lepidopteran insects with JcDNV (Jousset *et al.* 1990; Dumas *et al.* 1992) and GmDNV (*Galleria mellonella* DNV; work presented in the current Thesis) as genotypes. Subgroup B contains members with overall ambisense organization isolated from non-lepidopteran hosts, but differ in their fine organization of the coding sequence with PfDNV and AdDNV as members. The third subgroup C contains, so far, only one ambisense member, the CpDNV isolated from *Culex pipiens* (Jousset *et al.*, 2000). The second genus, *Iteravirus*, has so far only two members, *Casphalia extranea* densovirus, CeDNV (Fédière *et al.*, 2002) and *Bombyx mori* densovirus type 1, BmDNV1 (Li *et al.*, 2001) with monosense organization. The last genus, *Brevidensovirus*, contains viruses isolated from two distinct classes, Insecta and Crustacea. The insect brevidensoviruses are usually isolated from mosquitoes or their cultured cells (Afanasiev *et al.*, 1991; Boublik *et al.*, 1994), whereas, the crustacean

Genus Abbreviation	Insect species	Order	Reference
Densovirus			
Subgroup A:			
GmDNV	*Galleria mellonella*	Lepidoptera	Meynadier *et al.*, 1964
JcDNV	*Junonia coenia*	Lepidoptera	Rivers and Longworth, 1972
MlDNV	*Mythimna loreyi*	Lepidoptera	Fédière *et al.*, 1995
DsDNV	*Diataraea saccaralis*	Lepidoptera	Meynadier, 1977b
LdiDNV	*Lymantria dispar*	Lepidoptera	Grignon *et al.*, 1982
PiDNV	*Pseudoplusia includens*	Lepidoptera	Chao *et al.*, 1985
Subgroup B:			
PfDNV	*Periplaneta fuliginosa*	Dictyoptera	Suto *et al.*, 1979
AdDNV	*Acheta domestica*	Orthoptera	Meynadier *et al.*, 1977c
MpDNV	*Myzus persicae*	Hemiptera	Van Munster *et al.*, 2003a
PcDNV	*Planococcus citri*	Hemiptera	Thao *et al.*, 2001
Subgroup C:			
CpDNV	*Culex pipiens*	Diptera	Jousset *et al.*, 2000
Iteravirus			
BmDNV-1	*Bombyx mori*	Lepidoptera	Shimuzu, 1975
CeDNV	*Casphalia extranea*	Lepidoptera	Fédière *et al.*, 1983
Brevidensovirus			
AaeDNV	*Aedes aegypti*	Diptera	Lebedeva *et al.*, 1973
AalDNV	*Aedes albopictus*	Diptera	Jousset *et al.*, 1993
C6/36DNV*	*Aedes albopictus*	Diptera	Chen *et al.*, 2004
HeDNV*	*Haemogogus aquinus*	Diptera	O'Neill *et al.*, 1995
TaDNV*	*Toxorhynchites amboinensis*	Diptera	O'Neill *et al.*, 1995
		(Class Crustacea)	
PstDNV	*Penaeus stylirostris*	Decapoda	Lu *et al.*, 1989

Table 1A. Members of the subfamily *Densovirinae* (Tijssen *et al.*, 2004).

Abbreviation	Insect Species	Order	Reference
Class Insecta			
AvDNV	*Agriulis vanillae*	Lepidoptera	Kelly *et al.*, 1980
EaDNV	*Euxoa auxilliaris*	Lepidoptera	Sutter, 1973
PrDNV	*Pieris rapae*	Lepidoptera	Sun and Chen, 1981
SfDNV	*Sibine fusca*	Lepidoptera	Meynadier *et al.*, 1977a
BgDNV	*Blattella germanica*	Dictyoptera	Mukha and Schal, 2003
ApDNV	*Aedes pseudoscutellaris*	Diptera	Gorziglia *et al.*, 1980
SvDNV	*Simulium vittatum*	Diptera	Federici, 1976
LduDNV	*Leucorrhinia dubia*	Odonata	Charpentier, 1979
Class Crustacea			
CmDNV	*Carcinus mediterraneus*	Decapoda	Mari and Bonami, 1988
MrDNV	*Macrobrachium rosenbergii*	Decapoda	Anderson *et al.*, 1990
PmeDNV	*Penaeus merguiensis*	Decapoda	Lightner and Redman, 1985
PmoDNV	*Penaeus monodon*	Decapoda	Lightner and Redman, 1985
PoDNV	*Penaeus orientalis*	Decapoda	Lightner and Redman, 1985
PseDNV	*Penaeus semisculatus*	Decapoda	Lightner and Redman, 1985

Table 1B. Non-classified densovirus-like viruses.
* viruses isolated from cultured cells

densovirus isolates are usually from shrimps (Shike *et al.*, 2000) (Table 1). These viruses have also a monosense genomic organization.

Many members of the *Parvoviridae* family, both invertebrate and vertebrate viruses, contain complementary ssDNA molecules, of which both " + " or "-" strands are encapsidated in separate virions. These strands harbour terminal palindromes (hairpins) which serve as primers for DNA replication. Hence, parvovirus-like viruses isolated from *Bombyx mori* that were previously regarded as densoviruses, BmDNV2 (Bando *et al.*, 1992) and BmDNV3 (Gao and Cai, 1994), were excluded from this group. These viruses have a bipartite genome and lack the presence of terminal hairpins which implies a different replication mechanism (Tijssen and Bergoin, 1995; Bergoin and Tijssen, 2000).

PATHOLOGY OF INSECT PARVOVIRUSES

Symptoms of infection

Mortality due to densovirus infection takes effect between two to twenty days after infection depending on the inoculating virus concentration and the insect larval stage (Meynadier *et al.*, 1964; Vago *et al.*, 1964; Shimuzu, 1975). Infected insect larvae manifest clear symptoms which start by a delay in insect movement with a partial paralysis, body flaccidity and inhibition of molting followed by complete paralysis and death. The cuticle undergoes progressive depigmentation during the development of the infection, when the colour becomes more and more white accompanied by slow melanization and then turns dark by the death of insect (Meynadier *et al.*, 1964; Vago *et al.*, 1964; Kelly *et al.*, 1980; Chao *et al.*, 1985; Kawase, 1985). Insect larvae usually develop anorexia a few days after infection and stop feeding (Kawase *et al.*, 1990). The

infection can also affect the silk glands and decrease the secretion quality as in the case of GmDNV infection in the wax moth *G. mellonella* (Amargier, 1966). Larvae infected by densoviruses in early stages usually suffer molting problems, while infection in late stages can stop larval development in the pre-nymph stage under a high virus concentration. Larvae infected with low virus concentrations in late stages can continue to pupate but larval tissues within pupae can cause the infection to spread progressively into imaginal organs (Amargier *et al.*, 1965; Boemare and Brès, 1969).

Infected mosquito larvae manifest clear symptoms by losing their mobility and hanging near the water surface as distorted and curved comma-shaped bodies (Barreau *et al.*, 1996; Jousset *et al.*, 2000). Densoviruses can also fatally infect adults of the cockroach causing uncoordinated movement and paralysis in the hind legs and a clear swollen abdomen showing, when dissected, hypertrophied fat bodies with a high replication rate of virus (Suto, 1979).

Cytopathological and histopathological symptoms

Cytopathological symptoms of the infection by a densovirus were first described by Vago *et al.* (1964) and Amargier *et al.* (1965) as cells with hypertrophied nuclei accompanied by a strong reactivity to coloration with Feulgen. Nuclei of infected cells contain an electron-dense virogenic stroma after which the causative virus was identified as a densonucleosis virus, shortened later to densovirus or DNV (Vago *et al.*, 1966a). The histological studies on *G. mellonella* larvae infected by the densovirus GmDNV showed that the virus has a wide tissue tropism infecting the fat bodies, Malpighian tubules, hypodermal cells, hemocytes and peritracheal cells as well as the silk glands (Vago *et al.*, 1964; Amargier *et al.*, 1965; Amargier *et al.*, 1966, Bergoin and Brès, 1968). Ultrastructural changes within the infected cells consist of an increase in the number of

free ribosomes and formation of microbody-like structures as a result of the accumulation of small round bodies of a diameter of 17-20 nm inside the vesicles. The nucleolus is also marked by dramatic changes through the infection during which it undergoes hypertrophy.

As members of *Parvovirinae*, densoviruses have a ssDNA genome that varies between 4 to 6 Kb. Replicative as well as monomeric forms of their DNA can easily be detected within the infected cells using fluorescent microscopy after staining by acridine orange. A greenish-yellow colour indicates the presence of double stranded nucleic acid (replicative form) and a green to red colour indicates the presence of ssDNA (monomeric form) (Kurstak, 1972). Visible tumor lesions in the digestive tract can be associated with the infection of certain densoviruses as in the case of *Sibine fusca* and *Caspbalia extranea* caterpillars that suffer from intensified infection by densoviruses (Meynadier *et al.*, 1977; Fédière, 1983). These lesions were also observed within the hypodermal tissues, tracheal cells, muscles, and Malpighian tubules but were predominant in the intestine where it is a characteristic sign of the densovirus infection in these insects (Kawase *et al.*, 1990).

Morphology and biophysical properties of densovirus virions

In general, parvoviruses are among the smallest DNA viruses with non-enveloped icosahedral particles with a diameter ranging between 18-26 nm (Berns, 1996). Densoviruses observed by electron microscopy (Fig. 2), under negative contrast staining, show spherical particles of 22-25 nm (T value equal 1, Tijssen *et al.*, 1977). Particles of densoviruses have a molecular mass of about 5.5 to 6 MDa. The high content of DNA (20-30 %), in addition to proteins, but without lipidic envelopes, results in a high buoyant density in CsCl, 1.4-1.44 g/cm^3, and a sedimentation coefficient of

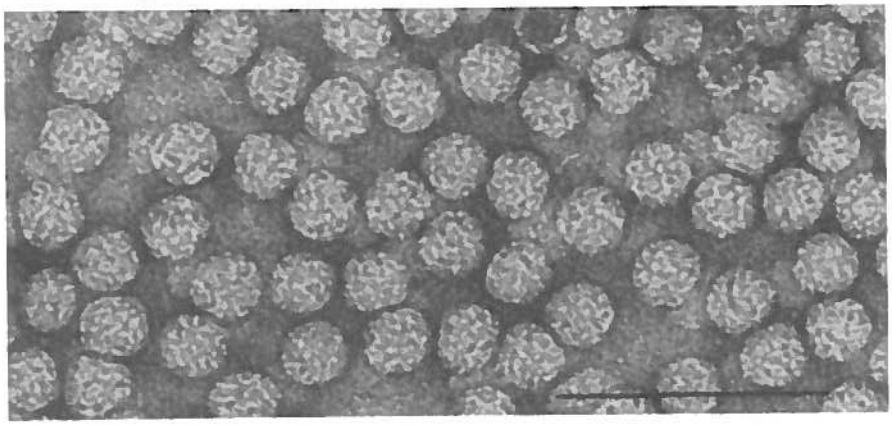


Fig. 2. Densovirus (JcDNV) as shown by electron microscopy. Bar represents 100 nm.

about 110S (Truffaut *et al.*, 1967; Longworth *et al.*, 1968; Meynadier *et al.*, 1977). Capsids of certain densoviruses such as GmDNV, JcDNV and BmDNV-1 have also been shown to harbour polyamines (spermine, spermidine and putrescine), of up to 1.5 % of the particle weight, bound to the DNA. These polyamines suffice to neutralize 25 % of the particle's DNA (Kelly and Elliott, 1977; Bando *et al.*, 1983). It is also probable that other densoviruses, or even vertebrate parvoviruses, may have polyamines but this has still not been reported.

Tijssen *et al.* (1977) showed that, upon purification of GmDNV on CsCl gradients, two populations of viral capsids can readily be distinguished: a fraction at a density of 1.40 g/cm3 with a sedimentation rate of 111 S and a second fraction at 1.44 g/cm3 with a sedimentation of 89 S. Both types contain single-stranded DNA which are identical in physiochemical properties. Another population of empty and defective-interfering particles with sedimentation of about 60S has been also reported (Longworth *et al.*, 1968; Meynadier *et al.*, 1977). The same polymorphism in the buoyant density of densovirus capsids has also been reported in the case of PfDNV (Suto, 1979) and BmDNV-1 (Nakagaki and Kawase, 1980). Interestingly, it was previously hypothesized that the presence of different populations of virions upon purification may result from the binding of bivalent cations to the capsid proteins which may affect the quaternary structure of the capsid (Tijssen *et al.*, 1977). Recent studies by Zádori and co-workers (2001) determined that the amino acids involved in the binding of Ca^{2+} (Ca^{2+} binding loop) are located within the viral protein VP1 unique region (VP1up). This region exhibits a phospholipase A2 activity and was shown to be critically needed for successful infection.

Virions of densoviruses as well as all parvoviruses are made up of 12 pentamers, each consisting of 5 units of structural proteins in a total of 60 monomers per particle

(Tijssen *et al.*, 1977). The crystal structures of particles from several vertebrate parvoviruses have been determined and have confirmed this prediction, including canine parvovirus (CPV) (Tsao *et al.*, 1991; Wu and Rossmann, 1993), B19 (Agbandje *et al.*,1994), MVM (Agbandje-McKenna *et al.*, 1998), FPV (Agbandje *et al.*, 1993), ADV (McKenna *et al.*, 1999), porcine parvovirus (PPV) (Simpson *et al.*, 2002) and AAV2 (Kronenberg *et al.*, 2001). In invertebrate parvoviruses, the GmDNV capsid was the first to be resolved at the near-atomic structure (3.7 Å resolution) making it possible to compare vertebrate and invertebrate parvoviruses (Simpson *et al.*, 1998). Comparison between the overall structure of GmDNV capsid and that of vertebrate parvoviruses shows that the former one is much smoother (Fig. 3). This structural difference may be attributed to different evolutionary pressures on both vertebrate and invertebrate viruses (Simpson *et al.*, 1998). In both viruses, the capsid ß strands are connected by long loops of up to 100 residues. In CPV, the subunit interactions between loop 3 and 4 form a type of spike at the three-fold vertices, where loop 3 forms the base and loop 4 forms the tip of the spike. The presence of such protruding spikes on the surface of vertebrate parvoviruses is a prominent feature and it has been shown in B19 to form the binding site for the receptor on the host cell (Chipman *et al.*, 1996). However, GmDNV lacks loop 4 and loop 3 is much shorter than that of CPV leading to the formation of ß-annulus type structure instead of the spike on the three-fold axes. Both the GmDNV and vertebrate parvoviruses share the presence of the ß-barrel in their capsid structure, which is common in viral proteins. However, Simpson *et al.* (1998) stated that the ß-barrel of GmDNV must be rotated by 7.4 Å and translated radially inwards by 9.7 Å to superimpose it on the ß-barrel of the capsid protein of canine parvovirus (CPV), when the rotational symmetry axes are superimposed.

The 3D-structure of AalDNV-2, from the *Brevidensovirus* genus, has also been solved to a resolution of about 16 Å by electron cryomicroscopy (Chen *et al.*, 2004).

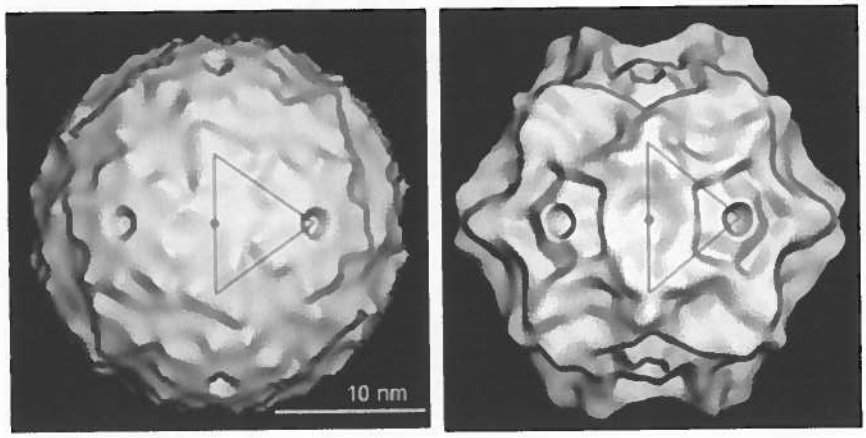


Fig. 3. Surface-rendered-image for GmDNV (left) in comparison to CPV (right) at 25 Å resolution (Simpson *et al.*, 1998).

The sequence identity of AalDNV-2 structural proteins with GmDNV is less than 20 % which is translated into some distinctive structural features. For example, AalDNV-2 particles display density ridges around the three-fold axes and prominent protrusions at the five-fold axes that are both absent in the GmDNV particle. Nevertheless, the two-fold proximal depression as well as the inner surfaces and the large cavities under the five-fold vertices are conserved. Similarly, inner surfaces were most conserved, as was observed among vertebrate parvoviruses (Simpson *et al.*, 2002).

Biochemical properties

Densoviruses are known to have a high stability against several harsh conditions such as high temperatures (56 °C for 60 minutes) and a wide range of pH (between 3-9). They can be preserved for prolonged periods in non-purified forms; however, they rapidly lose their infectivity once purified (Boemare *et al.*, 1970; Suto, 1979; Siegl *et al.*, 1985). Densoviruses are resistant to treatment with organic solvents since they do not contain essential lipids and they can also resist treatment with DNase. Yet, treating the virus preparations with formaldehyde, β-propriolactone, hydroxylamine, or oxidizing agents can readily inactivate the virus (Siegl *et al.*, 1985).

Such high resistance is highly desirable when dealing with an insect pathogen that will be used for field application as a biopesticide since it may be exposed to a variety of environmental conditions. The more resistant a pathogen is, the fewer applications are required for pest control.

Genome nature and organization

Densoviruses package a monopartite linear single-stranded DNA molecule

(ssDNA) of 4-6 Kb within their virions in either a negative or a positive polarity. The two viral strands, "-" and " + ", thus anneal upon extraction in high salt conditions (50-100 mM NaCl) to form double-stranded DNA molecules (Barwise and Walker, 1970; Kurstak *et al.*, 1971; Kelly *et al.*, 1977). The proportion between the encapsidated negative and positive strands of DNA varies according to the virus genus. In *Densovirus* and *Iteravirus* genera, both strands are encapsidated in an equimolar proportion sharing that characteristic with vertebrate dependoviruses (AAVs) (Kelly *et al.*, 1977; Berns and Adler, 1972). In contrast, in *Brevidensovirus*, AaeDNV and AalDNV, the negative strand is preferentially encapsidated (90 %) similarly to vertebrate parvoviruses such as MVM and PPV (Tijssen and Bergoin, 1995; Berns, 1996). Cloning of the complete genome of several densoviruses and hence, determination of their sequence has provided a prediction of the genomic organization of this group of viruses. Up to now, there are 17 densoviruses whose genomes have been sequenced and that fall within the three densovirus genera. The emerging number of densoviruses and determination of their sequence identity and genome organization led to a reconsideration of their taxonomy in several instances (Tijssen and Bergoin, 1995; van Regenmortel *et al.*, 2000; Tijssen *et al.*, 2004).

Members of the *Densovirus* genus have genomes of 5.5 to 6 Kb with an ambisense organization which is unique within the family of *Parvoviridae*. The classical densoviruses isolated from Lepidoptera (butterflies) such as GmDNV and JcDNV, with genomes of 6 Kb, share a high level of sequence identity that reaches around 90 %. Both of their complementary DNA strands have the capacity to code for proteins through a limited number of large open reading frames (ORFs) distributed within the 5'-half of the two complementary strands. One strand codes for structural proteins, usually 4, VP1-VP4 (Tijssen *et al.*, 1976), whereas the complementary strand codes for three non-structural proteins or Rep proteins; NS1-NS3 (Fig. 4A). The NS1 and NS3 reading frames are

separated by only one TAA stop codon, while the sequence of NS2 overlaps the N-terminal of NS1 and is translated from an overlapping reading frame. This group of viruses is very distinct from all other parvoviruses by the presence of the longest terminal repeats (ITRs) of over 500 nts (Tijssen, 1990; Dumas *et al.*, 1992). The ORFs of the NS and VP genes in these classical ambisense densoviruses are separated by about only 30 nucleotides. Since these genes are on opposite strands, this may lead to the production of antisense mRNA in this region which may play a role in the regulation of expression of NS and VP genes (Bergoin and Tijssen, 2000).

Other lepidopteran densoviruses such as DsDNV (Boublik *et al.*, 2004: GenBank accession number NC 001899), *Pseudoplusia includens* densovirus PiDNV (Tijssen, non-published data) share the same characteristics and high sequence identity with classical densoviruses. However, non-classical ambisense densoviruses isolated from orders other than Lepidoptera (Table 1), such as Dictyoptera (PfDNV), Orthoptera (AdDNV), Diptera (CpDNV) and Hemiptera (MpDNV and PcDNV) are not homogenous in genome identity and organization (Fig. 4B and C). These viruses have a shorter genome of around 5.5 Kb and structural protein molar masses as well as relative amounts that are distinct from the classical members. They also have ITRs of about 200-320 nts that are different in size and structure from those of classical densoviruses (see below). This may be due to the evolutionary distance between the different insect orders.

The other two genera of insect parvoviruses, *Brevidensovirus* and *Iteravirus*, possess a monosense genomic organization. They have a cassette of two NS genes instead of three in the case of the *Densovirus* genus and a second cassette for VP proteins, with each cassette controlled apparently by only one functional promoter (Fig. 4D and E) (Bergoin and Tijssen, 1998). However, these two genera differ in the genome length, with 5 Kb in *Iteravirus* members but only 4 Kb in *Brevidensovirus* genomes.

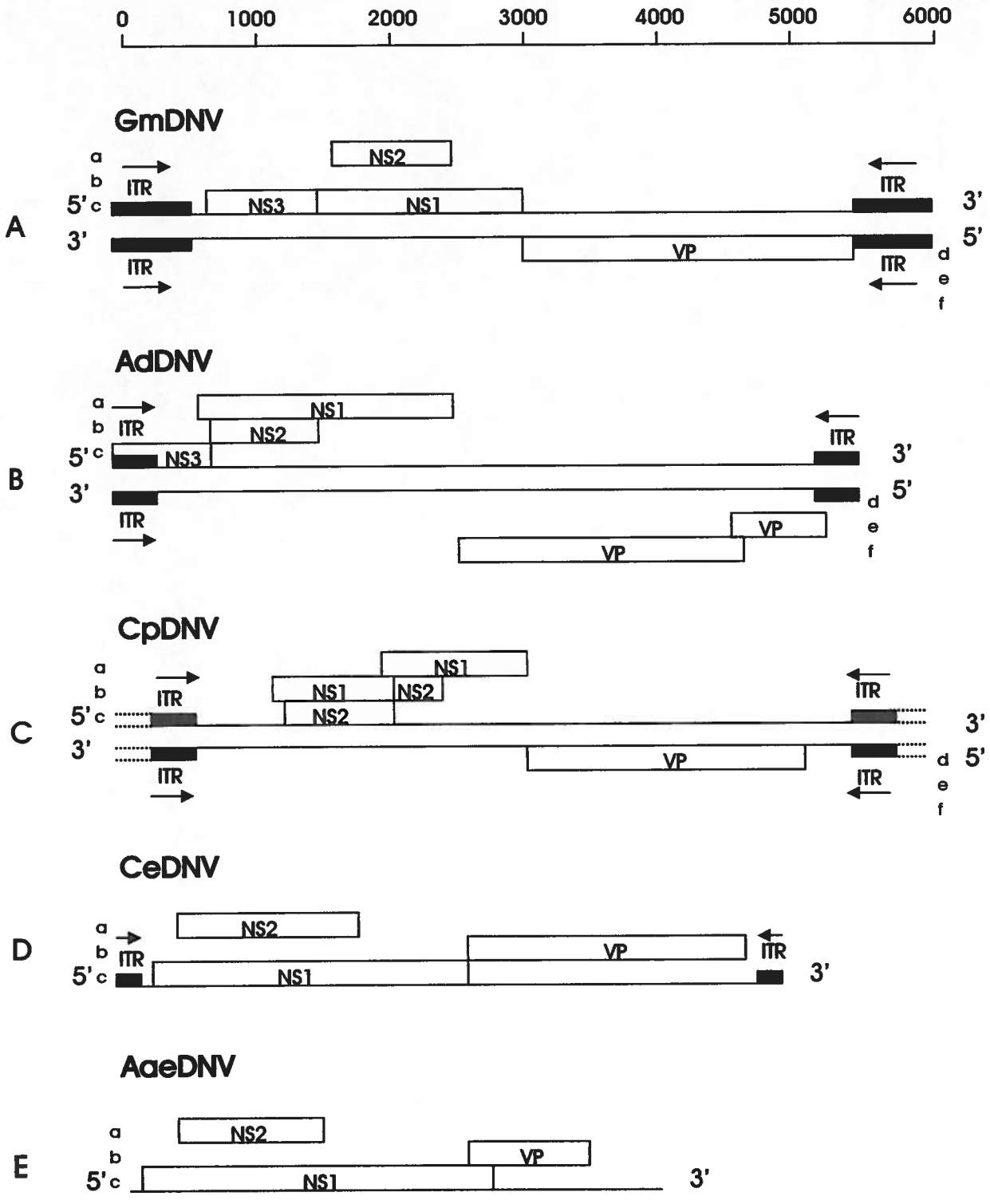


Fig. 4. Genomic organization of densoviruses from the three genera. Subgroups A, B and C: members of the *Densovirus* genus (GmDNV, AdDNV and CpDNV, respectively), D: *Iteravirus* genus (CeDNV) and E: AaeDNV, *Brevidensovirus* genus (Bergoin and Tijssen, 2000).

They also differ in genomic terminal structures. Iteraviruses (BmDNV and CeDNV) have ITRs with J-shaped hairpins of 230 nts (Li *et al.*, 2001; Fédière *et al.*, 2002). On the other hand, brevidensoviruses (Aedes densoviruses) have unlike palindromic structures with T-shaped hairpins at the two ends (Afanasiev *et al.*, 1991; Boublik *et al.*, 1994; Shike *et al.*, 2000).

Terminal structures of densovirus genome

Priming a DNA molecule during the replication process is a key step. DNA viruses have evolved a number of unusual mechanisms to prime the replication of their genome such as RNA priming, DNA priming and even protein priming (Flint *et al.*, 2000). Parvoviruses are unique among all DNA viruses by harbouring palindromic sequences, or so-called terminal hairpins, on their 3' and 5' extremities of both complementary strands. These terminal hairpins serve for DNA replication as inboard cis-acting primers for genome extension by cellular DNA polymerase(s) using oneself as a template (Tattersall and Ward, 1976; Lusby, *et al.*, 1980; Bando *et al.*, 1990; Berns, 1996). In densoviruses, the presence of these terminal palindromic structures has been proven by both direct observation using electron microscopy as well as by direct sequencing for several viruses and prediction of their secondary structures (Kelly and Bud, 1987; Bando *et al.*, 1990; Tijssen, 1990; Dumas *et al.*, 1992; Li *et al.*, 2001; Fédière *et al.*, 2002). In addition to their role in viral DNA replication, they allow the rescue of the cloned genome during experimental transfections, both *in vitro* and *in vivo* (Jourdan *et al.*, 1990). However, the size and the secondary structure of these terminal sequences vary considerably among densoviruses as well as among the different members of *Parvoviridae*.

Classical densoviruses within the *Densovirus* genus have the longest identical

ITRs. Dumas *et al.* (1992) showed that the JcDNV has ITRs of 517 nts long, which exceeds the size of all known parvovirus ITRs, such as B19 of 383 nts (Deiss *et al.*, 1990) and AAV2 of 145 nts (Lusby *et al.*, 1980). The distal 96 nucleotides form a Y-shaped hairpin which is typical for parvovirus termini with the flip and its complement flop configurations (Fig. 5A). This terminal dimorphism results from the hairpin transfer during the DNA replication (Bando *et al.*, 1990; Bergoin and Tijssen, 2000). Nevertheless, non-classical densoviruses have shorter ITRs with a different structure. For PfDNV, the ITRs are 202 nts long with the distal 120 nts forming the hairpin which is a simple form of folding back (Yamagishi *et al.*, 1999). Another densovirus isolated from the cockroach *Blattella germanica* (BgDNV) (Mukha and Schal, 2003) has also ITRs of 216 and 217 nts at the two ends from which the distal 192 nts form an I-shaped hairpin.

Iteraviruses, BmDNV and CeDNV, the only two known viruses in this genus, have short ITRs of 230 nts that occupy around 9% of their genomes. Their terminal repeats, existing in an imperfect palindrome, forming a J-shaped hairpin of 159 nts, can be found in either form, flip or flop (Fig. 5B) (Li *et al.*, 2001; Fédière *et al.*, 2002). Brevidensoviruses, including densoviruses isolated from mosquitoes or their cell lines, AaeDNV (Afanasiev *et al.*, 1991), AalDNV (Boublik *et al.*, 1994) and C6/36DNV (Chen *et al.*, 2004) have the smallest genomes among all parvoviruses (4009, 4176 and 4094 nts, respectively). Their terminal repeats form unlike hairpins on their 5' and 3' termini, therefore, lacking ITRs, and resembling the majority of vertebrate autonomous parvoviruses. However, these terminal hairpins can fold back on themselves into T-shaped structures with 134 nts and 182 nts at either ends (Fig. 5C). Brevidensoviruses share, with several vertebrate parvoviruses, the property to predominantly encapsidate strands with negative polarity. This may be a consequence of the absence of ITRs and the presence of putative packaging signals at one of the two unlike terminal hairpins

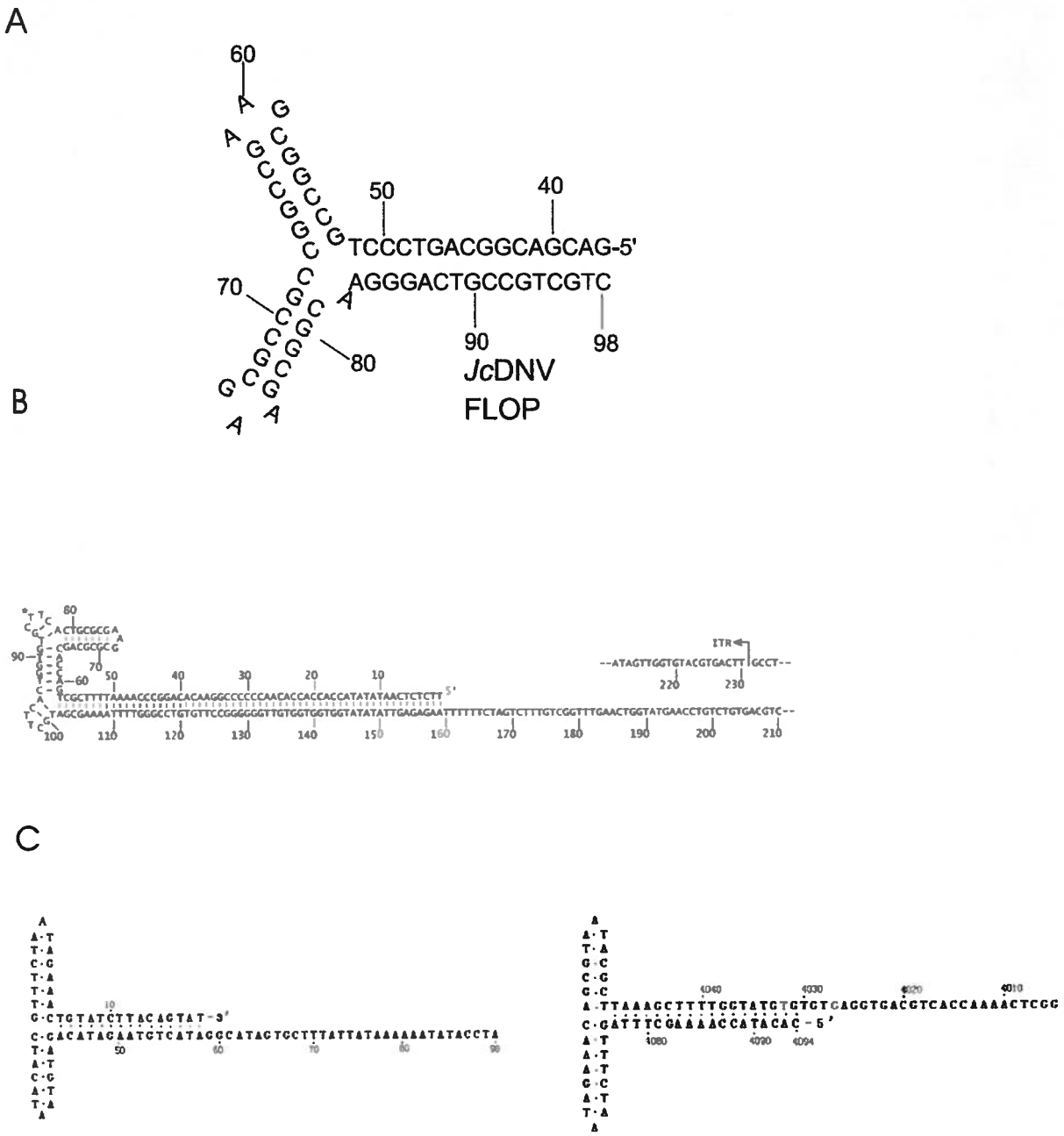


Fig. 5. Comparison between the terminal hairpin secondary structure of members from the different genera under the *Densovirinae* subfamily: A) JcDNV for *Densovirus* genus (Dumas *et al.*, 1992), B) BmDNV-1 for *Iteravirus* genus (Li *et al.*, 2001), and C) C6/36DNV for *Brevidensovirus* with unlike hairpins at 5' to the right and 3' to the left (Chen *et al.*, 2004).

(Cotmore and Tattersall, 1989). Other putative brevidensoviruses were isolated from shrimps, such as IHHNV (Lighthner *et al.*, 1983; Kalagayan *et al.*, 1991). However, the terminal sequence obtained thus far from this virus does not form the typical Y- or T-shaped hairpin, which may be the result of a partially cloned genome since it is only 3873 nts long (Shike *et al.*, 2000).

VIRAL PROTEINS

Structural proteins

Densovirus capsids are assembled from 60 copies of essentially one structural protein. This protein can have different N-terminal extensions, as shown by peptide mapping (Tijssen *et al.*, 1976, Tijssen and Kurstak, 1981) as well as by X-ray crystallography (Simpson *et al.*, 1998). This has also been demonstrated by an in-frame insertion of the β-gal reporter gene in the C-terminal region of the VP coding sequence which resulted in VP2-, VP3- and VP4-β-gal fusion proteins (Giraud *et al.*, 1992). Thus, two to five different isoforms of VPs, which usually have molecular masses between 40 and 100 KDa, can be detected in densoviruses. The production of these N-terminal isoforms of viral proteins is a strategy employed by densoviruses to maximise the coding capacity of their genomes which are limited to 4-6 Kbs. About half of the genome is dedicated to produce 4 structural proteins coded by one gene. However, the roles of these N-terminal extensions are thus far unknown.

Classical densoviruses, all isolated from lepidopteran insects, have genomes of around 6 kb and an ambisense organization. Sequence identities between the different members; GmDNV (Tijssen *et al.*, 1976), JcDNV (Dumas *et al.*, 1992), MlDNV (Fédière *et al.*, 1995) and PiDNV (Tijssen, non-published data) is about 80-90 % and they have

strong serological cross-reactivities among their structural proteins. Their virions are made up of 4 VPs, with molecular masses of about 47, 53, 58 and 89 KDa, that are produced from a unique open reading frame (ORF). The four proteins are usually found in different ratios within the viral particle and the most abundant one is VP4. In GmDNV, the ratio between VP1, VP2, VP3 and VP4 is 2:15:15:68, respectively (Tijssen *et al.*, 1976 and 1977) while it is 14:26:21:39 in JcDNV (Fédière, 1983). This composition can change from batch to batch of the same virus (Tijssen and Kurstak, 1981). Interestingly, VP4, the smallest and the most abundant capsid protein, can form a virion or the virus-like particle by itself when expressed in a baculovirus system (Croizier *et al.*, 2000). Therefore, N-termini of VP1-3 seem to have accessory roles, for example during cell entry. The capacity of virus-like particles, formed by one viral protein, to package a full genome size as well as their biological activity was recently addressed (Abd-Alla, 2003). *In vitro* transfection using a mutated JcDNV genome in which the first three ATGs corresponding to the initiation codons of VP1-VP3 were knocked-out, thus producing only VP4, showed that VP4 particles were able to package genome-size DNA. Nevertheless, *in vivo* transfection by the same truncated genome did not result in a viable infection.

Non-classical ambisense subgroup B and C densoviruses such as PfDNV (Hu *et al.*, 1994), AdDNV (Meynadier *et al.*, 1977), MpDNV (van Munster *et al.*, 2003b), PcDNV (Thao *et al.*, 2001) and BgDNV (Mukha and Schal, 2003) isolated from orders other than Lepidoptera, have shorter genomes of around 5.5 Kb with a different genome organization. They express their structural proteins from two ORFs and employ splicing to connect these ORFs (ORF A and B in the case of PfDNV, yielding five VPs of 52, 56, 79, 82 and 105 KDa) (Yamagishi *et al.*, 1999). Such strategies, employed by PfDNV and PcDNV, are unique among *Densovirus* members and may lead to its re-classification into a separate genus within the *Densovirinae* (Guo *et al.*, 2000; Thao *et al.*, 2001). Another

striking difference is the low sequence homology among the different members as well as the variations within the molecular masses of their structural proteins. A subgroup C densovirus (CpDNV) isolated from mosquitoes *Culex pipiens* (Diptera: Culicidae) shares with members of subgroup A the genome size of 6 Kb and the presence of 4 structural proteins. However, these proteins are different sizes (90, 64, 57 and 12 KDa). CpDNV is also more serologically related to JcDNV than to mosquito Brevidensoviruses that are mainly isolated from Diptera (Jousset *et al.*, 2000).

The Iteraviruses, BmDNV1 and CeDNV, produce five structural proteins, VP1, 2, 3, 3' and 4 of 77, 70, 57, 56, 50 kD and 76, 65, 56, 55, 48 KDa, respectively (Li *et al.* 2001; Fédière *et al.*, 2002) instead of previously reported four proteins (Nakagaki and Kawase, 1980; Fédière *et al.*, 1986).Translation of VP3 starts at two closely-spaced ATGs by a leaky scanning mechanism resulting in two forms,VP3 and VP3', which are almost identical in their molecular mass. VP2 and VP4 are the most abundant in the icosahedral particle making up to 52 molecules out of the 60, whereas the remainder are formed by VP1, VP3 and VP5 (Fédière *et al.*, 1986). Members of the third genus, *Brevidensovirus,* produce a limited number of different structural proteins varying between only one, as in the case of IHHNV (Bonami *et al.*, 1990) to two structural proteins of around 40-70 KDa in AaeDNV and AalDNV (Afanasiev *et al.*, 1991; Jousset *et al.*, 1993).

Role of the N-terminal extension of the structural proteins

In order to maintain and to reproduce themselves, viruses have to protect their genomes from damage, a characteristic which relies on the virion proteins. Flint *et al.* (2000) concluded that the virion proteins are multi functional. They can act as a carrier vehicle protecting the virus genome during its delivery into appropriate cellular

compartment. They can be involved in the process of attachment to cell receptors and fusion and, in certain cases, interaction with cellular components to ensure the right transport to intracellular site for replication.

Densoviruses as well as vertebrate parvoviruses have a limited number of coding genes due to their short and compact genomes, and thus, a limited number of protein products. This, in part, leads to an organization of overlapping genes to maximize the use of the limited genetic material, and furthermore implies the production of multi-functional proteins. The VP1 was previously reported to harbor a conserved region, or the so-called PGY motif, of 70-98% identity within a stretch of 39 a.a. in both vertebrate and invertebrate parvoviruses (Dumas *et al.*, 1992; Tijssen and Bergoin, 1998). This domain was later shown to have a phospholipase A2 (PLA2) enzymatic activity. This PLA2 domain resides within the N-terminal region of VP1 (VP1up) of almost all known parvoviruses and is required for successful infection (Zádori *et al.*, 2001). This VP1 N-terminus, which was previously shown for some parvoviruses to be protruded out of the capsid shell without disassembly during virus entry (Cotmore *et al.*, 1999), might be required for the transport of virions from the late endosomes/lysosomes to the nucleus (Zádori *et al.*, 2001). The PLA2s form a superfamily of key enzymes involved in physiological and pathological processes such as lipid membrane metabolism, signal transduction pathways, inflammation, acute hypersensitivity and degenerative diseases (Dennis, 1997; Kramer and Sharp, 1997; Balsinde *et al.*, 1999). The Ca^{+2} binding loop (GPGN) as well as the catalytic site of PLA2 (DxxAxxHDxxY) were found to be conserved among both vertebrate and invertebrate parvoviruses (Fig. 6). However, the specific activity of the PLA2 of *Densovirus* members was significantly lower than that of the *Iteravirus* or vertebrate parvoviruses (Zádori *et al.* 2001; Li *et al.* 2001; Fédière *et al.* 2002). Alteration of JcDNV capsid protein by deleting the VP1 N-terminus abolished virus infectivity, most probably because of the lack of PLA2.

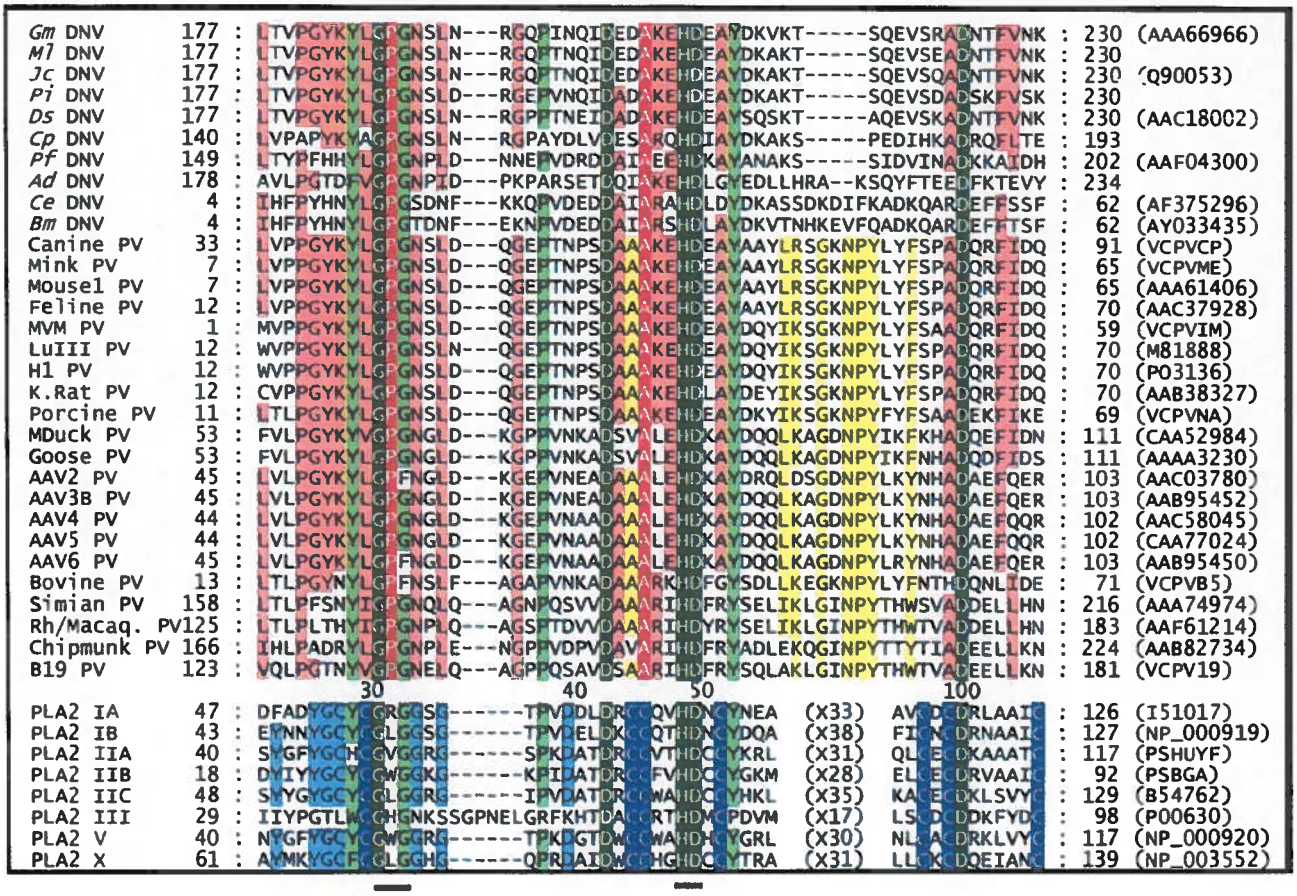


Fig. 6. The PLA2 conserved motif within the VP1N-terminus of *Parvoviridae* members. The Ca^{2+}- binding loop and the catalytic HD site are underlined (Zádori *et al.*, 2001).

However, production of DNA-containing particles by this altered virus could be achieved after transfection (Abd-Alla, 2003). It is noteworthy that viruses in the third densovirus genus, *Brevidensovirus*, do not possess such enzymatic activity within their VP proteins which may be a result of divergence during the evolution.

A second conserved motif on the VP coding sequences has been found in the N-terminal region of VP2 of vertebrate parvoviruses and VP4 of densoviruses (Fig. 7A). This motif, which is S-T/G-rich is believed to be involved in the N-terminal delivery of VP2 or VP4 to the surface of the virus capsid through the five-fold channel (Tsao *et al.*, 1991; Bergoin and Tijssen, 1998; Simpson *et al.*, 1998).

Nonstructural proteins

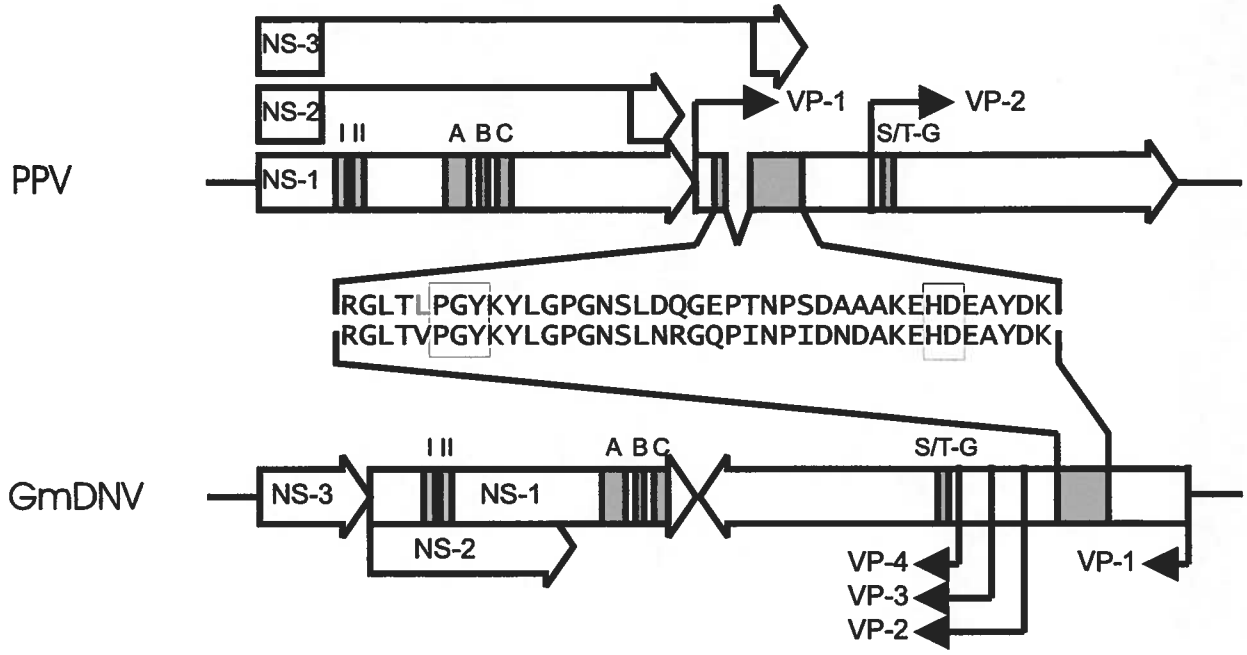
As in the case of viral capsid proteins, densoviruses have a limited number of nonstructural proteins, usually two. However, classical ambisense densoviruses are unique among vertebrate and invertebrate parvoviruses by having three NS proteins produced from three ORFs distributed on the 5' half of one of the two complementary strands and controlled by most likely one promoter located within the long ITR (Tijssen and Bergoin, 1995). These proteins are likely to be involved in viral DNA replication, but may also have other functions. In vertebrate parvoviruses, it is well known that NS proteins (NS1 and NS2) are involved in both viral DNA replication and regulation of gene expression (Cotmore and Tattersall, 1995; Berns, 1996). NS1 is a multifunctional protein that exhibits site-specific DNA binding, ATPase, helicase and nickase activities which are all required during viral DNA replication (Cotmore and Tattersall, 1995). It is also involved in the regulation of P4 and transactivation of P38 promoters which control the expression of nonstructural and capsid proteins, respectively (Cotmore and Tattersall, 1987; Doerig *et al.*, 1988; Doerig *et al.*, 1990; Vanacker *et al.*, 1996). Moreover,

NS2 protein was shown to be needed for efficient nuclear egress of progeny virions through interaction with nuclear Crm1, and may, therefore, participate in virus spread (Eichwald *et al.*, 2002).

On the other hand, some vertebrate dependoviruses (AAVs) encode four NS proteins, Rep78, Rep68, Rep52 and Rep40. The Rep78 and Rep68 have been extensively studied and have biochemical activities similar to the NS1 protein of autonomous parvoviruses, such as DNA binding, site-specific and strand-specific endonuclease activities (Im and Muzyczka, 1989; 1990; 1992), and DNA helicase activities (Wonderling *et al.*, 1995). Rep78 and Rep68 are also required for mediating targeted integration of the AAV genome into the host cell chromosome during persistent infections, which occur in the absence of helper virus infections (Samulski, 1993; Shelling and Smith, 1994; Balague *et al.*, 1997; Surosky *et al.*, 1997)

The *in vitro* transcription of the three JcDNV NS ORFs by bacteriophage T7 RNA polymerase followed by analysis of the translation products revealed three NS proteins of molecular masses of 20, 30 and 68 KDa, of which the 20 and 30 KDa proteins are the most abundant (Li, 1993) . Among the three NS proteins, the NS1 is the most conserved in ambisense densoviruses, up to 90-95% in subgroup A densoviruses but less than 28% with subgroups B and C. NS1 carries several sequence motifs that are conserved among densoviruses as well as among vertebrate parvoviruses (Tijssen and Bergoin, 1995). As indicated in Fig. 7 B, two important sets of protein motifs were found to be shared by NS1 of both vertebrate parvoviruses and densoviruses (Tijssen and Bergoin, 1995). The first set has two conserved motifs called initiator protein motifs I & II, which are believed to be involved in the initiation and termination of the rolling circle replication (RCR) (Ilyina and Koonin, 1992). The second set has motifs that contain sequences homologous to the phylogenically conserved Walker A- and B-site

A)



B)

Initiator (replicator) protein motifs

```
MVM     (125-217) GWHCHVLIG-51-LLTYKHKQTKKDYTKCVLFGNMIAYYFLTKKKI
PPV     (126-218) GYHCHVLLG-51-LLTYTHKQTKKQYTKMTHFGNMIAYYFLNKKRK
B19     ( 79-148) GYHIHVVTG-28-VKLKFLPGMTTKGKYFRDGEQFIENYLMKKIPL
GmDNV   (131-192) GDHIHVIHD-20-SVQKTGKPVKYIWEFKRTDWYDVFIYFFVRKRG
BmDNV   (538-619) FKHIHGIPW-41-TSATTSSVANANAQYPIDVFHFDEAYET-NYGI
AeDNV   (315-374) GDHIHILFS-19-SATSAGSAEATITFSKVKFLRNYILYCI-RYGI
        Consensus   H&H&&&                                &  Y&  K
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NS1 helicase superfamily III motifs

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PPV     (394-486) ILFHGPASTGKSIIAQHIANL-23-LIWIEEAGNF-30-TPVIMTTNE
B19     (324-416) LWFYGPPSTGKTNLAMAIAKS-23-LVVWDEGIIK-30-VPVVITSNG
GmDNV   (402-496) FLVMSPPSAGKNFFFDMIFGL-25-VLLWNEPNYE-30-TPVIILTNN
BmDNV   ( 70-164) FQIVSPPSAGKNFFIETVLAF-25-VNYWDEPNFE-30-TPVIITANY
AeDNV   (563-656) MVLEGITNAGKSLILDNLLAM-24-SILFEEPMIT-31-TPTWITTAT
        Consensus &&& GPPgTGKT&&a T&GK&     &&&&DD&GK&      P&&& TTN&
```

Fig. 7. Conserved motifs within the NS and VP proteins of vertebrate and invertebrate parvoviruses. A: localization of the conserved motifs on the viral genome, B: description of NS conserved motifs (Tijssen and Bergoin, 1995).

motifs that comprise the ATPase activity of the helicase superfamily III (Gorbalenya, 1989; Gorbalenya and Koonin, 1989; Koonin, 1993). The Walker A-site consists of GxxxxGK(T/S) and the B-site, which follows a variable spacer region, consists of uuuu(D/E)(D/E), where u is a hydrophobic residue. However, GmDNV, JcDNV, PfDNV and DsDNV have putative motifs which are divergent from the consensus Walker A-and B-sites, SxxxxGKNfff-x31-vllwnEp. Nevertheless, Ding *et al.* (2002) demonstrated the ATP-dependent DNA helicase activity of this NS1. They also clearly showed the involvement of NS1 protein in viral DNA replication. JcDNV NS1, expressed as a maltose-binding fusion protein, binds specifically to densovirus terminal palindrome sequence to a site of (GAC)4 located on the A-A' region. It then catalysis the cleavage of single-stranded DNA within the putative origin of replication, primarily at two sites in the motif 5'-G°TAT°TG-3' (Fig. 8). Such activities are important in the generation of monomeric genomic units from the replicative form during the replication of densovirus DNA.

The exact role played by the other two types of NS proteins, NS2 and NS3, remain to be elucidated. However, Abd-Alla *et al.* (2004) showed that NS3 is critical for JcDNV genome replication. In JcDNV infectious clone with partial or complete deletion in the NS3, viral DNA replication was abolished. The replication could be restored by the NS3 protein expressed under the control of *B. mori* actin 3 promoter introduced in *trans* through a co-transfection.

Conserved sequences between densoviruses and non-parvovirus members

Recent reports on the full sequence of two members of the family *Baculoviridae*, *Cryptophlebia leucotreta* granulovirus (CrleGV) (Lange and Jehle, 2003) and *Neodiprion lecontei* nucleopolyhedrovirus (NeleNPV) (Lauzon *et al.*, 2004) showed an interesting

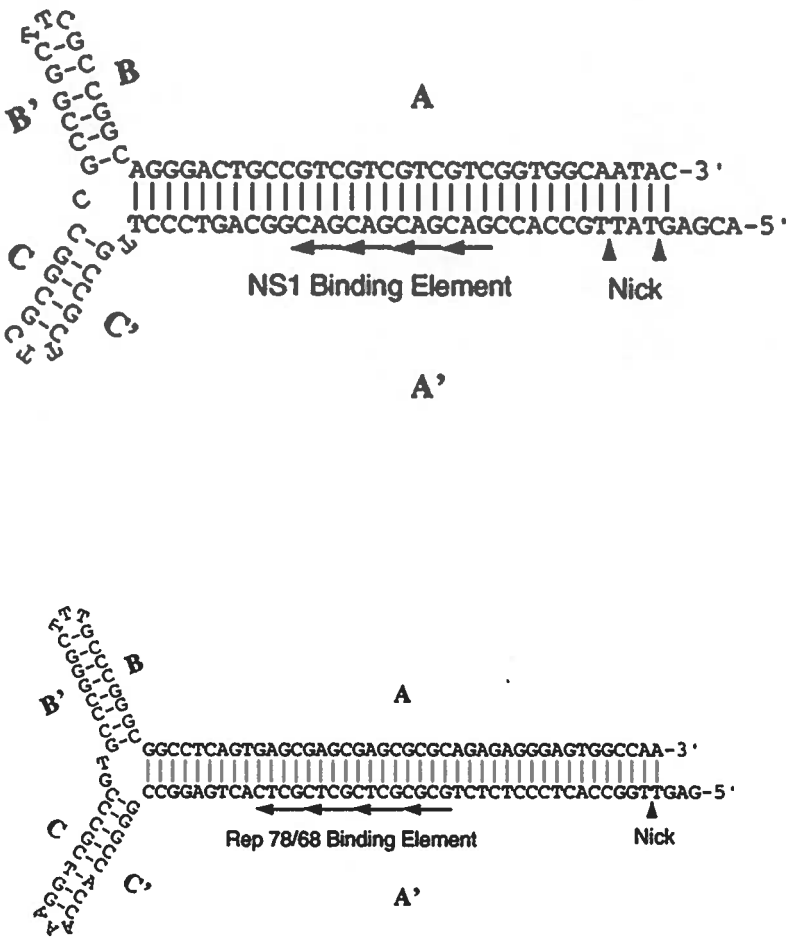

Fig. 8. Binding and nicking sites of the JcDNV NS protein on the viral hairpin (upper) relative to AAV-2 (lower) (Ding *et al.*, 2002).

homology with densoviruses. The CrleGV has 126 ORFs from which there are 5 ORFs with no homology to other members of the *Baculoviridae* (Crle9, Crle18, Crle22, Crle48, and Crle49). However, unique Crle9 ORF showed a remarkable homology to JcDNV NS3 as well as to the BmDNV2 nonstructural protein. In a similar finding, Lauzon and Jehle described a significant homology of 30.2% between the NeleNPV's ORF N181 and structural proteins from CeDNV. It has also 20.4% of homology with PfDNV as well as 24.8% to a putative *B. mori* densovirus 5 (GenBank accession number AB042597). Yet, it is not known whether or not these two unique ORFs found in CrleGV and NeleNPV code for proteins. The authors concluded that the presence of these homologues within two distantly-related virus groups may occur as a result of recombination or transposition during DNA replication or simply as an early horizontal transmission during evolution.

REPLICATION OF DENSOVIRUSES

Parvovirus replication is restricted to the nucleus and is dependent on certain helper functions from the host cell. The cell must undergo the S phase of mitosis for viral replication to occur. The single-stranded DNA genome of the virus needs to be converted to a double-stranded intermediate in order to start transcription and translation of the viral genome and proteins, respectively (Berns, 1990). The DNA polymerase δ, the most probable cellular enzyme responsible for the synthesis of the complementary strand, is expressed in cells only during the S phase of the cell cycle (Cossons *et al.*, 1996). The large dependence of vertebrate parvoviruses on the cell machinery for transcription and replication can also be seen in bacteriopahges such as the phiX-174 and M13. These bacteriopahges contain circular ssDNA of a similar size to parvovirus genomes and which replicate through a double-stranded DNA intermediate (Sinsheimer, 1968; Marvin and Hahn, 1969).

Several models for vertebrate parvovirus replication have been proposed based on experimental results (Tattersall and Ward, 1976; Hauswirth and Berns, 1977; Cotmore and Tattersall, 1995; Berns, 1996; Cotmore and Tattersall, 1998). Not much attention has been paid to the mode of replication of densoviruses. However, densoviruses share common genomic structures with vertebrate parvoviruses, implying that they might share similar strategies for their genome replication. The most prominent feature is the presence of terminal hairpins, which work like inboard-primers to initiate the replication as the only possibility since there was no evidence for other priming factors such as RNA or Okazaki fragments (Berns, 1996). Another feature shared with vertebrate parvoviruses is the conserved domains within NS1 protein, initiator protein motif and NS1 helicase superfamily III motifs, which are involved in the Rolling Circle Replication model, RCR (Tijssen and Bergoin, 1995). These features lead Bergoin and Tijssen (2000) to hypothesize a replication model for members from the genera *Densovirus* and *Iteravirus* with typical ITRs on both genomic termini. In this model, both replication and transcription are inter-regulated and are concentration-limited to NS1 protein. When an excess of NS1 protein is available in the replication environment, nicking activity by NS1 will occur at all the nicking sites of the replicative-intermediate DNA, resolving monomeric genomic units. However, in conditions of a low concentration of NS1, only a limited number of nicking sites will be resolved, leading to the accumulation of multimeric DNA molecules which serve for more viral gene transcription (Fig. 9).

GENE EXPRESSION IN DENSOVIRUSES

Densoviruses have been accorded little attention for the expression of their genes compared to well-known strategies of vertebrate autonomous and dependent parvoviruses that employ alternative splicing to regulate expression of the different

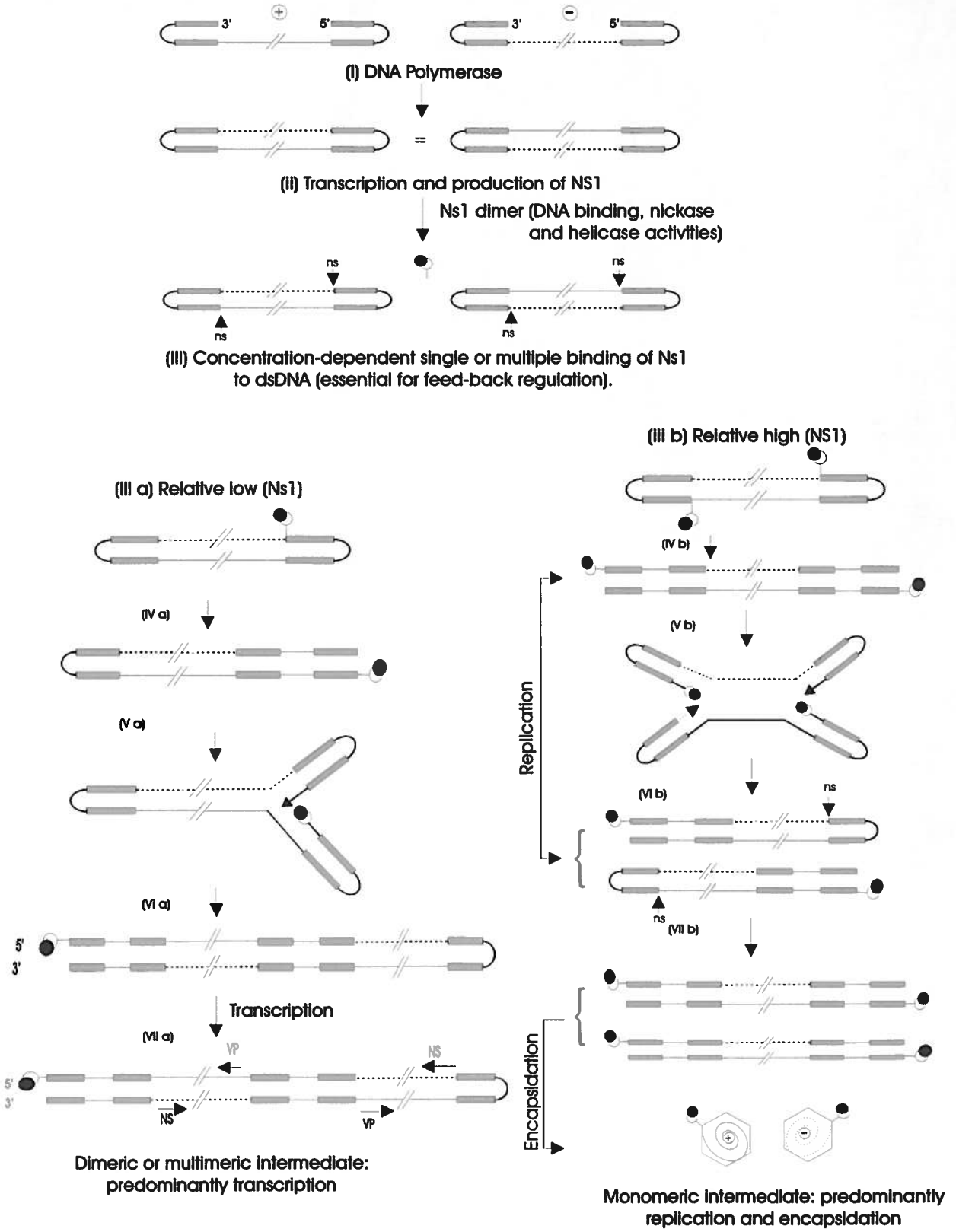

Fig. 9. Schematic representation for the densovirus replication model. ns: nicking site, NS: non-structural proteins and VP: viral structural proteins (Bergoin and Tijssen, 2000).

genes. Autonomous vertebrate parvoviruses usually have two cassettes of overlapping genes on only one viral strand. The regulatory proteins (NS proteins) reside to the left of the viral strand and are controlled by one functional promoter located at map unit 4, upstream of the NS genes. This cassette of NS yields different co-terminal transcripts at cleavage polyadenylation/ signal AATAAA, at map unit 95-96, downstream from the VP gene. On the right of the genome, resides the coding sequence for the capsid proteins VP1 and VP2 which are translated using two distinct initiation codons as a result of differential splicing of the precursor messenger RNA controlled by a promoter at map unit 38 to 40 (Bergeron *et al.*, 1993; Pintel *et al.*, 1995; Berns, 1996; Hirt, 2000). In certain cases, VP2 undergoes proteolytic cleavage in the N-terminal 20-25 amino acids, resulting in the VP3 protein that takes place only on complete DNA-containing virions (Clinton and Hayashi, 1976; Tattersall *et al.*, 1977; Paradiso, 1981; Weichert *et al.*, 1998). The core promoter element usually contains a TATAA consensus sequence and an upstream SP1 binding site with a GC rich motif (Lorson *et al.*, 1996). The activity of this promoter is NS1 dependent (Rhode *et al.*, 1987; Cotmore *et al.*, 1995; Vanacker *et al.*, 1995). Fig.10 illustrates the transcription mapping of the autonomous parvovirus PPV (Porcine parvovirus) with two primary large transcrpits, PT4 and PT40 (Bergeron *et al.*, 1993). By employing alternative splicing, these transcripts generate three NS proteins and 2 VP structural proteins.

The overall structure of monosense organization is shared by members of both *Iteravirus* and *Brevidensovirus* from insect parvoviruses (Tijssen and Bergoin, 1995). The Iteraviruses, however, use a leaky scanning mechanism to produce up to five structural proteins from a unique transcript. It was shown by Li *et al.* (2001) and Fédière *et al.* (2002) that the 5 potential ATGs of the VP cassette could drive the expression of wild-type-identical proteins when expressed in the baculovirus system. The individual proteins had the capacity to auto-assemble into virus-like particles.

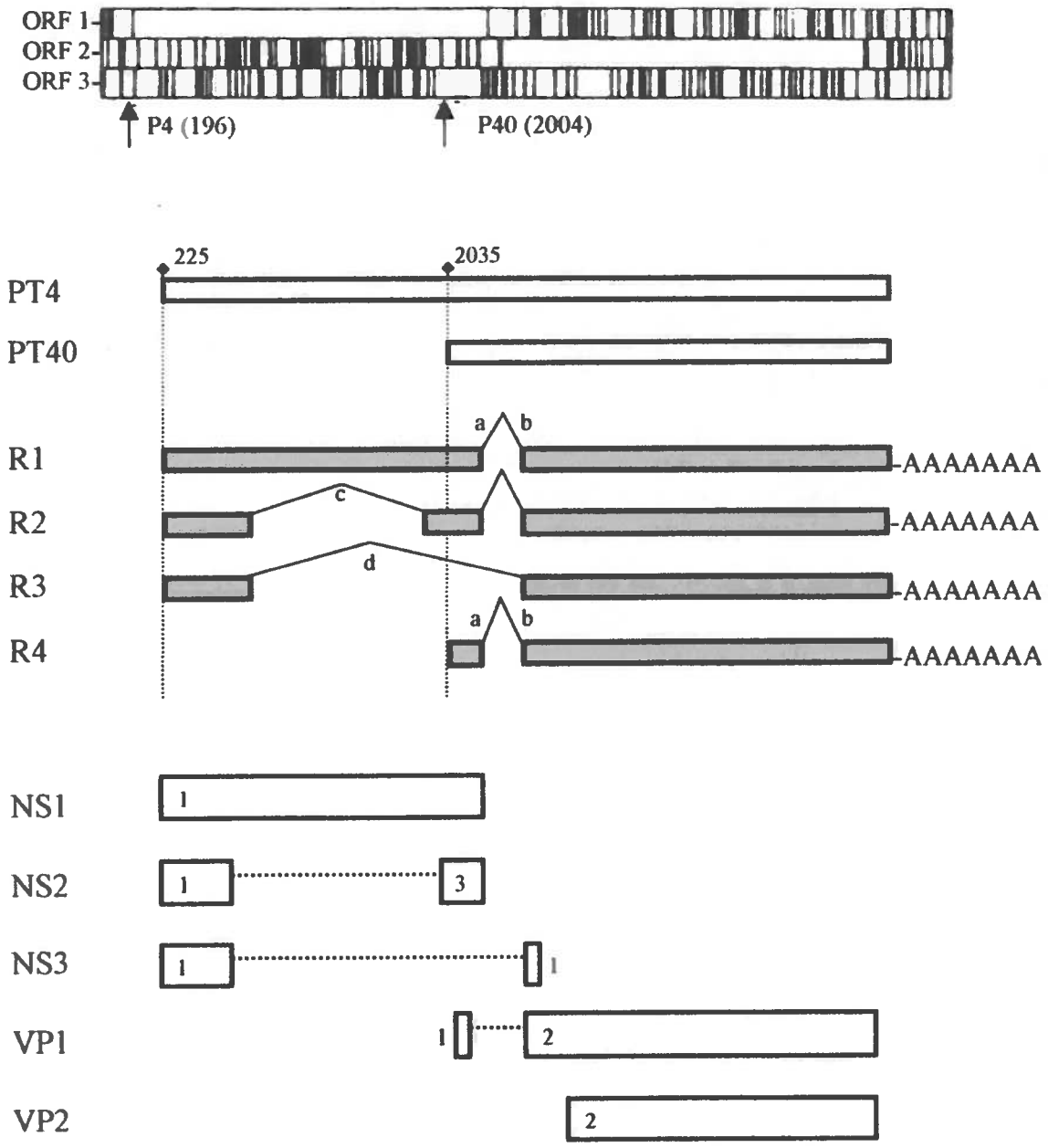

Fig. 10. Transcription mapping of porcine parvovirus as a model for vertebrate parvoviruses. Two large transcripts: Pt4 and Pt40 that undergo alternative splicing to generate two VPs and three NS proteins. Splicing sites are indicated by the inverted conical lines (Bergeron *et al.*, 1993).

However, the expression mechanism of the NS gene is not yet clear.

On the other hand, Subgroup A densoviruses from the *Densovirus* genus, which carry an ambisense organization, have putative promoters situated in their ITRs, with the TATA-box within the terminal repeats and the CAGT-box in the unique sequence (Bergoin and Tijssen, 1998). Early studies on JcDNV, the prototype of this genus, showed the presence of two active promoters residing within the ITRs (P9 and P93). These promoters drive the expression of two unspliced populations of mRNA which produce VP and NS proteins (Li, 1993). However, recent studies on the transcription of JcDNV NS gene showed that the virus employs alternative splicing to produce its nonstructural proteins. Two forms of mRNA could be detected after transfection of the JcDNV genome in insect cells; a non-spliced form of 2.7 kb serving to produce NS3, and a spliced one of 1.7 Kb to produce NS1 and NS2 (Abd-Alla, 2003). The activity of the P9 promoter in insect cells could also be assayed by driving the expression of a selective genes, *neo*^{r}, and a non-selective reporter gene, β-gal (Giraud *et al.*, 1992; Rolling, 1992; Royer *et al.*, 2001). The GmDNV has two putative promoters that correspond in genome localization to those of JcDNV. The insertion of the chloramphenicol acetyl transferase (*CAT*) gene downstream of these VP and NS promoters, creating pVP-CAT and pNS-CAT fusion proteins, respectively, led to the expression of the reporter genes *in vivo* (Tal and Attathon, 1992). Detection of *CAT* activity in larval homogenate after transfecting *G. mellonella* by both vectors indicated the transient expression driven by functional VP and NS promoters.

The PfDNV, which is still regarded as a putative member of the *Densovirus* genus, employs however a different expression strategy which would justify its classification in a separate genus. The genome has an overall ambisense organization similar to classical densoviruses, exemplified by GmDNV (Fig. 4), but expresses its VP proteins

through two open reading frames, instead of only one in classical densoviruses. After transcription, the two ORFs are connected by splicing and reorganized by frame-shifting in order to produce the largest VP protein (Yamagishi *et al.*, 1999). Different termination sites for the VP transcripts, by alternative polyadenylation, were also observed in PfDNV expression, a molecular phenomenon which is unique among densoviruses. Another difference resides on the presence of two potential promoters for controlling the expression of the NS proteins at map unit 3 and 18, P3 for ORFβ and P18 for ORFα and or γ.

In *Brevidensovirus*, AaeDNV, three potential promoters were observed and their activity was assayed by transient expression of the β-gal reporter gene. Two of them, P7 that drives the expression of NS2 (map unit 7) and P61 that controls the VPs transcription (map unit 61), were shown to be highly active, whereas the third one, P0.5, needed the trans-activation of NS proteins (Afanasiev *et al.*, 1991). Nevertheless, Ward *et al.* (2001) showed that the VP transcript of AaeDNV initiated at a consensus sequence, CAGT, which is 60 nts upstream from the previously predicted P7 TATAA sequence. Furthermore, deletions in the CAGT sequence decreased the expression by 93%, whereas, deletions in the TATAA had a very limited effect. The exact mechanism of their expression remains to be elucidated.

VIRUS TROPISM

Insect parvoviruses share with their vertebrate counterparts the limited number of coded genes and a high dependence on host cellular factors for replication. Their host tropism seems odd since a large difference could be found between closely related members. GmDNV and JcDNV are two members of the densovirus genus with high

sequence identity of up to 90 % and a similar genomic organization. Despite the close serologic relationship between these two viruses, GmDNV has a restricted host range, infecting only its original host, *G. mellonella*. Yet, it is polytropic, infecting almost all tissues of its host, except the midgut cells (Amargier *et al.*, 1965; Kurstak and Vago, 1967; Garzon and Kurstak, 1968). On the other hand, JcDNV was reported to infect, in addition to its original host of isolation, *Junonia coenia*, several other hosts: *Mamestra brassicae (Noctuidae), Mamestra oleracea, B. mori, Lymantria dispar, Aglais urticae, Scotia ipsilon, Spodoptera exigua, Spodoptera littoralis* and *Chrysodeixis chalcites*. But it does not infect *G. mellonella* (Rivers and Longworth, 1972; Fédière, 1996; Kawase *et al.*, 1996). In a similar manner, MlDNV (a major subject in the study of the current thesis) possesses also a comparable genome size and serological relationship to the former two viruses but has a broad host range that differs from both GmDNV and JcDNV. Several hosts were reported for MlDNV: *Mythimna loreyi, Sesamia cretica, Spodoptera littoralis, Chilo agamemnon, Ostrinia nubilalis* and *Pictinophora gossypiella*, as well as *G. mellonella*, all within the order of Lepidoptera (Fédière *et al.*, 1996). *Periplaneta fuliginosa* densovirus (PfDNV) infects three other insect species within the same family (Blattidae: Blattodea), *P. australasiae, P. brunnea* and *P. japonica* (Suto, 1979). Thus, cross-infectivity to related insect species and in some cases insect families, occurs within these densoviruses but none of them was able to cross the insect order.

CeDNV from the *Iteravirus* genus is also restricted to its host, the oil-palm pest *Casphalia extranea* (Limacodidae: Lepidoptera) but can infect cultured cells of *B. mori* (Bombycidae: Lepidoptera) *in vitro* (Fédière *et al.*, 1990). The second member of *Iteravirus* genus, BmDNV-1, is restricted to its host *B. mori* but it can infect different strains. In contrast to GmDNV, it multiplies preferentially in the columnar cells of the midgut epithelium (Maeda and Watanabe,1978; Watanabe, 1981; Seki and Iwashita, 1983). However, it has been shown that Bombyx strains that are resistant to DNV-2 (a

bipartite parvo-like virus that has been eliminated from densovirus classification) are indeed susceptible to DNV-1 and vice versa (Watanabe and Maeda, 1981; Seki, 1984). On the other hand, the host range of members of the third genus, *Brevidensovirus*, seems less restricted within the arthropod host spectrum. AaeDNV can infect *Aedes vexans*, *A. geniculatus*, *A. caspius dorsalis*, *A. cantans*, *A. albopictus*, *Culex pipiens pipiens*, *Culex pipiens molestus* and *Culiseta annulata* (Lebedeva *et al.*, 1973).

Tropism of densoviruses *in vitro*

Despite the fact that densoviruses are highly pathogenic and cause lethal diseases to their hosts *in vivo*, they lack the typical lytic cycle during experimental infections in cell cultures. Therefore, cultured cells infected with densoviruses do not manifest clear cytopathic effects, which prevents the development of precise methods for quantification, a dominant problem in densovirus research (Bergoin and Tijssen, 1998; Li *et al.*, 1996). Notably, several mosquito cell lines were shown to be chronically infected by densoviruses without any prominent cytopathic effect (CPE). Jousset *et al.* (1993) isolated a densovirus, AalDNV, from an established cell line of *Aedes Albopictus*, C6/36, that causes no CPE. When inoculated *in vivo* into mosquito larvae *Aedes aegypti*, the virus caused a typical infection. Interestingly, Chen *et al.* (2004) isolated another densovirus from the same cell line C6/36 that seemed healthy and called this virus C6/36 DNV. The new virus was more closely related to AaeDNV in sequence identity, but more closely related to AalDNV in genomic organization. Similarly, O'Neill *et al.* (1995) used a PCR detection assay to screen mosquito cell lines *Toxorhynchites amboinensis* (TRA-284) and *Haemagogus equinus* (GML-HE-12) for densovirus infections. Indeed, their analysis showed two new putative densovirus members (TaDNV and HeDNV) that chronically infected these cultured cells. The avirulent infection was confirmed by electron microscopy and immunofluorescence. Together, these observations suggest that

densoviruses are often involved in silent infections of mosquito cell lines and present a useful model to study their evolution in relation to the host. However, early successful attempts to adapt a densovirus *in vitro* were carried out by adding GmDNV to primary cultures derived from *B. mori* ovarioles and *G. mellonella* fat bodies, ovarioles and silk glands (Vago and Luciani, 1965; Vago *et al.*, 1966b; Quiot *et al.*, 1970). The JcDNV was also reported to be infectious *in vitro* for two cell lines derived from *Spodoptera littoralis* (Mialhe *et al.*, 1984). CeDNV, in a similar way, initiated an asymptomatic infection on a continuous cell line from *B. mori*, SPC-BM40, that was only detectable by immunofluorescence and electron microscopy (Fédière *et al.*, 1990).

Recent efforts were made to develop a quantitative assay that relies on the indirect detection of densovirus infection in cells by immunofluorescence (IF). Li *et al.* (1996) adapted the IF technique to determine the JcDNV titer by calculating the tissue culture 50 % infectious dose ($TCID_{50}$) on four different cell lines: the SPC-SL 52 from *Spodoptera littoralis*, the SPC-PL 40 and the SPC-PL 65 cells derived from *Spodoptera litura* ovaries and hemocytes, respectively, and the SC-LD 135 from *Lymantria dispar*. The SPC-PL 65 was the most susceptible to JcDNV infection among the different cell lines, giving a titer of 10^7 $TCID_{50}$/ml, whereas SPC-SL 52 was most suitable and sensitive to transfection using a JcDNV infectious clone. In both cases, the population of infected cells did not exceed 50 % and decreased dramatically after a few passages, in which the newly produced virus was used to infect monolayer cells (Li *et al.*, 1994; Li *et al.*, 1996). It is noteworthy that insect cell lines are limited in number and are usually derived from lepidopteran, dipteran or orthopteran insects (Bergoin and Tijssen, 1998). These cell lines are thus not efficient for sub-cloning. The mixture of populations could in part explain the limited number of susceptible cells during infection and transfection assays.

Factors that control virus tropism

The following section will review the tropism determinants of vertebrate parvoviruses since no such details are available for densoviruses. The only known case of a molecular basis for controlling densovirus tropism was found with BmDNV1 and BmDNV2. Resistance to either virus was shown to be controlled by host genes. Resistance to BmDNV1 infection is controlled by two recessive insect genes: Nonsusceptibility to BmDNV1, *nɔd-1* (Watanabe and Maeda, 1981) and Non-infection to densovirus gene, *Nid-1* (Eguchi *et al.*, 1986). By the same manner, resistance to BmDNV2 is controlled by a third gene, *nɔd-2* (Seki, 1984). *Nɔd-1* was localized on chromosome 21 at a position 8.3 cM (cM for centimorgan, a chromosome mapping unit) (Eguchi *et al.*, 1991), whereas, *Nid-1* was localized to chromosome 17 at 31.1 cM (cited by Ogoyi *et al.*, 2003).

Determinants that dominate the tropism of vertebrate parvoviruses have been studied in some detail. The data point to the importance of the capsid surface in controlling tissue tropism, pathogenicity and antigenicity among different parvoviruses and among the strains of a particular virus (Mckenna *et al.*, 1999; Hueffer and Parrish, 2003). An interesting study has been carried out on canine parvovirus (CPV) and feline panleukopenia virus (FPV) host range interchange. CPV first emerged in the late 1970s as a host range variant of FPV as shown by phylogenetic analysis (Siegl *et al.*, 1985; Parrish *et al.*, 1988; Truyen *et al.*, 1995). FPV normally infects cats with no known tropism to dogs, whereas the original isolate of CPV [designated CPV-2 to distinguish it from a related minute virus of dogs (Schwartz *et al.*, 2002)] was able to infect dogs and both canine and feline cells *in vitro* but not cats. After a few years, new variants of CPV-2 regained infectivity to cats (Appel *et al.*, 1979; Carmichael and Binn, 1981; Parrish *et al.*, 1988; Parrish *et al.*, 1991; Truyen and Parrish, 1992; Truyen *et al.*, 1996).

FPV and CPV-2 share a high sequence identity, of over 99 %, and as few as two amino acid changes within the VP2 capsid protein were sufficient to alter the tropism of one of them to the other. Using directed mutagenesis on FPV capsid protein, the change in residue 93 of FPV from Lys to Asn and 323 from Asp to Asn clearly extended FPV tropism to canines. Whereas, the opposite change on CPV-2 greatly decreased the virus replication in canine cells or completely abolished it (Chang *et al.*, 1992). The two amino acid changes were also responsible for the transfer of a CPV-specific antigenic epitope into FPV as well as being involved in the pH dependence of hemagglutination difference between both viruses (Chang *et al.*, 1992; Horiuchi *et al.*, 1994). Another report showed that the change of CPV-2 amino acid residue alanine at position 300 to aspartic acid led the CPV particles to escape neutralizing antibodies and was solely responsible for causing a loss in canine host range (Llamas-Saiz *et al.*, 1996). Together, the position of these amino acids, 90, 300 and 323, on the three-fold spike of capsid protein, in harmony with the surrounding structures, plays an important role in the determination of the antigenic properties that define the host range of CPV-2 (Strassheim *et al.*, 1994; Llamas-Saiz *et al.*, 1996). Yet, other studies raised the importance of amino acid residues at 299, 300, 301 and 387 that reside on the shoulder of the three-fold spike of the CPV-2 capsid, showing that they have a specific conformation required to retain canine host range (Parker and Parrish, 1997). It was also shown that both CPV-2 and FPV use feline transferrin receptors (TfRs) to infect feline cells (Parker *et al.*, 2001). However, Hueffer *et al.* (2003) showed that the ability of CPV-2 to infect canine cells is dependent on the canine transferrin receptors and that the top and the side of the three-fold spike of the capsid surface controlled specific TfR binding and the efficiency of binding to feline and canine cells. Interestingly, mutating the residues 90, 300, and 323 clearly affected the interaction of CPV-2 to canine but not to feline TfR, which suggests that the specific interaction with the canine TfR involves residues that are not important for the feline TfR binding (Govindasamy *et al.*, 2003).

Another interesting example in parvovirus tropism has been the identification of the host range determinants of porcine parvovirus (PPV). Several PPV isolates with different pathogenicity have been recovered from various infected tissues (Molitor and Joo, 1990). One virus strain, NADL-2, is successfully used as an attenuated vaccine due to its limited pathogenecity and its failure to cross the placental barrier in experimental infections (Paul and Mengeling, 1980). Early studies on the host range of PPV showed that the virus, NADL-2 strain, can efficiently be taken up by host and non-host cells. Thus, it seems plausible that restriction of the virus tropism is dependent on intracellular events such as DNA replication, RNA synthesis and viral antigen expression (Ridpath and Mengeling, 1988; Oraveerakul *et al.*, 1992).

Other recent studies showed that the restriction of the host range of PPV strains is controlled by a few amino acid differences on the capsid protein. NADL-2 is very closely related to the virulent Kresse strain (Kresse *et al.*, 1985) with respect to sequence identity and genome organization (Bergeron *et al.*, 1996). When the Kresse strain sequence was compared with virulent field strains, five amino acids within the VP1/VP2 coding sequence were consistently different. However, these amino acids were not identical with NADL-2 strain. Interchanging the *Bgl*II restriction fragment that contains three of the five different amino acids (D378, H383 and S436 of NADL-2) between the two strains, reverts the acceptor virus tropism to that of the donor (Bergeron *et al.*, 1996). Resolving the 3-D structure of empty capsids of NADL-2 by X-ray crystallography permitted the localization of these amino acid residues onto the PPV capsid protein (Simpson *et al.*, 2002). Residue 436 (Ser in NADL-2 to Pro in Kresse) is located at the top of the 3-fold spike, whereas 378 (Asp to Gly) and 383 (His to Gln) are located on the edge of the 2-fold dimple, with 383 being nearer to the surface (Fig. 11). According to the analysis of the surface structure, alteration of residues 378 and 383 would provoke conformational changes that harm the putative receptor attachment site.

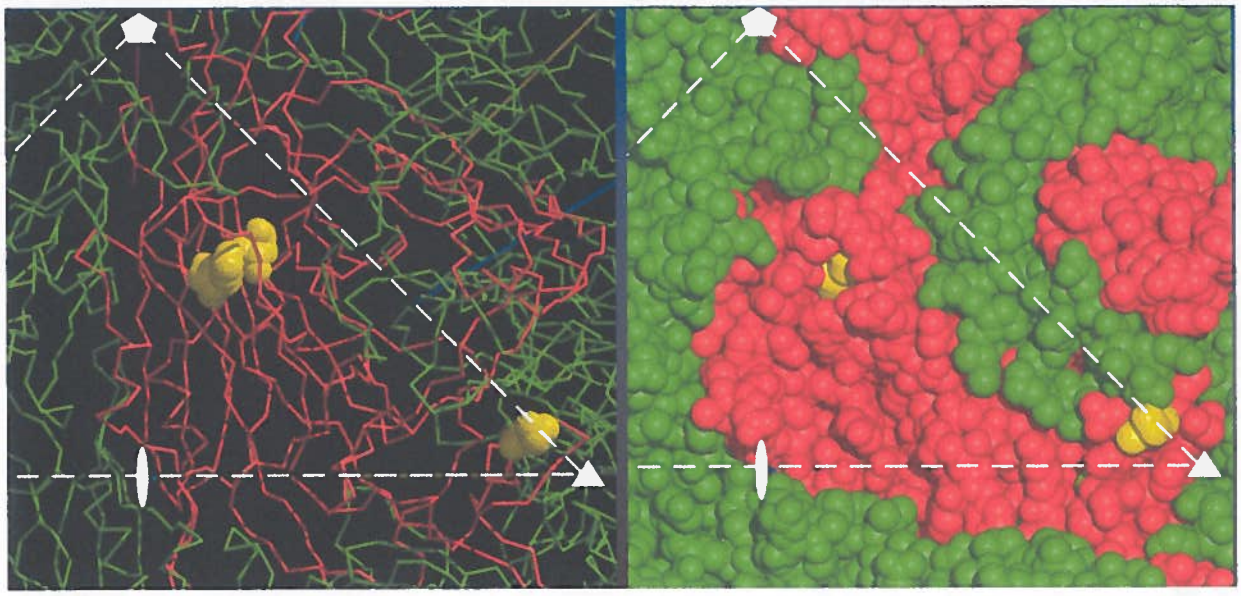

Fig. 11. Surface view of PPV capsid protein. The position of the three amino acids (a.a.) involved in the control of the virus tropism are shown. Residue 436 is near the 3-fold spike. Whereas, residues 378 and 383 are closed to the edge of the 2-fold dimple (all three a.a. in yellow). To the left, is a van der Waals representation and to the right is an atomic view to show the accessibility of these a.a. to the exterior of the capsid structure (Simpson *et al.*, 2002).

Mutation of residue 436 would create little distortion of the polypeptide backbone.

Similarly, the tropism determinants of helper-dependant adeno-associated virus were mapped to the capsid protein. AAV1 and AAV2 are two of the many known serotypes of adeno-associated virus (Murphy *et al.*, 1995). AAV1, however, is distinguished by its more efficient tropism into muscle cells than AAV2 (Xiao *et al.*, 1999; Chao *et al.*, 2000). The capsid genes of both serotypes share up to 90 % identity, with the remaining 10 % amino acid difference being distributed throughout the VP1 gene (Srivastava *et al.*, 1999; Xiao *et al.*, 1999). Replacing the capsid amino acids of AAV2 VP1 between 350 to 736 by the corresponding amino acids from AAV1 extended the AAV2 tropism to muscle cells (Hauck and Xiao, 2003). It was previously shown by neutralization assays of AAV2 by monoclonal antibodies that the receptor binding site on the virus capsid is located between residues 493 and 502 and between residues 601 and 610 (Wobus *et al.*, 2000). Hence, it seems plausible that the extension of AAV2 tropism to muscle cells required changes in these domains. Interestingly, AAV2 tropism could be managed to a broader host range by experimental insertion of desired ligands such as the L14 epitope (Girod *et al.*, 1999) and a single chain variable fragment directed against human CD34 into the virus capsid protein (Yang *et al.*, 1998).

Likewise, the capsid protein plays a comparable role in the cellular and tissue tropism of other parvoviruses such as minute virus of mice (MVM) and Aleutian mink disease (AMD) (Gardiner and Tattersall, 1988a; Bloom *et al.*, 1993; Bloom *et al.*, 1998). In both cases, the altered tropism was mapped to sequence regions coding for protein domains on or near the three-fold spike on the viral capsid (Agbandje-McKenna *et al.*, 1998; Mckenna *et al.*, 1999). But unlike CPV and FPV, the role of the MVM capsid protein in determination of the tropism seems to be more critical in intracellular compartments rather than on the cell surface, suggesting the implication of cellular

factors (Gardiner and Tattersall, 1988b). In this regard, Davis *et al.* (2003) suggested a function for cellular transcription factors in the activation of the MVM P4 promoter and hence, determination of the tissue tropism. The NS proteins of MVM were also reported to restrict the host range of MVM through the interaction with recruited cellular factors in a cell-type dependent manner that may work at post-encapsidation steps of viral maturation (Rubio *et al.*, 2001).

INTEGRATION OF THE VIRAL GENOME: POTENTIAL USE OF DENSOVIRUSES AS EXPRESSION VECTORS

Exogenous DNA can become integrated into cellular chromosomes by two means, homology-dependent and homology-independent mechanisms. While the second mechanism of random integration is poorly understood, the former integration takes advantage of similarities between the incoming DNA and the targeted locus to induce homologous recombination (Doerfler *et al.*, 2001; Würtele *et al.*, 2003). Several viruses have been shown to integrate their genomes into host chromosomes that allows the virus to go into a latent form within infected cells such as in parvovirus AAV2 (Richardson and Westphal, 1981), Epstein-Barr virus (Ohshima *et al.*, 1998; Takakuwa *et al.*, 2004), adenovirus (Groneberg *et al.*, 1977; Stabel *et al.*, 1980) and retroviruses, through their proviral DNA copy of RNA genomes (Asante-Appiah and Skalka, 1997; Colicelli and Goff, 1988; Cereseto and Giacca, 2004). According to their capacity to establish long-term expression in infected cells, most of these viruses are being studied for gene therapy goals (Lundstorm, 2003; Grimm and Kay, 2003)

The integration process in the vertebrate parvovirus adeno-associated virus (AAV2) is one of two major strategies marking its life cycle. In the absence of a helper virus infection (adenovirus or herpesvirus) or in some cases genotoxic stimuli, the AAV2

genome integrates in a specific order into the q arm of human chromosome 19 within a site called AAVS1, from which rescue occurs following a helper virus infection (Richardson and Westphal, 1981; Berns and Linden, 1995; Kotin, 1994; Kotin *et al.*, 1996; Samulski *et al.*, 1991; Samulski, 1993). The site-specific integration is mediated by the presence of the genome telomeres, the ITRs, in a *cis*-acting manner and the viral coded Rep proteins, Rep78 and its C-terminal spliced version Rep68. This complex of proteins binds at the Rep binding elements (RBEs) situated in both AAV ITRs and at the AAVS1 site on host cell DNA, a 4 Kb region on chromosome 19 (Giraud *et al.*, 1994; Kotin et al., 1992; Weitzman *et al.*, 1994). However, in the absence of Rep proteins, the ITRs alone can initiate random integration of AAV into the host genome (Walsh *et al.*, 1992; Kearns *et al.*, 1996; Xiao *et al.*, 1997; Young *et al.*, 2000). AAV vectors are promising, since they lack pathogenicity or toxicity to humans in addition to their long-term expression. However, some difficulties include the limited number of cells that could be targeted and possible neutralization with anti-AAV antibodies that are highly prevalent in the human population (Grimm and Kay, 2003).

Cloning of the entire genome of several densoviruses and the capacity of cloned DNA to rescue and to initiate typical infection both *in vivo* and *in vitro* led also to the use of densoviruses in the field of insect gene transfer (Jourdan *et al.*, 1990; Giraud *et al.*, 1992; Afanasiev *et al.*, 1999; Bergoin and Tijssen, 1998). The transgenesis strategy depends on the introduction of foreign genes under the control of densovirus active promoters, usually the VP promoter. It needs also to be complemented by densovirus accessory proteins, NS and VP, through a replication-deficient vector, in *trans*, in order to produce pseudocapsids that serve for the transfer. The cloned genome of JcDNV (Jourdan *et al.*, 1990) has been successfully used in a such a strategy to express the reporter genes *CAT* and *LacZ* in the cultured cells SPC-SL52. High expression levels were achieved through the insertion of the exogenous protein under the control of the

VP promoter p9 and conserving the NS coding sequence and the terminal repeats (Giraud *et al*, 1992; Rolling, 1992). Long-lasting expression of the reporter gene *LacZ* was observed and explained as an early integration of the recombinant JcDNV genome into the host cell DNA (Rolling, 1992). The integration of recombinant JcDNV DNA was further investigated *in vivo* by injecting the replicative-defective vector, pJlacZNS3, that has been deleted in NS3, into *Drosophila melanogaster* eggs (Royer *et al.*, 2001). Interestingly, strong β-galactosidase expression was observed after metamorphosis in adult tissues, which means a persistence of the vector sequence within the host DNA. This was subsequently confirmed by Southern blotting. The integration of JcDNV vectors was further investigated by Bossin *et al.* (2003). They inserted the GFP reporter gene under the control of the p9 promoter accompanied by different deletions within the viral NS genes. Introduction of these recombinant clones into Sf9 cells by transfection and subsequent cloning of GFP-fluorescent cells for over 30 passages did not alter the maintenance of the VP-GFP chimeric proteins expression under the control of the P9 promoter. Sequences of the cloning vector, pBR322 plasmid, were maintained along with the JcDNV recombinant sequence in all the clones. The 3' terminal junction sequence of JcDNV with the plasmid was intact, whereas the 5' sequence has undergone several rearrangements suggesting that the JcDNV 5' ITR is the primary site for recombination.

Densoviruses from the *Brevidensovirus* genus do not have ITRs in contrast to classical densoviruses. However, their potential use as transfer vehicles for foreign genes into insect cells has been promising especially that they can be used in the transgenesis of medically important Dipteran insects (Afanasiev *et al.*, 1994). In a similar strategy to that employed with JcDNV vectors, the cloned genome of AaeDNV was used to transfer and to drive the expression of β-galactosidase in mosquito cells C6/36. The β-galactosidase protein, fused to the capsid protein of AaeDNV, could be readily

transferred into the nucleus and be packaged within the viral capsids (Afanasiev *et al.*, 1994). The exogenous protein can also be fused to the NS protein without altering the biological activities of both proteins. GFP (green fluorescent protein) fused into the C-terminal of AaeDNV NS1 could successfully be expressed in mosquito larvae *in vivo* and became detectable 48 h postinfection (Afanasiev *et al.*, 1999). In a strategy different from that used in packaging recombinant JcDNV vectors into viral capsids, Allen-Miura *et al.* (1999) used a Sindbis virus expression system to produce AaeDNV pseudocapsids. The pseudocapsids were then introduced by infection into cultured C6/36 cells transfected with AaeDNV vector harboring the GFP gene fused to NS1. Such a strategy would circumvent the production of wild-type virus through possible recombinations between the transducing construct and the helper vector which produces the capsid proteins.

The use of densovirus capsid protein as a platform for the introduction of medically important virus epitopes as a safe vaccine has also been addressed (David, 2001). An epitope constituted of 15 amino acids from the surface gylcoprotein G of the human respiratory syncytial virus (RSV), a causative agent of severe lower respiratory tract infections in young children (Glezen *et al.*, 1973), was introduced to the C-terminal region of the abundant GmDNV-VP4. The recombinant protein was then expressed in a baculovirus expression system producing large quantities of densovirus-like particles carrying the G protein epitope on the surface. However, introduction of this pseudovirus into a mouse model did not promote an immune response against RSV (David, 2001).

CHAPTER II :
MATERIALS AND METHODS

Cells and medium

Four established insect cell lines were used in the current study: (I) Sf9 cells derived from *Spodoptera frugiperda* ovaries (Vaughn *et al.*, 1977) maintained in serum-free Sf-900 II SFM medium (GIBCO-BRL) and supplemented by 1% [v/v] antibiotic mixture of penicillin (50 U/ml), streptomycin (5 mg/ml), (II) LD652 cells from *Lymantria dispar* ovaries (Goodwin, 1985), also maintained in Sf-900 II SFM with additional 10% Fetal Bovine Serum (FBS) and antibiotic mixture, (III) T-ni cells from *Trichoplusia ni*, adapted to Express Five® SFM medium supplied by Invitrogen and (IV) Sl52 from *Spodoptera littoralis* ovaries (Mialhe *et al.*, 1984), maintained in TC-100 medium (Invitrogen, GIBCO) with 10% FBS and 1% antibiotic mixture. Cells were kept at 28 ± 1 °C and regularly passed before they reach 100 % confluence (usually around 4 to 5 days) by simple mechanical detachment using a cell scraper.

Four mammalian cell lines were also used: (I) L929 from mouse connective tissue fibroblast (Earle, 1943), (II) 293 from transformed embryonal human kidney cells (Graham *et al.*, 1977), (III) COS-7 from transformed African green Monkey kidney (Gluzman, 1981) and (IV) PT from Porcine Testis (Bergeron *et al.*, 1993). These cells were maintained in Dulbecco's Modified Eagle Medium, DMEM (GIBCO) with the addition of 1% [v/v] antibiotic mixture, 1% [v/v] L-Glutamine, and 7% [v/v] FBS. Cells were kept at 37 °C in a humified incubator supplied with 5% CO_2. To subculture the cells, PBS was used to wash the monolayers followed by 1 minute treatment with trypsin solution (0.01 % [w/v] trypsin and 0.04% [w/v] EDTA) and resuspension in fresh medium.

Insects

In order to evaluate the infectivity of the cloned densovirus genomes we used a laboratory colony of the insect host *G. mellonella* (Lepidoptera: Noctuidae). The insect was reared on artificial medium composed of: A) 200 g of wax, 900 g of honey and 240 ml glycerol and B) 1110 g of Pablum, 260 g of maize corn flower and 40 g of inactivated dried yeast. Ingredients in group A were mixed together, placed in a microwave oven at low power for 10-15 min until melting then left at room temperature in order to cool down to around 50 °C. The yeast in Group B was diluted in 400 ml regular tap water and placed at 37 °C for 30-40 min. All ingredients were then mixed well and then kneaded into spheres of about 5 cm diameter and transferred to -20 °C until usage.

Vectors

During the current work two densovirus vectors were exclusively used in the study of virus tropism. One is the pGm4, a non-infectious clone encompassing the complete coding sequence of GmDNV cloned within pUC19 vector but lacking the half of the ITR from each side, around 280 nucleotides. The second is pMl28, an infectious clone of MlDNV, cloned in pEMBL^{19+} vector, with one intact ITR on the VP side with a deletion of 100 nucleotides from the left hand ITR, NS side. Genomic sub-clones were all obtained using the phagemid vector pBluscript KS II^{+} (Stratagene). The pQBI25 (Quantum Biotechnologies) carrying the green fluorescent protein (GFP) gene was used in control transfection. The pSV2*neo* vector (Clontech) was used as a source for the neomycin resistance (neo^{r}) gene (Southern and Berg, 1982).

Virus stocks

The densovirus *Mythimna loreyi* (MlDNV) was produced by Dr Gilles Fédière at the viral production unit of the Faculty of Agriculture, Cairo University, using 4th instar larvae of *Spodoptera littorarlis* (Lepidoptera: Noctuidae). Infected larvae were collected and squashed in 0.1 mM Tris, pH 7.8 and filtered through Mosly Cloth, centrifuged at 5K rpm to remove debris and supernantant was then centrifuged at high speed (35K) for 120 minutes in Beckman rotor Ti55. Virus pellets were resuspended in Tris buffer and layered onto sucrose gradient 15-45 % (w/w) and centrifuged at 24K rpm in a Beckman Swing Rotor (SW 28) for 150 minutes. Virus bands were recovered from SW28 tubes, diluted in 10 volumes Tris buffer and re-centrifuged at 35 rpm.

Highly purified stocks were obtained by extra purification on cesium chloride (CsCl) gradients. The virus preparations were layered onto CsCl gradients of 1.33-1.45 g/ml and run overnight at 35K rpm in SW41 rotor (Beckman). Virus bands were collected, diluted in Tris buffer and precipitated as mentioned above, and the final pellet was resuspended in 22 µm-filtered Tris buffer.

Infection and transfection

Viral titers were determined as the fluorescent forming unit (ffu) per ml. Susceptible insect cells LD652 were cultured at a density of 10^3 cells per well in 96 wells plates for 20-24 hrs prior to infection. Cells were incubated with different exponential dilutions of the virus stock prepared in cell recommended medium in final volume of 50 µl for 6 hrs, then washed and covered by 200 µl fresh medium for 72 hrs. Comparative experimental infections on insect and mammalian cells were carried out on cells cultured in 6 well plates at densities of $7x10^5$ cells per well according to the previously described

conditions.

Transfection methods were adapted to fit with the cell type in order to achieve high efficiency. Three different kits of lipid based reagents were used: (I) Lipofectin, (II) LipofectAmine reagent (GIBCO-BRL) and (III) DOTAP reagent (Roche). In mammalian cells, the kit efficiency was measured by control transfection using the GFP-gene harboring-vector pQBI25. Transfection was carried out by incubating 2 µg DNA with 8 µg lipid in 150 µl of serum-free medium for 20 minutes then applied in droplets onto 20-24 hrs pre-cultured cells, incubated for 4-6 hrs then washed and covered by fresh medium. However, in insect cells, efficiency was directly monitored by the number of virus-infected cells revealed by immunofluorescence as will be described below.

Indirect immunofluorescence assay

Medium was removed from infected cell boxes and the attached monolayers were then washed one time with PBS and fixed with 3% formaldehyde in IF buffer (Sanofi Diagnostic Pasteur) for 30 min at room temperature (RT). After one time wash with PBS, cells were permeabilized with 3% Triton-X 100 prepared in IF buffer (IFB) for another 30 min at RT. The first antibody, produced in rabbits, against the GmDNV capsid proteins and diluted as 1/500 in IFB was then added for 30 minutes. Fluorescence was developed by incubating the cells for 30 minutes with the secondary anti-rabbit antibodies labelled with fluorescein isothiocyanate accompanied with 1/1000 dilution of Evans blue (Sanofi Diagnostic Pasteur). In a second set of tests, the Alexa-Fluor 488 goat anti-rabbit (Molecular Probes) diluted 1/500 in IFB was used as a secondary antibody. UV microscopy was used to observe and to count the fluorescent cells.

Flow cytometric analysis

In order to quantify viral infection, cells were submitted to indirect immuno-staining and detection by FACS analysis as described by Morris and Miller (1993) with a few modifications as follows.

Cells were detached from flasks by vigorous washing with PBS using a pipette, collected in V-bottom 15 ml tubes and centrifuged at low speed (500 g) for 3 min. Fixation was carried out by the addition of 1 ml of 0.5% formaldehyde, in PBS, and incubation for 30 min at room temperature. After centrifugation at low speed , cells were permeabilized with 0.3% Triton-X 100 for 30 min and then incubated with antiviral antibodies (polyclonal antibodies produced in rabbit against GmDNV capsid proteins) diluted in PBS to 1/2000 for 60 min at 4 °C. Secondary antibody (goat anti-rabbit labelled with Alexa-Fluor 488) was added to PBS-washed cells in a 1/2000 dilution and incubated at 4 °C for 60 min. Finally, cells were collected at low speed, resuspended in PBS at a density of 10^4 cells/ml and subjected to data collection by Fluorescence-Activated Cell Sorter (FACS) analysis using Coulter XL flow cytometer with EXPO32 software.

Cloning protocol and preparation of competent cells

Restriction enzymes as well as DNA modifying enzymes are indicated in the results section. Two types of bacterial cells were used in the current study for the transformation purpose, DH5∝ and Sure (Stratagene). DH5∝ chemical competent cells were prepared with $CaCl_2$ according to the protocol of Sambrook and Maniatis (1989). However, Sure competent cells were prepared as follows: 5 ml of broth medium (Sambrook *et al.*, 1989) were inoculated with a single colony and incubated overnight

at 30 °C. The second day, the 5 ml were diluted in 500 ml fresh medium and incubated at 30 °C with shaking (200-225 rpm) to an optical density (OD 600) of 0.5 to 0.6. Bacterial flasks were transferred into an ice-water bath and kept for 10 min, then divided into 2 centrifuge tubes of 250 ml pre-chilled in ice. Cells were then collected by centrifugation at 5000 rpm in Beckman GSA rotor for 15 min at 4°C, washed one time with 500 ml ice-cold dH_2O and re-centrifuged. A second step of washing and centrifugation was carried out before discarding the supernatant and resuspension of the bacterial pellet in 2 ml of ice cold 10 % glycerol in water. Cells were divided into 100 µl aliquots in cold 1.5 ml microfuge tubes and transferred immediately to -80 °C.

Electroporation of competent cells

Prior to electroporation, competent cells were thawed rapidly between hands, placed on ice and mixed gently with 1 µl of the ligation reaction mixture by swirling with pipette tip then transferred into transformation cuvette. Electroporation took place at 2.5 KV in the (BTX, Genetronic Inc.) apparatus. Cells were rapidly diluted with 1 ml of SOC medium (Sambrook *et al.*, 1989) and incubated at 30 °C for 30 min with shaking at 250 rpm (round per minute). One hundred µl of cell suspension were then spread on LB-agar plates and incubated overnight at 30 °C.

The *CAT* assay

The chloramphenicol acetyl transferase (*CAT*) assay (Gorman *et al.*, 1982), was used to test the activity of MlDNV and GmDNV promoters. Three days post-transfection, the cells were washed three times with 2 ml of PBS then collected in 1 ml PBS into Eppendorf tubes and centrifuged at 1000 xg for 3 minutes. PBS was then removed and cells were resuspended in 100 µl of 200 mM Tris-HCl, pH 7.8. The cells

were disrupted by three cycles of freezing and thawing at -80 ^{0}C and a 37^{0}C water bath, respectively. After centrifugation of the cell debris in the microcentrifuge for 5 minutes at 4 ^{0}C, the supernatant was utilized directly for the *CAT* assay. Alternatively, the supernatant were stored at -80^{0}C. To quantitate the *CAT* activity, an assay mixture was prepared by combining 10 µl of an appropriate amount of a cell extract (diluted with 200 mM Tris-HCl, pH 7.8), 10 µl of 1 M Tris-HCl, pH 7.8, 4 µl of 4 mM acetyl CoA (freshly prepared), and 1 µl ^{14}C-chloramphenicol (NEN: 60 mCi/mmol, 50 µCi/ml). This mixture was incubated at 37 ^{0}C for various times (usually 60 minutes). The ^{14}C-chloramphenicol and its acetylated derivatives were then extracted into 150 µl of ethyl acetate and the organic phase was collected and evaporated to dryness in a speed-vac. The residue was resuspended in 20 µl of ethyl acetate and spotted in 5 µl aliquots on a silica gel thin layer chromatography plate (EM Science). The plate was developed in chloroform : methanol (9:1) until the solvent front had ascended approximately 10 cm and the radioactive chloramphenicol was visualized by autoradiography using the PhosphoImager.

Electrophoretic mobility shift assay (EMSA)

In order to study the viral DNA interaction with cellular proteins, we employed the EMSA technique (electrophoretic mobility shift assay). The EMSA technique is based on the observation that protein :DNA complexes migrate slower than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis (Garner and Revzin, 1981; Fried and Crothers, 1981).

A) Preparation of cellular extract

Cultured cells were harvested and washed two times with PBS, collected by low

speed centrifugation and stored at -80 °C. When ready to use, cells were resuspended in 3 volumes of HYPB buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA in addition to 0.75 mM spermidine, 1.5 mM spermine, 2 mM DTT, 1 mM PMSF, added immediately before use) well mixed and then centrifuged at 3000 rpm for 10 min at 4 °C. The cell pellet was resuspended in 4 volumes of HYPB, left on ice to swell for 20 min and then transferred into Dounce homogenizer and undergone 10-12 strokes followed by centrifugation for 15 min at 4000g at 4 °C. The nuclear pellet was resuspended in SUCB (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, with 0.75 mM spermidine, 1.5 mM spermine, 2 mM DTT and 1 mM PMSF added immediately before use) and layered onto 30% sucrose cushion and allowed to spin for 45 min at 75,000 g, 4°C. The pure nuclear pellet was then resuspended in 4 volumes of EXTRB (20 mM HEPES, pH 7.9, 0.45 mM KCl, 0.1 mM EDTA, 5 mM MgCl2, 20% glycerol and immediately before use an extra 2 mM DTT and 1 mM PMSF were added). Extraction was carried out by mild mixing at 4°C for 30 min followed by centrifugation at 20,000 g for 20 min at 4°C.

The supernatant was recovered and ammonium sulfate was added to a final concentration of 25%, mixed well then centrifuged at 20 000 g for 20 min, 4°C. Ammonium sulfate was added again to the recovered supernatant to a final concentration of 75% and incubated for 2 hrs at 4°C followed by another cycle of centrifugation. The nuclear extract was then dissolved in 3 ml DIAL buffer (20 mM HEPES, pH, 7.9, 1 mM EDTA, 50 mM KCl, 1 mM DTT, and 0.5 mM PMSF). Dialization was carried out in 500 ml DIAL buffer 3 times, 4 hrs each, with the addition of 20% glycerol at the third time. Dialized extract was loaded onto a column containing 5 ml heparin-agarose (HA). The column was then washed with 7 volumes of DIAL buffer and the elution was carried out using 3 volumes of step gradient (100 mM, 300 mM, 600 mM and 1M of KCl prepared in DIAL buffer). The different collected

fractions of the step-gradient were concentrated by centrifugation in Centricon-30 to a final volume of 500-1000 µl. Concentrated fractions were dialized again in 500 ml DIAL buffer with three time changes of the buffer (the last one contained 20% glycerol). The extract was then divided into 50 µl aliquots and kept at -80°C.

B) DNA labelling

The designed DNA primers were labelled at their 5' end using gamma ^{32}P-ATP as follows:

2 µl of primer (10 pmol/µl)

4 µl of 5X reaction buffer

10 µl dH_2O

3 µl gamma-^{32}P-ATP

1 µl of T4 polynucleotide kinase

Incubation took place at 37 °C for 60 min, then inactivated at 75 °C for 15 min. The forward primer, 2.5 µl, was then added at room temperature. The labelled DNA was further purified from non-incorporated labelling material using Sephadex G-25 columns and precipitated by 0.1 volume of Na Acetate and 3 volumes of absolute ethanol then kept at -80°C.

C) The binding reaction

The protein was mixed with the labelled DNA in the following order:

4 µl of binding buffer 5X (100 mM HEPES, pH 7.9, 250 mM KCl, 25 mM $MgCl_2$, 2.5 mM EDTA, 5 mM DTT, 500 µg/ml BSA and 20% Glycerol)

6 µl of protein extract

1 µl of 1mg/ml poly(dI.dC) as a non-specific competitor DNA

6 µl dH_2O

2 µl labelled primer (10 000 cpm/µl)

Incubation took place at 28°C for 30 min, then quickly loaded onto gel

D) Preparation of non-denaturing polyacrylamide gels

The 10% gel was prepared as follows:

2.5 ml 10X TBE (45 mM Tris-Borate, 1 mM EDTA, pH 8)

26 ml dH_2O

12.5 ml 40% A-bA (Acrylamide : bis-Acrylamide as 29:1)

8.6 ml of 2% A-bA

350 µl of 10% amonium persulfate

50 µl TEMED

The gel was left to polymerize for 3 hrs, then was activated by 2 hrs run (without samples) in 0.5 X TBE. This pre-run was important to remove all traces of ammonium persulfate (used to polymerize the polyacrylamide gel) and to ensure a constant gel temperature. Prepared samples were then loaded and the run took place at 100V for 3 hrs.

E) Scanning radiolabelled gels

After the run, the gel was dried for 1 hr at 80°C followed by exposure to the PhosphorImager screen overnight and then removed. The screen was then inserted into the PhosphorImager stage and scanned using ImageQuant software.

CHAPTER III : RESULTS
ARTICLE I

ORGANIZATION AND EXPRESSION STRATEGY OF THE AMBISENSE GENOME OF DENSONUCLEOSIS VIRUS OF *GALLERIA MELLONELLA*

PREFACE

Originally, GmDNV was cloned and sequenced by Dr. Peter Tijssen in the late 1980s. This sequence was submitted to the GenBank under the accession number L32896 in 1994. The original complete clones were lost in the lab move and new clones were obtained and sequenced again, a work done by Dr. Jozsef Szelei and Micheline Letarte, M.Sc. These new clones, indeed, confirmed the earlier sequences without exception indicating that this virus has a very high genetic stability. The phospholipase A2 activity attributed to the virus capsid protein has been studied by Dr. Zoltan Zádori, whereas the expression of densovirus proteins in the baculovirus system has been done by Dr. Yi Li, then a research associate in the lab of Dr. Tijssen. In this work, I contributed by studying the transcription mapping of GmDNV, which was critical for my thesis project in order to compare it with that of MlDNV. This led us to deduce the expression mechanism employed by GmDNV, the first detailed mechanism described for insect parvoviruses. This was a prerequisite before proceeding to densovirus tropism and safety projects, described in the following chapters. My contribution can be summarized by work represented in Fig. 2 (page 81) including the Northern blots, purification of viral mRNA and production of cDNAa, and determination of 5' and 3' ends of viral transcripts. Description and significance of results in Fig. 2 are explained in the text in page 78 under the title "Mapping of viral transcripts". I also participated in the bioassay experiments, done by the virus infectious clone, both *in vitro* and *in vivo*. These data are detailed in the last paragraph of the Results section in page 82 under the title "Transfection with cloned DNV".

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Organization and Expression Strategy of the Ambisense Genome of Densonucleosis Virus of *Galleria mellonella*

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The expression strategy of parvoviruses of the *Densovirus* genus has as yet not been reported. Clones were obtained from the densonucleosis virus of *Galleria mellonella* (*Gm*DNV) that yielded infectious virus upon transfection into LD652 cells. Its genome was found to be the longest (6,039 nucleotides [nt]), with the largest inverted terminal repeats (ITRs) (550 nt) among all parvoviruses. The distal 136 nt could be folded into hairpins with flop or flip sequence orientations. In contrast to vertebrate parvoviruses, the gene cassettes for the nonstructural (NS) and structural (VP) proteins were found on the 5′ halves of the opposite strands. The transcripts for both cassettes started 23 nt downstream of the ITRs. The TATA boxes, as well as all upstream promoter elements, were localized in the ITRs and, therefore, identical for the NS and VP transcripts. These transcripts overlapped for 60 nt at the 3′ ends (antisense RNAs) at 50 m.u. The NS cassette consisted of three genes of which NS2 was contained completely within NS1 but from a different reading frame. Most of the NS transcripts were spliced to remove the upstream NS3, allowing leaky scanning translation of NS1 and NS2, similar to the genes of RNA-6 of influenza B virus. NS3 could be translated from the unspliced transcript. The VP transcript was not spliced and generated four VPs by a leaky scanning mechanism. The 5′-untranslated region of the VP transcript was only 5 nt long. Despite the transcription and translation strategies being radically different from those of vertebrate parvoviruses, the capsid was found to have phospholipase A_2 activity, a feature thus far unique for parvoviruses.

Densonucleosis viruses (densoviruses or DNVs) are parvoviruses of invertebrates (30). Thus far, about 25 DNVs have been isolated from different species of insects belonging to at least six insect orders, shrimps, and possibly crabs (6, 13, 27). All characterized DNVs package complementary, linear single strands of the DNA into separate virions, as do several parvoviruses of vertebrates (e.g., B19 and AAV [11]), and replicate autonomously. DNVs have little sequence identity with the vertebrate parvoviruses (9), and, consequently, they have been classified as a separate subfamily, the *Densovirinae*, in the family of *Parvoviridae* (34). Further studies indicated that there are at least three distinct DNV groups, one genus (*Densovirus*), exemplified by *Junonia coenia* DNV (*Jc*DNV) (12, 19), a second genus (*Brevidensovirus*) by the *Aedes* (1, 8) and shrimp (27) DNVs, and a third genus (*Iteravirus*) by the *Casphalia* DNV (14) and *Bombyx mori* DNV type 1 (22). Most DNVs remain unclassified, since few are characterized with respect to their molecular biology.

The DNV from the greater waxmoth, *Galleria mellonella* (*Gm*DNV), was the first DNV to be isolated (25). Its particles yield four clearly distinct polypeptide bands upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (between 90 and 45 kDa and relative amounts increasing in the order (from least to greatest) VP1, VP3, VP2, and VP4 [32]). These structural proteins have a strong identity upon peptide mapping, and this suggested a common gene (31). Restriction mapping confirmed a close relationship between *Gm*DNV and *Jc*DNV (19). Sequencing of the *Jc*DNV genome demonstrated large open reading frames (ORFs) in the 5′ halves of opposite strands (12), but the expression strategy of this genus of viruses has, as yet, not been published.

The near-atomic structure of *Gm*DNV was solved by X-ray crystallography (28). Since this is the only DNV thus characterized, the structure cannot be compared to that of other DNVs, but some striking differences with the structure of vertebrate parvoviruses were observed. Although the β-barrel of the capsid proteins is highly conserved among virus structures, the *Gm*DNV capsid protein β-barrel must be rotated by 7.4 Å and translated radially inwards by 9.7 Å to superimpose it on the β-barrel of the capsid protein of canine parvovirus when the rotational symmetry axes are superimposed. Among other, surprising differences with the vertebrate parvoviruses was the absence of loop 4 in the *Gm*DNV capsid protein, resulting in a β-annulus type structure instead of a spike around the threefold axis. In *Gm*DNV, the βA strand is linearly extended across the twofold axis from the βB strand, allowing it to form hydrogen bonds with the βB strand of the neighboring subunit. In contrast, the βA strand folds back to its own subunit in vertebrate parvovirus structures thus far solved. The outside of the *Gm*DNV capsid is much smoother than that of vertebrate parvoviruses (28), perhaps as a result of a different evolutionary pressure.

The purpose of this study was (i) to obtain an infectious clone of the virus and a tissue culture host that supports it, (ii) to determine the genome organization of *Gm*DNV, and (iii) to investigate the strategy of transcription and expression. It was found that this virus has an ambisense genome organization, confirming results obtained by in vitro translation of hybrid-selected mRNA (17), with long inverted terminal repeats (ITRs). The expression strategy was found to differ from

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that of all other parvoviruses analyzed thus far. The capsids were also found to contain a domain with phospholipase A_2 activity. Finally, the roadmap of its capsid surface was compared with that of *Jc*DNV, since other studies (e.g., see references 16 and 29) have indicated that parvovirus allotropic determinants reside on or near the virion surface.

MATERIALS AND METHODS

Production and purification of *Gm*DNV and isolation of viral DNA. *Gm*DNV was obtained by infection of *G. mellonella* larvae and purification in CsCl gradients as described previously (31). DNA was extracted from virus particles banding at 1.40 g/ml after dialysis against a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM EDTA. In the original DNA extraction method, proteinase K was added to a final concentration of 50 μg/ml and SDS was added to a final concentration of 0.5%. The sample was incubated for 1 h at 56°C and then diluted with an equal volume of cold water and extracted three times with phenol (equilibrated with 50 mM Tris-HCl [pH 8.0]), once with phenol-chloroform (1:1), and once with an equal volume of chloroform. The phenol phase with interphase was back extracted several times with the buffer. The DNA was precipitated with ethanol from the combined suspensions, resuspended in 1× TE (10 mM Tris-HCl [pH 8.0], plus 1 mM EDTA), and stored at −20°C. Alternatively, the High Pure plasmid isolation kit from Roche Molecular Biochemicals (catalog no. 1754785; Laval, Canada) was adapted to improve quantitatively and qualitatively the extraction of viral DNA. Lysis buffer (300 μl of 6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% Triton X-100 [pH 4.4] containing 80 μg of poly(A) carrier RNA/ml) and 200 μl of sample were mixed and incubated for 10 min at 70°C. The sample was vortexed after adding 125 μl of isopropanol, and the DNA was then purified on the spin columns according to the supplier's instructions.

Cloning of viral DNA. Complete DNA was blunt ended using Klenow and T4 DNA polymerase (10 U each with 300 ng of DNA and 35 μM deoxynucleoside triphosphates in 50 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, and 10 mM dithiothreitol), heated for 15 min at 70°C, and then ethanol precipitated. Either complete DNA or restriction fragments were cloned by standard methods. Terminal fragments (of about 1 kb) were obtained by digestion with *Hind*III and cloned into *Hind*III/*Sma*I-digested pUC19 (pGmH series of clones). A *Bam*HI restriction site was present at about 285 nucleotides (nt) from either end, and the large middle fragment of about 5.5 kb was also cloned in pUC19 (pGm3 and pGm4, respectively, forward and reverse orientation). These two sets of clones served to obtain pBluescript II(KS) clones, which contained the complete viral genome (pGm1 and pGm2, respectively, forward and reverse orientation).

Sequencing and analysis of *Gm*DNV DNA. Originally, M13 subclones, which formed overlapping nested sets, were sequenced by the dideoxy method (Sanger) using the Sequenase (U.S. Biochemicals) or T7 sequencing (Pharmacia) kits. Recent resequencing was by primer-walking using an ABI310, according to the manufacturer's instructions. Both directions, from independent clones, were sequenced. The terminal hairpins yielded compressions which were difficult to read; however, prior treatment with the restriction enzymes (*Alw*NI and *Bst*UI in separate experiments) that cut in that region yielded clean sequence reads.

Isolation and characterization of viral RNA. Total RNA was isolated from infected larvae and transfected, LD652 cells, 48 h postinfection, by the method of Chomczynski and Sacchi (10). Total RNA extracted was subjected to an mRNA purification using an mRNA isolation kit (Roche).

Northern blots. The mRNA was then submitted to electrophoresis on a 1% formaldehyde-agarose gel and blotted on positively charged nylon membranes (Roche). The blotted membranes were incubated overnight at 65°C with the digoxigenin-labeled probes (the 2-kb nonstructural (NS) probe was obtained by PCR amplication between nt 635 and 2658, whereas the 1.3-kb VP probe was amplified between nt 3151 and 4422 of the *Gm*DNV genome). Hybrids were identified with the chemiluminescence kit (CSAPD; Roche) and exposure for 3 h of BioMax film (Kodak) to the membrane.

Mapping of 5′ and 3′ ends of viral transcripts. From the open reading frames obtained by sequence analysis, the most probable location of the transcripts was predicted. The 3′-rapid amplification of cDNA ends system (15) was used to characterize the 3′ ends of the polyadenylated transcripts. A primer (RNA-TAG, 5′-GGG TCT AGA GCT CGA GT [17]) was synthesized which would recognize poly(A) tails [all *Gm*DNV transcripts bear poly(A) tails (18)] and serve as the primer for reverse transcription. Primers upstream of putative AATAAA polyadenylation sites and the ADAP primer (as RNA-TAG but lacking 3′-terminal 16 T) were then used to amplify the 3′ ends of the viral transcripts. For the 5′ end of the transcripts, primers at different distances from the putative transcription starts were used to generate mRNA using AMV reverse transcriptase. After degradation of the RNA by RNaseH, the cDNA was dA tailed using terminal transferase. Using internal DNV-specific primers, reagents, and oligonucleotides and the ADAP primer, amplification by PCR was carried out according to standard methods (15). Restriction sites appended to some primers (such as in RNA-TAG) and just downstream of other primers were used to clone the amplicons. The cloned segments were then submitted to dideoxy sequencing as described above.

Expression of structural proteins by the baculovirus system. In the sequence (below), the potential initiation codons were identified for the VPs (the first five AUGs in ORF4). Each of the potential coding sequences was cloned into the *Autographica californica* nuclear polyhedrosis virus downstream of the polyhedrin promoter by the Bac-To-Bac Baculovirus expression system (GIBCO-BRL) developed by Luckow et al. (23) according to the supplier's instructions.

The following primers were synthesized in order to obtain by PCR amplification the potential structural protein genes (homologous viral sequence are in caps): DNVP1 gtgggatccaaTTAGTCACT*ATG*CTTTCTTCAAAAATC, DNVP2 gtgggatccaca*ATG*TCCCGTCATATTAAT, DNVP3 gtgggatccaca*ATG*CAAGAAGCAACGAAACG, DNVP4a gtgggatccGCT*ATG*TCATTAC CAGGAACTGGC, and DNV4b gtgggatccACA*ATG*GCTATaTCATTACCA GGA as forward primers (italics indicate initiation codons) and DNVPR CACGTCGACTTGCTTTTCAAGAAGCTCCG as the reverse primer. The expressed proteins were analyzed by SDS-PAGE (21) and Western blotting (33) and compared to the VPs from the virus. The ability of the individually expressed capsid proteins to form intact viral capsids was assessed by electron microscopy.

Amino-acid sequencing. Structural proteins from *Gm*DNV were separated by SDS-PAGE (21) on 10% polyacrylamide gels and in the presence of thioglycolate, electroblotted onto polyvinylidene difluoride membranes (Westran; Schleicher & Schuell, Keene, N.H.) (33), and sequenced according to the method of Matsudaira (24).

Transfection of *Gm*DNV clones. Transfection of insect cells (LD652) was carried out using the DOTAP transfection kit (Roche). For this purpose, 2.5 μg of viral DNA was precipitated with 10 μg of DOTAP in 1 ml of serum-free medium for each well of six-well plates containing the same cell densities as were used for infection.

Nucleotide sequence accession number. The GenBank sequence accession number for *Gm*DNV is L32896.

RESULTS

Purification of *Gm*DNV and isolation of DNA. The yield of virus was about 5 μg of DNV per larva. The widely used SDS-proteinase K method for extraction of DNA yielded rarely more than 5 to 10% maximum recovery, even when combined with several back extractions. The extraction conditions were chosen so that the complementary single-stranded DNA annealed upon release from the virion. Electrophoretic analysis of the extracted DNA showed, in addition to the expected 6-kb band, a fuzzy band corresponding to about 4 kb, several bands at high molecular weights, and a general strong background over the whole lane. Most of the DNA stayed in the sample well and may have formed large complexes. The final yield of monomeric double-stranded DNA (<1 μg of DNA/mg of virus) was reproducibly much lower than for comparable DNVs from *Pseudoplusia includens*, *Mythimna loreyi*, *Acheta domesticus*, *Casphalia extranea*, *Spodoptera littoralis*, and *Chilo agamemnon* (unpublished observations). In contrast, the recovery of DNA from *Gm*DNV was virtually complete with the spin column method. Electrophoretic analysis of this DNA did not demonstrate extra visible bands or background, while very little DNA stayed in the sample well.

Cloning and stability of clones. Preliminary single or multiple digestions with restriction enzymes revealed useful sites for cloning. Since a *Bam*HI site was found only near both ends, first a large *Bam*HI-*Bam*HI fragment (5.48 kb) from the viral genome was cloned into pUC19 (two orientations, pGm3 and

pGm4). Both clones served for further restriction and sequencing analysis. Cloning of complete viral DNA was unsuccessful. Therefore, viral DNA was blunt ended, digested with *Hind*III, and cloned into pUC19 vectors digested with *Sma*I and *Hind*III in order to obtain the terminal fragments of the viral genome. The bacterial host and the temperature of incubation of the cultures determined the stability of the inserts. Sure cells, but not JM101, DH5α, XL-1 blue, or Stbl2 cells, with an incubation at 30°C (instead of 37°C), were found to confer stability to the clones over at least five successive overnight cultures. After *Pvu*II digestion, clones obtained using XL-1 blue or Stbl2 bacteria usually yielded inserts that had undergone recombination. These clones yielded a doublet containing the expected 602-nt and an additional 505 (deleted) nt fragments indicating that only a fraction of the population of that clone had not yet undergone recombination). After a few overnight passages all clones had undergone deletions in the hairpins.

Sequencing of DNA. The complete sequence of the viral genome was determined for both strands (Fig. 1). The total length of the genome was 6,039 nt (the longest parvovirus genome described to date) and contained ITRs of 550 nt, although many clones were found up to 4 nucleotides shorter from either end. The convention of Armentrout et al. (2) to present parvoviral sequences with the 3′ end of the viral genome (minus strand) to the left could not be adopted since both strands are packaged and both contain large open reading frames (ambisense genome, see below). Since all parvoviruses following this convention have the genes coding for the nonstructural proteins in the left half of the genome, it was decided to define the strand of the *Gm*DNV genome containing the ORFs for the NS genes as the "+" strand so that the genes would be similarly located.

The distal 136 nt of the ITRs could be folded into the Y-shaped hairpin terminals typical of many parvoviruses (Fig. 2A). In the stem, the first 52 nt are perfectly complementary to nt 85 to 136, whereas nt 53 to 84 can be folded into two asymmetrical GC-rich arms and are present in either of two alternative sequences of 32 nucleotides, "flip" (unpaired pyrimidines in tips of arms in 5′ to 3′ orientation and unpaired purines in the 3′ to 5′ orientation) and its reverse complement, "flop" (Fig. 2A).

Large ORFs. Both complementary strands contain large ORFs in their 5′ halves, one strand with three ORFs (ORF1 to ORF3) of which two are overlapping and one strand with a 2.5-kb ORF (ORF4 [Fig. 1]). Excluding frameshifting, only the latter would be large enough to code for the largest structural protein, VP1. Since all structural proteins have common sequences (29), this suggested that the other structural proteins, VP-2, VP-3, and VP-4, are coded by ORF4 as well. ORF1 to ORF3 would then code for the nonstructural proteins. This hypothesis was reinforced by the observation that ORF1 contains the NTPase motif found in the NS1 of other parvoviruses (3) and that ORF4 contains the phospholipase A_2 motif recently identified in the structural proteins of most parvoviruses (14, 22, 36).

Mapping of viral transcripts. The initiation codon ATG for VP1 was found to start 28 nt after the ITR, suggesting that most of the upstream promoter elements would be located within the ITR. Consequently, the identical promoter elements would be present in the ITR upstream of the NS transcripts. The inboard ends of the ITRs contained potential TATA boxes (Fig. 1). Northern blotting revealed two NS transcripts (about 2.5 and 1.8 kb) and 1 VP transcript (about 2.6 kb) (Fig. 2C). Both NS transcripts were sequenced to further study the mode of expression.

The VP transcript was found to start 23 nt upstream of the 3′ ITR at nt 5467, and the starts of both NS transcripts were at nt 573, also 23 nt downstream of the 5′ ITR. The sequence context of both starts corresponded reasonably well with the consensus sequence for Inr boxes (TCAGTG). The 5′-leader sequence in the VP mRNA to the putative VP1 AUG was therefore only 5 nt long, whereas for the two NS transcripts the 5′-untranslated regions were 82 (1.8-kb transcript) and 84 nt (2.5-kb transcript) long.

The 3′ ends of the transcripts were identified at nt 2954 for the VP transcript and at nt 3013 for both NS transcripts (Fig. 1), i.e., both 13 nt downstream of their canonical AAUAAA polyadenylation signals that start at nt 2971 and nt 2995, respectively. Consequently, the VP transcript overlapped the NS transcript by 60 nt, but it was not clear whether this antisense RNA had any regulatory function. Recently we have observed this phenomenon also with transcripts from other DNVs in the same genus (unpublished observations).

No splicing was observed in the VP transcripts. However, the 1.8-kb NS transcript was found to be a spliced form of the 2.5-kb unspliced NS mRNA (Fig. 1). As estimated by Northern blotting, about 50 to 75% of the NS transcripts were spliced (Fig. 2C). The first initiation codon in the unspliced 2.5-kb NS transcript at nt 657 was in a reasonably favorable context and would yield the 232-amino-acid NS3 protein. This NS3 coding sequence was completely removed in the spliced NS transcript. A single 707-nt splice was observed between nt G(654), 2 nt upstream of the AUG start codon of NS3, and A(1362) of the AUG initiation codon of NS1, 7 nt downstream of the stop codon for NS3 (Fig. 1). The two large downstream ORFs could thus be translated by a leaky scanning mechanism from the same transcript as a result of this splicing. The first initiation codon in this transcript, in the NS1 ORF, starting at nt 1362, was, after splicing, not in a favorable context, which might lead to leaky scanning and explain additional translation from the initiation codon starting at nt 1369, which has a consensus Kozak sequence. The two largest NS proteins (544 and 274 amino acids, respectively) would therefore be generated from the smaller NS transcript.

Predictions of the molecular masses of the four structural proteins from the first four in-frame AUGs in the VP ORF corresponded well with the values seen in SDS-PAGE (32) and suggested a leaky scanning mechanism for this gene as well. The very short untranslated 5′-leader sequence and the less favorable contexts of VP2 and VP3 AUG initiation codons would support such a mechanism. Nevertheless, it would be possible that the set of VPs is generated by proteolysis of the largest VP (VP1), translation downstream of (an) internal ribosome entry site(s), or ribosomal shunting.

Translation products. The capsid proteins were submitted to N-terminal sequencing in order to confirm that these proteins start with the methionines predicted for translation initiation. This was successful only for VP3, since the other N termini were blocked. The sequence obtained for VP3, MQEATK* KADSP (asterisk indicates ambiguous amino acid), indicated

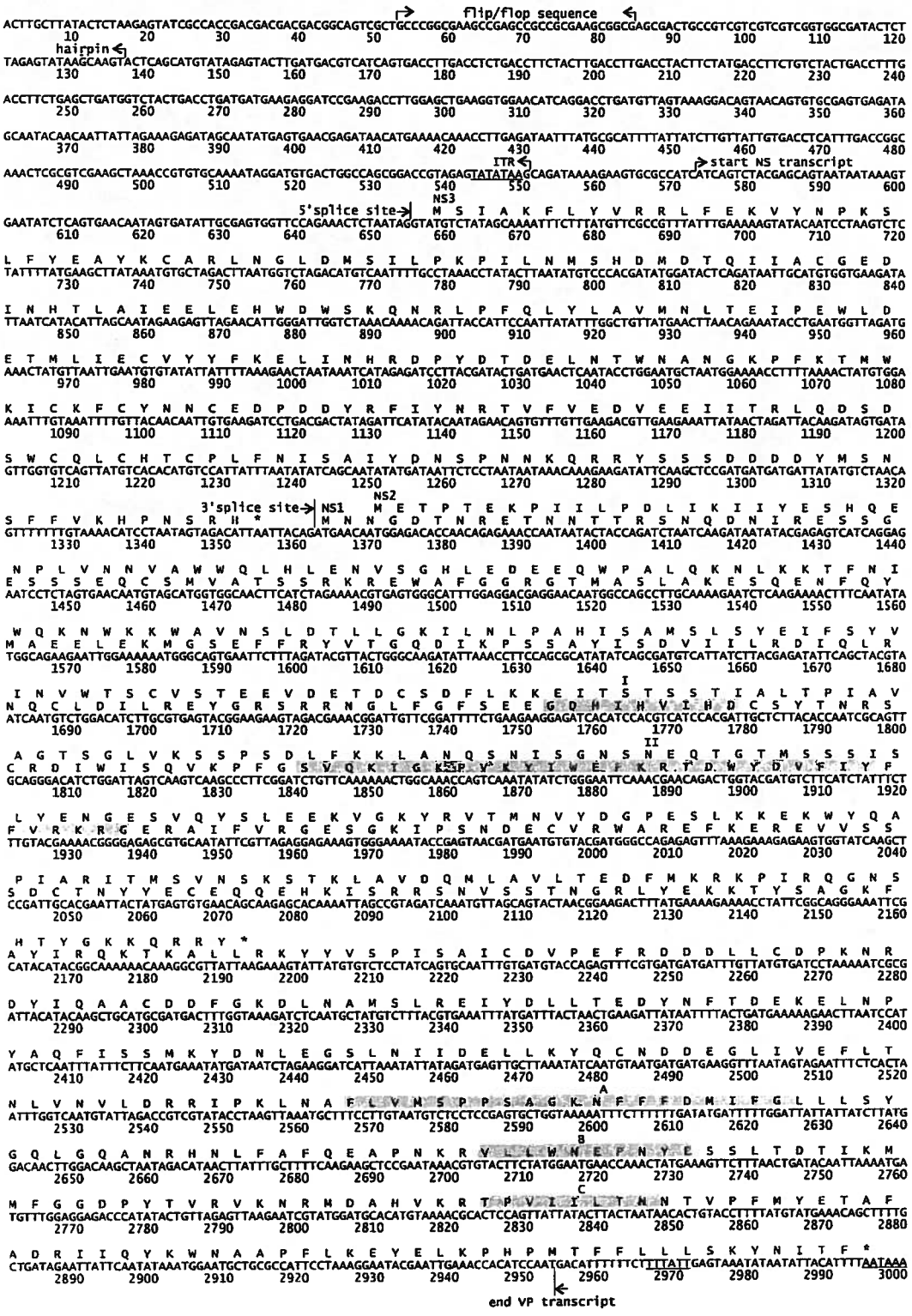

FIG. 1. Sequence of the *Gm*DNV genome. The first 136 nt in the 550-nt ITR can be folded in a typical parvovirus Y-shaped hairpin in which the middle 32 nt occurred in both forward and reverse complementary orientations (flip/flop). The transcripts started 23 nt downstream of the ITRs with the TATA box just inboard of the ITRs. The forward transcription and translation domains are indicated above the sequence, whereas those from the complementary strand are underneath the sequence. The NS and VP transcripts were found to be antisense between positions 2954 and 3013. Domains conserved with other DNVs are shaded (I, II, putative replication initiation domains; A, B, and C, putative DNA-dependent ATPase motifs; and PLA2, phospholipase A_2 motif).

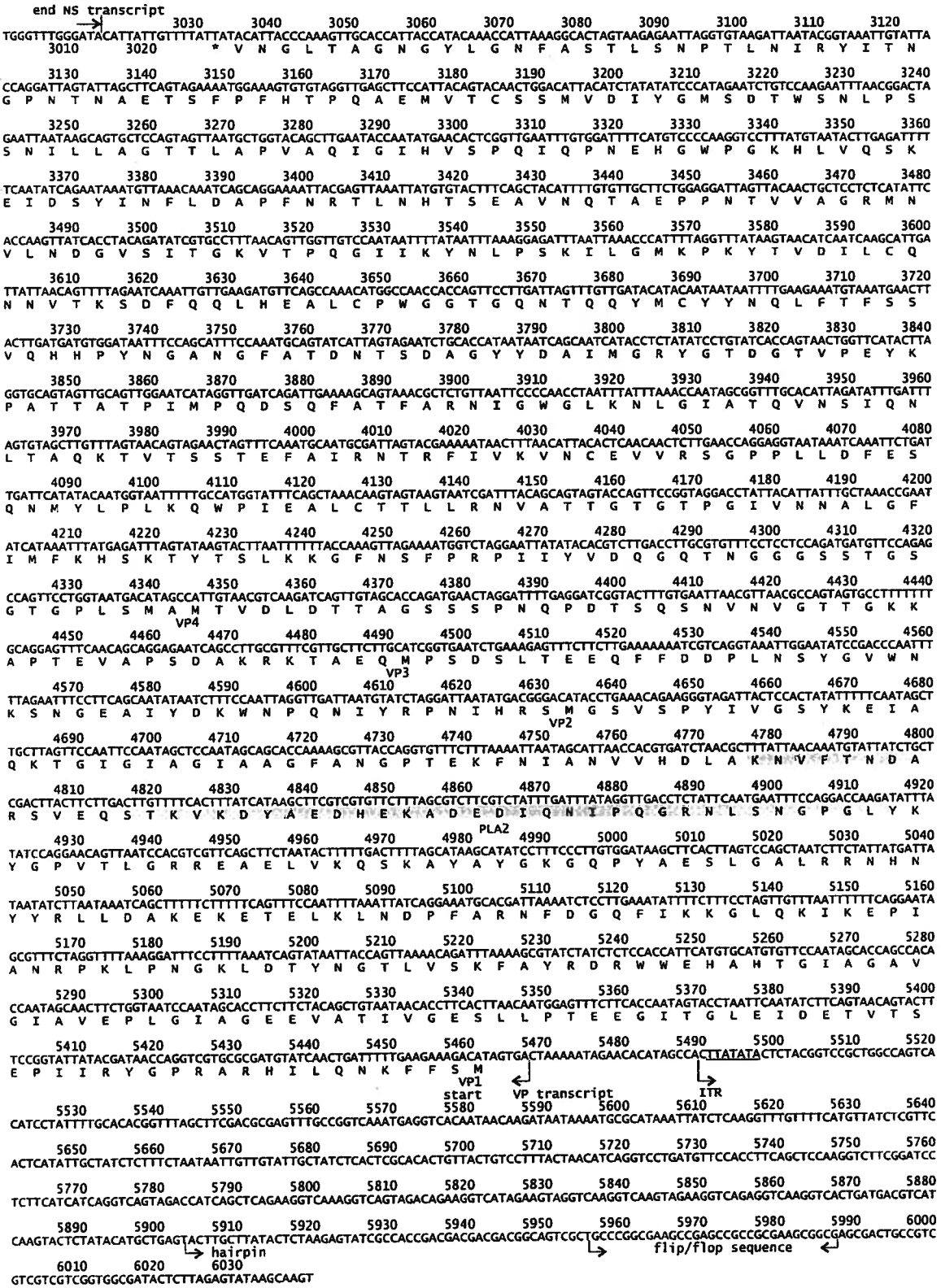

FIG. 1—*Continued.*

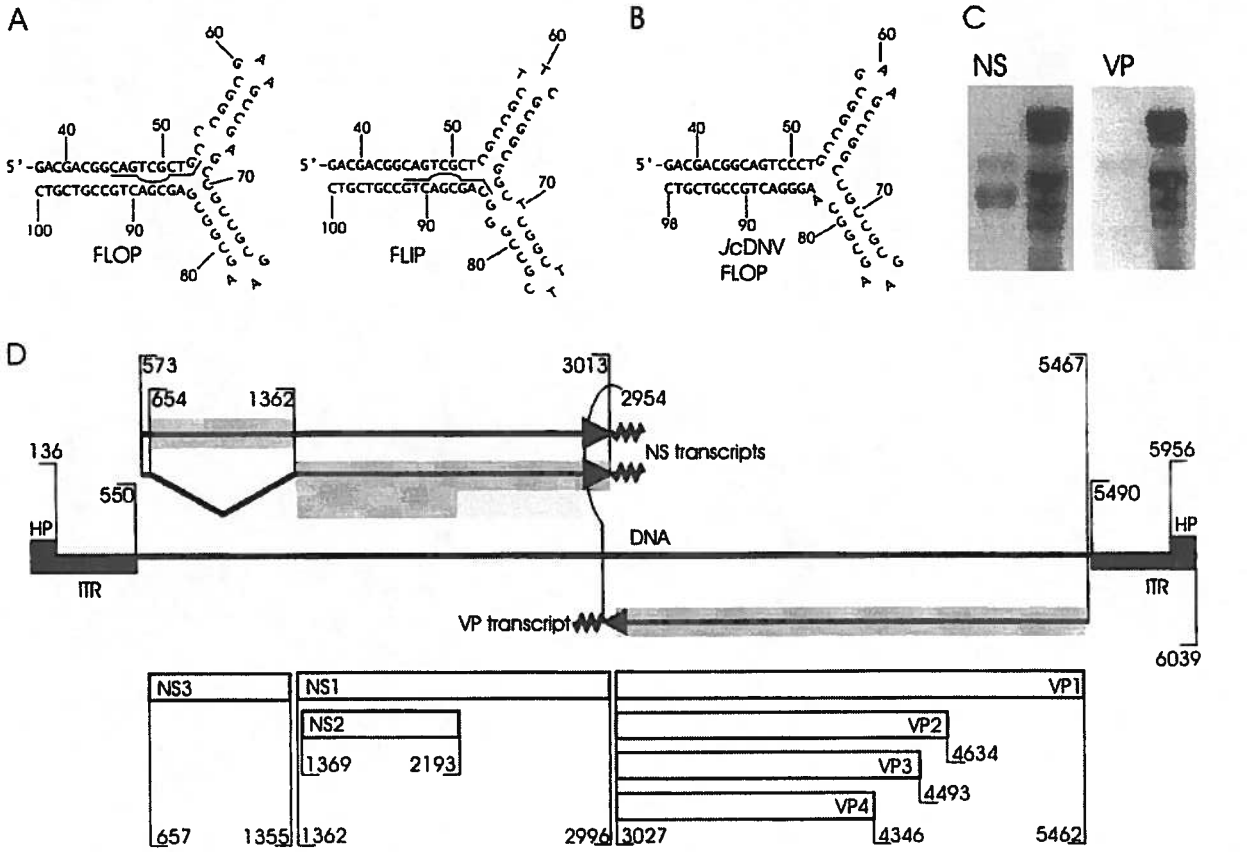

FIG. 2. Organization of the *Gm*DNV genome. (A) Folding of the genome termini of *Gm*DNV into a flip and flop (its reverse complement) orientation. The bipartite *Alw*NI recognition site is underlined. Since one of its nucleotides is located in the flip/flop side-arm, digestion with this enzyme readily distinguishes flip and flop orientations. (B) Structure of the *Jc*DNV hairpin. (C) Northern blots with NS and VP probes. (D) Summary of the *Gm*DNV genome organization.

that its translation started at AUG3 at nt 4493. Previously, Dumas et al. (12) reported for *Jc*DNV that VP4 also starts with an AUG (starting at 4340). This suggested that VP4 initiates at the downstream AUG of the *AUG*CTG*AUG* sequence. It remains possible, however, that the N terminus of another VP4 translation product, initiated at the upstream AUG codon, would be blocked and undetected.

In order to investigate further the translation process, the ORF for the VP and 5′-truncated forms that started just upstream of the different candidate initiation codons were inserted into a baculovirus expression vector in order to eliminate regions that could be involved in internal ribosome entry site or shunting activities. The expression products were analyzed by electron microscopy and Western blotting (Fig. 3). Expression of the VP-1 yielded the same four proteins as for the virus, with similar relative amounts, despite a much longer 5′ untranslated leader sequence in this mRNA than in the case of the viral mRNA (91 versus 5 nt). These proteins autoassembled into virus-like particles that could not be distinguished from virus particles. The context of the candidate AUGs for VP2 and VP3 initiation was improved by changing them to the context found for VP4. Whereas for VP4 hardly any leaky scanning was observed, both VP2 and VP3 initiation codons still showed some leakiness in the baculovirus expression system, although the large majority of translation products now started at the 5′-proximal initiation codon. The size of the proteins that were obtained corresponded to that obtained for proteins from the virus. VP4 was expressed using either of the potential initiation codons between nt 4340 and 4349 by mutating the other. Western blotting showed that strong initiation was obtained from both AUG codons, and electron microscopy revealed that all expression experiments yielded virus-like particles.

Phospholipase A_2 activity. Previously we identified a phospholipase A_2 (motif between amino acids 176 and 230 of the unique region of VP1, and we demonstrated that the expressed protein domain (amino acids 2 to 378) has weak phospholipase A_2 activity, i.e., a $(k_{cat}/K_m)_{app}$ of about $4 \times 10^3\ M^{-1} \cdot s^{-1}$, i.e., about 30% of the enzyme activity from DNVs from the *Iteravirus* genus (14, 22, 36). To investigate whether the domain that was chosen had an impact on the activity, proteins were expressed with domains covering amino acids 1 to 276, 1 to 323, 176 to 323, and 176 to 276,. The activities obtained in the mixed micelles assay at 30°C (highest activity) were similar for all proteins and decreased within 2 weeks to virtually undetectable, whereas the phospholipase A_2 activity of the verte-

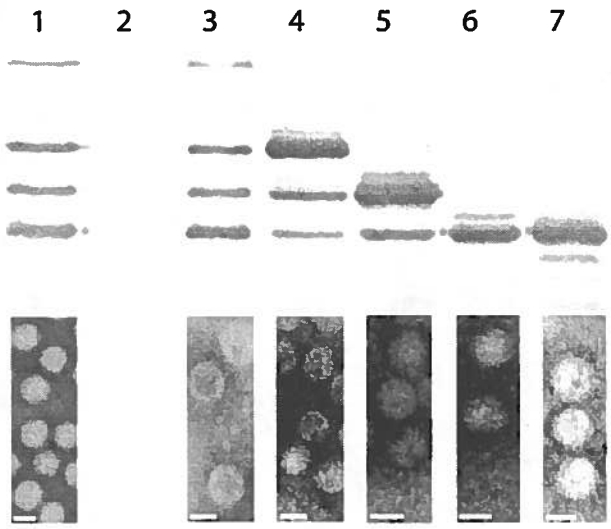

FIG. 3. Expression in baculovirus of *Gm*DNV structural protein from the first five initiation codons of the ORF (3 to 7). Their size corresponded well with the size in the native virus (1). Nonrecombinant baculovirus was used as a control (2). All expressions yielded virus-like particles (bars, 20 nm).

brate parvoviruses remained stable. The proteins were not degraded, suggesting that an incorrect folding caused this low activity.

Transfection with cloned DNV. *Gm*DNV was found to infect LD652 cells from *Lymantria dispar* but not SPC-SL52 from *S. littoralis*. Transfection of LD652 cells with either pGm1 (containing the complete viral genome) or pGm4 (lacking the ITRs from the *Bam*HI sites to the extremities) resulted in positive immunofluorescence in the nuclei of LD652 cells, indicating VP production, but not after mock transfection. Supernatants were collected from pGm1-, pGm4-, and pBluescript-transfected LD652 cells 3 days after transfection, and 5 μl was injected into larvae. Only the supernatant from the pGm1-transfected cells contained infectious virus, as indicated by the mortality of the injected larvae in a dose-dependent manner and the production of virus in these larvae as confirmed by electron microscopy. Injection of larvae with supernatants from pGm4- or mock-transfected cells did not result in mortality, and these larvae underwent normal moulting.

DISCUSSION

Cloning and sequencing of *Gm*DNV genome. Cloning of the full-length genome of *Gm*DNV and prevention of rearrangements in bacteria proved to be much more difficult than the preparation of infectious clones from other DNVs (14, 22) or porcine parvoviruses (4, 5). Both the use of Sure bacteria and culturing at 30°C instead of 37°C were found to be essential. The use of recombination-defective bacteria was previously shown to confer increased stability (7). Five successive overnight cultures in other bacterial hosts, even in Stbl2, or at 37°C all led rapidly to deletions by recombination.

When the sequence of the *Gm*DNV genome was initially determined, several important differences with the *Jc*DNV sequence reported by Dumas et al. (12) were observed: (i) the perfect Y-shaped structure of the *Jc*DNV terminal palindrome (no mismatches in side-arms) was not obtained for *Gm*DNV; (ii) the putative TATA boxes for the NS and VP proteins were for *Gm*DNV inside the ITRs, in contrast to *Jc*DNV, and made it difficult to predict how temporal regulation of expression would be achieved; (iii) the ITRs of *Gm*DNV had identical lengths and thus identical hairpins, in contrast to *Jc*DNV, which, compared to *Gm*DNV, lacked 109 nt at the NS end (no terminal hairpin) and 16 nt at the VP end, which raised the question of whether these ITR differences were somehow involved in temporal regulation of transcription; and (iv) the predicted NS3 of *Gm*DNV was substantially longer than the NS3 of *Jc*DNV. We adopted the same genome orientation and position numbering for *Jc*DNV as for *Gm*DNV to facilitate comparison.

Recently we obtained more independent clones, and clean sequencing reads of the hairpins were obtained with the modified procedure instead of the notoriously difficult-to-interpret compressions usually associated with parvoviral ITRs. This showed that the *Gm*DNV sequence that was originally submitted to GenBank (L32896) was correct. The *Jc*DNV ITR at the VP end in the pBRJ clone was also sequenced and was also found to be correct, indicating structural differences between the ITRs of *Gm*DNV and *Jc*DNV (Fig. 2A and B) caused by an insertion of unpaired bases at positions 55 and 67 in the flop orientation of *Gm*DNV. This is surprising, since for other DNVs a very strong conservation is found in the flip/flop region (cf. *Bm*DNV and *Ce*DNV [14, 22]). The ITRs of *Jc*DNV were found to be as long as those of *Gm*DNV but with a deletion of G258 and mispairings at 540 and in the TATA box at 546 and 548 (numbers correspond to those of *Gm*DNV). The ITRs reported for *Jc*DNV are shorter than those of *Gm*DNV, probably because termini lacking hairpins may have been preferentially cloned or deletions may have occurred due the bacterial host or during subcultures at 37°C. For *Gm*DNV, cloning into XL-1 cells at 37°C gave mostly deleted ITRs, whereas most ITRs obtained using Sure cells and at 30°C were complete. Whereas for *Jc*DNV only a flop orientation was obtained at one end, we obtained both flip and flop orientations at both ends.

The difference in lengths of the predicted NS3 proteins was found to be caused by a sequencing error in *Jc*DNV, i.e., an insertion of 1 nt, corresponding to a position between nt 686 and 687 of *Gm*DNV, thus causing a frameshift. The sizes of NS3 of the two viruses were very similar after this correction.

In contrast to a previous report (17), both spliced and unspliced NS transcripts served for translation, a strategy reminiscent of that of vertebrate parvoviruses. However, for vertebrate parvoviruses with multiple NS products the splicing occurs within the major NS ORF, resulting, after translation, in proteins with different C-terminal domains, whereas splicing of *Gm*DNV NS transcripts resulted in the expression of alternate genes. Unlike vertebrate parvoviruses, the *Gm*DNV VP and spliced NS transcripts direct the synthesis of proteins from multiple initiation codons via a leaky scanning mechanism.

The spliced, bicistronic NS transcript directs the synthesis of NS1 and NS2, initiated, respectively, at the first (NS1) and

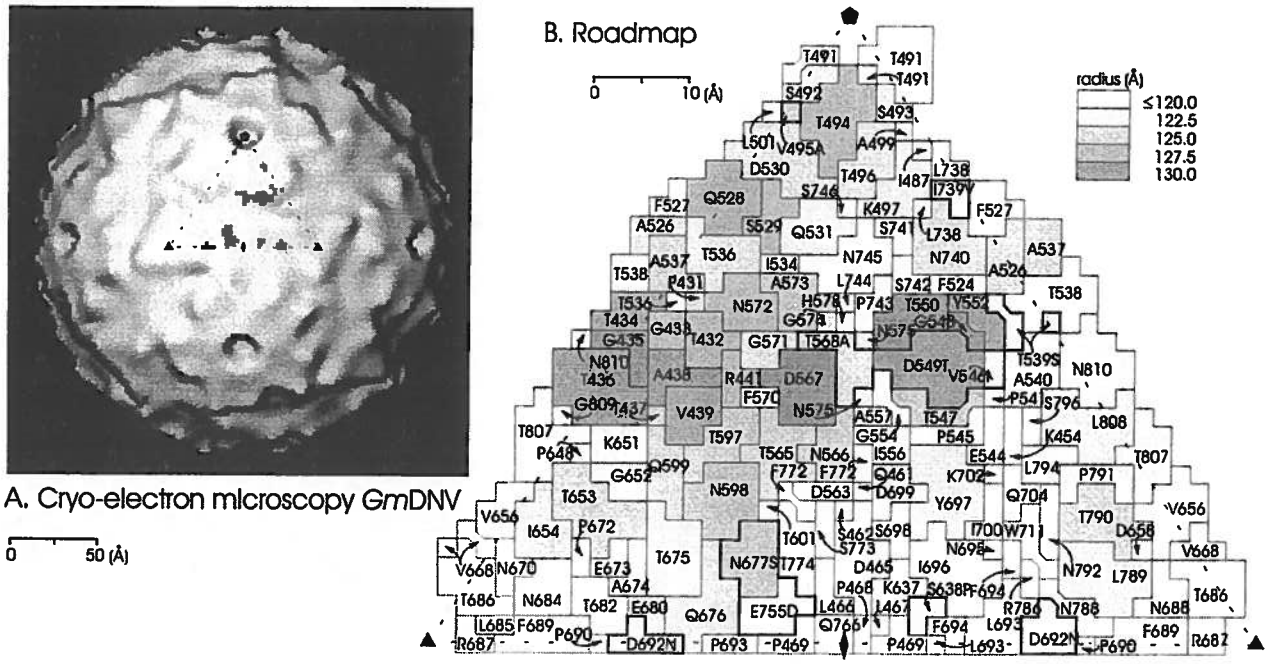

FIG. 4. Roadmap of amino acids in one of the asymmetric units on the surface of the *Gm*DNV particle. Locations of amino acids that differ from those of *Jc*DNV are surrounded by thick lines and indicated on the virus particle. These highlighted amino acids probably are responsible for the phenotypic differences, such as host range, between these two viruses. The penton indicates the fivefold axis, the triangle indicates the threefold axis, and the biface symbol indicates the twofold axis.

second (NS2) AUG codons that are spaced by 4 nt (*AUG*AA CA*AUG*). Interestingly, RNA-6 of influenza virus B also directs the synthesis of two proteins (integral membrane glycoprotein and neuramidase) from two initiation codons by leaky scanning with an identical spacing sequence of AACA (26). It was suggested that linear scanning by the 40S ribosomal subunit may break down if two AUGs are in close apposition, since increasing the spacing to 46 nt prevented initiation from the second AUG (35). However, Kozak (20) provided evidence of adherence to the first-AUG rule and a context-dependent leaky scanning mechanism. It is also interesting that the context of the first AUG in influenza virus RNA-6, with an A in position −3, is adequate (GenBank no. NC_004284). Although the *Gm*DNV NS1/NS2 transcript has a long leader sequence, it has a suboptimal context of the 5′-proximal AUG codon. The better context of the NS2 initiation codon probably explains why the 30-kDa NS2 was found to be the most abundant translation product after in vitro translation of viral RNAs (17).

Removal of potential internal ribosome entry sites or ribosomal shunting domains by expression of the VP-coding domains without upstream sequences into baculovirus did not change the production of multiple VPs. Translation by a leaky scanning mechanism, in which the 40S ribosomal subunits bypass initiation codons, is rare, in particular for more than two translation products (20). Nevertheless, the DNVs of the *Iteravirus* (14, 22) as well as the *Densovirus* genus, as exemplified by *Gm*DNV, use this strategy to generate their sets of structural proteins. In contrast, parvoviruses from vertebrates use alternative splicing. Interestingly, if the very short untranslated 5′ region (5 nt) of the *Gm*DNV VP transcript was replaced by a long untranslated region in the baculovirus system, leaky scanning was not significantly different, suggesting that other factors contribute to this mechanism. The production of the relative amounts of the four viral proteins may, however, differ slightly from batch to batch.

The allotropic determinant of some parvoviruses has been located in the structural proteins (5, 18) and is present on or near the surface of the virion (29). Preliminary results in our laboratory, using chimerae obtained by exchanging restriction fragments between the infectious clones of *Gm*DNV and *Ml*DNV, indicated that the allotropic determinants of these viruses are also located in the VP gene. Some of the limited number of amino acids on the surface of the virion that differ with those on the *Jc*DNV particle, as predicted from the *Gm*DNV structure (28) and the sequence differences between *Gm*DNV and *Jc*DNV, could thus be responsible for the host range of each virus (Fig. 4). This polymorphism could also be responsible for other phenotypic properties that distinguish these closely related DNVs. This roadmap shows that there are only 10 amino acid differences on the surface of each protein of the viral capsid, some of which are adjacent in the tertiary structure but not in the primary structure (e.g., N677 and E755, T568 and D549). Nevertheless, it remains possible that some of the N-terminal extensions of the structural proteins are somehow involved in tropism. Currently, the impact of these amino acid differences on the host-range phenotype is further investigated.

ACKNOWLEDGMENTS

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CHAPTER IV : RESULTS
ARTICLE II

EXPRESSION STRATEGY OF DENSONUCLEOSIS VIRUS *FROMMYTHIMNA LOREY/*

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

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Fédière G, El-Far M, Li Y, Bergoin M, Tijssen P. Expression strategy of densonucleosis virus from Mythimna loreyi. Virology. 2004 Mar 1;320(1):181-9. DOI : http://dx.doi.org/10.1016/j.virol.2003.11.033

PREFACE

The densovirus MlDNV has been isolated and characterized with respect to host range in the mid 1990s by Dr. Gilles Fédière, who was then leading a research group at the "Institut de la recherche pour le développement (formerly ORSTOM, France)" at the Faculty of Agriculture ofCairo University. He obtained several partial and complete clones of the viral genome during his sahhatical year in our lahoratory ofiNRS - Institut Armand-Frappier (1997-1998) and sequenced up to 90o/o of the genome. I reconfrrmed the earlier sequence and obtained the remaining hairpin sequences that were stilllacking (Fig. 2A). 1 also carried out ali the bioassay tests and the transcription mapping (Fig 1. and Fig. 2B and 3, respectively). Dr. Yi Li contributed by helping in the construction and testing the baculovirus expression system to study the mechanism of viral protein production (Fig. 4).

CHAPTER V : RESULTS

CHARACTERIZATION OF A NEW DENSOVIRUS FROM *HELICOVERPA ARMIGERA*, HADNV

Introduction

Densoviruses are widespread in different orders, families and species of insects. They are associated with their hosts whether in acute or occult forms. Isolation and reintroduction of these densoviruses into insect populations usually meets a large degree of success in controlling these pests (Bergoin and Tijssen, 1998; Fédière, 2000). Local isolation of DNVs is of great importance since it increases the repertory of bioagents useful to combat and to control these pest insects to levels below the economic threshold without having to import foreign pathogens.

Isolation of new viruses, cloning of their genome and sequencing and studying their biology, both *in vivo* and *in vitro*, are all important aspects that need to be addressed before they can be added to the biological control arsenal. Since the late 1980s, the Entomovirological Research Group in Cairo (Faculty of Agriculture of Cairo University, Egypt), the IRD (Institut de la recherche pour le développement, France) and INRS - Institut Armand-Frappier have developed a program to obtain new DNV isolates from the agricultural fields. This program led to the isolation of the MlDNV, studied in the previous chapters of the current thesis. Recently, this research also yielded a new densovirus-like virus from the heliothis, *Helicoverpa armigera* (Noctuidae: Lepidoptera), one of the most important pests of sweet corn and cotton. In this chapter, we will describe the cloning and sequencing of this virus, named HaDNV, as well as comparison of its genome and conserved domains with that of other parvoviruses.

Cloning of the complete genome of HaDNV

The HaDNV DNA was extracted from the virus in conditions of high ionic strength and yielded both full-length dsDNA as well as shorter dsDNA fragments. The

virus has a DNA genome of around 6 Kb as shown by agarose gel electrophoresis. This DNA was then blunt-ended by a mixture of DNA polymerase I (Klenow Fragment) and T4 DNA polymerase that has a 3'-5' exonuclease activity. The blunt-ended DNA was cloned into the *Eco*RV site of pBluescript vector. Nevertheless, even with this approach cloning of the full length genome remained difficult. However, several subclones carrying small fragments were obtained. In fig. 12A (right panel), three subclones (H2A, H6A and H10A) are shown, however, a number of other small clones created by the same method were also recovered (data not shown).

In a second set of experiments, the viral DNA was analyzed by several restriction enzymes (RENs), *Bam*HI, *Eco*RI, *Eco*RV, *Hin∂*III and *Bgl*II. The RENs profiles indicated a close relationship to both MlDNV and GmDNV. Unfortunately, no clean pictures are available for these gels. One of the most interesting profiles was the *Bam*HI pattern. Digesting the viral DNA with this enzyme gave rise to a large fragment of about 5.5 Kb and a small fragment of ≃0.3 Kb. This small DNA fragment is probably a doublet of 0.3 Kb, resulting from the existence of symmetrical *Bam*HI sites within the viral putative ITRs, similar to some of the other densoviruses. This observation helped to clone the viral DNA by methods that were successfully employed for GmDNV and MlDNV. The first method consisted of digesting the DNA by *Bam*HI followed by cloning into pBluescript *Bam*HI site. Two large clones were recovered from one cloning assay by this method, pHa2 and pHa8 (Fig. 12A, left panel). Considering that the cloning was carried out by *Bam*HI digestion, incubating the two new clones with *Bam*HI should give only two fragments: 5.5 Kb, corresponding to the viral DNA, and a 2.9 Kb fragment of the linearized vector. The pHa2 clone yielded the expected fragment sizes when analyzed by RENs, whereas, pHa8 carried, after *Bam*HI digestion, an additional small fragment of about 0.4 Kb (Fig. 12B). Taking in account the size of the cloning vector's DNA as well as the lack of the densovirus terminal sequences from the pHa2 clone, the different

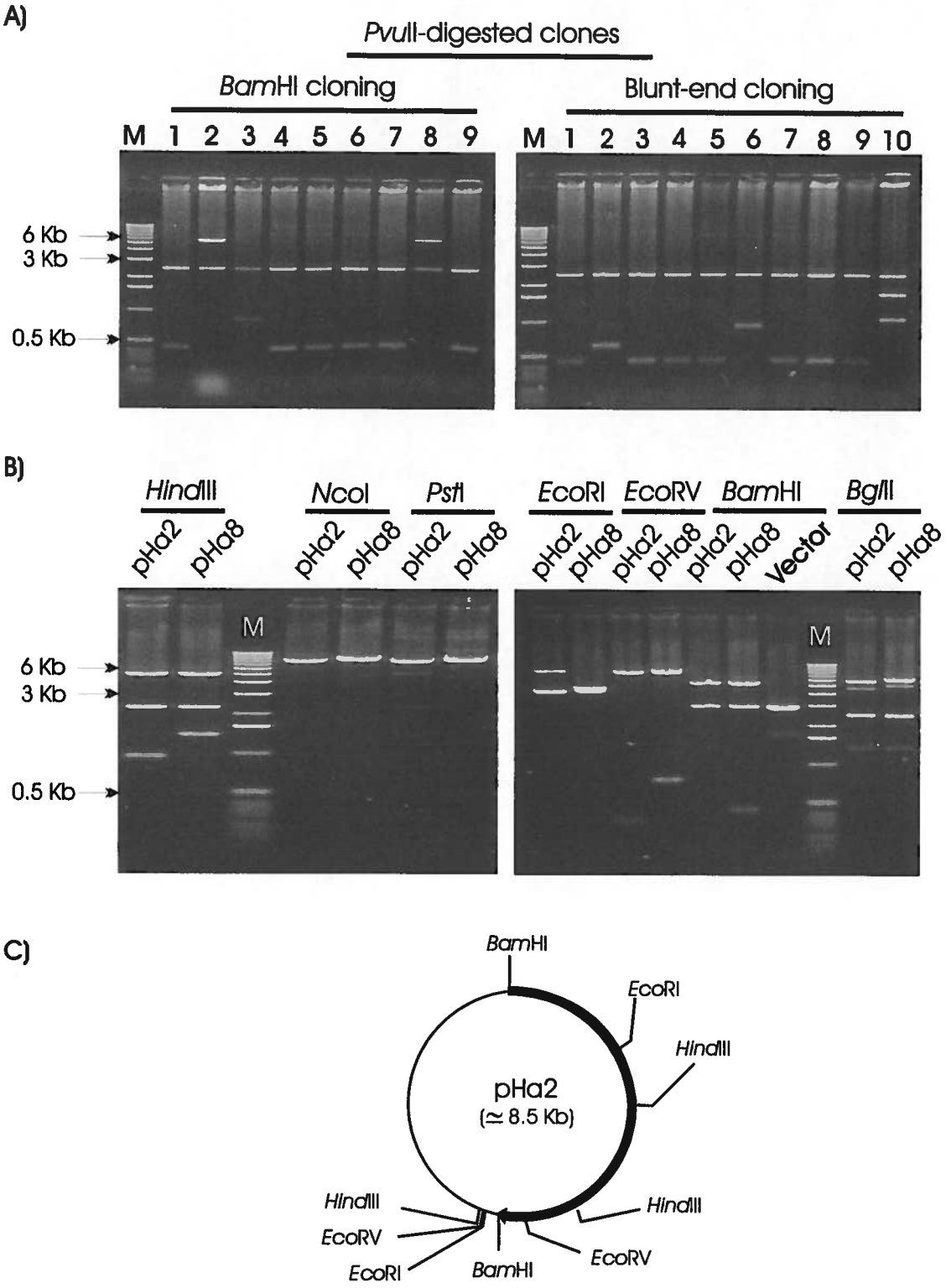


Fig. 12. Cloning of HaDNV genome into pBluescript vector. A: to the left, partial clones obtained by *Bam*HI digestion of native viral DNA and cloning into the vector *Bam*HI site. This cloning resulted in two large clones, pHa2 and pHa8, 2&8, respectively. To the right, sub-clones created by artificially blunt-ended DNA cloned into the vector *Eco*RV site that resulted in H2A, H6A and H10A, lanes, 2, 6 and 10, respectively. B: Large clones, pHa2 and pHa8 digested by different restriction enzymes (REN). C: predicted locations of the different REN, used in B, onto the cloned viral DNA, pHa2 clone.

REN profiles of this clone (Fig. 12B) corresponded well to homologous profiles of native viral DNA digested with the same restriction enzymes. Therefore, the sequence of pHa2 is authentic to HaDNV DNA.

Sequencing of cloned genomic sub-fragments

The two large clones, pHa2 and pHa8, were subjected to sequencing using the primer-walking method. pHa2 was sequenced and the *Bam*HI-*Bam*HI fragment was found to be 5470 nts long of which the distal 267 nts are perfect complementary inverted terminal repeats. Sequencing of pHa8 showed that it carries the same large *Bam*HI insert of HaDNV, but with an extra 300 nts that correspond in sequence to the terminal hairpin of classical densoviruses. This extra fragment was cloned in an opposite direction and is probably a cloning artifact. Sequencing of the small blunt-ended subclones, H2A, H6A and H10A showed that H2A and H10A carry fragments that are identical to internal sequences of pHa2 and pHa8. However, H6A contained 541 nts from which the distal 126 nts formed the typical Y-shaped hairpin of classical densoviruses. From the other end, this fragment had also 263 nts overlapping the terminal sequences of the *Bam*HI-*Bam*HI viral fragment in the pHa2 clone, confirming that it contains one of the two viral ITRs. Reconstruction of the two sequences, pHa2 and H6A, by computer analysis yielded the complete viral sequence of 6039 nts with ITRs of 545 nts (Fig. 13). Thus, the virus genome size was similar to that of the GmDNV, the longest genome size within the family of *Parvoviridae*.

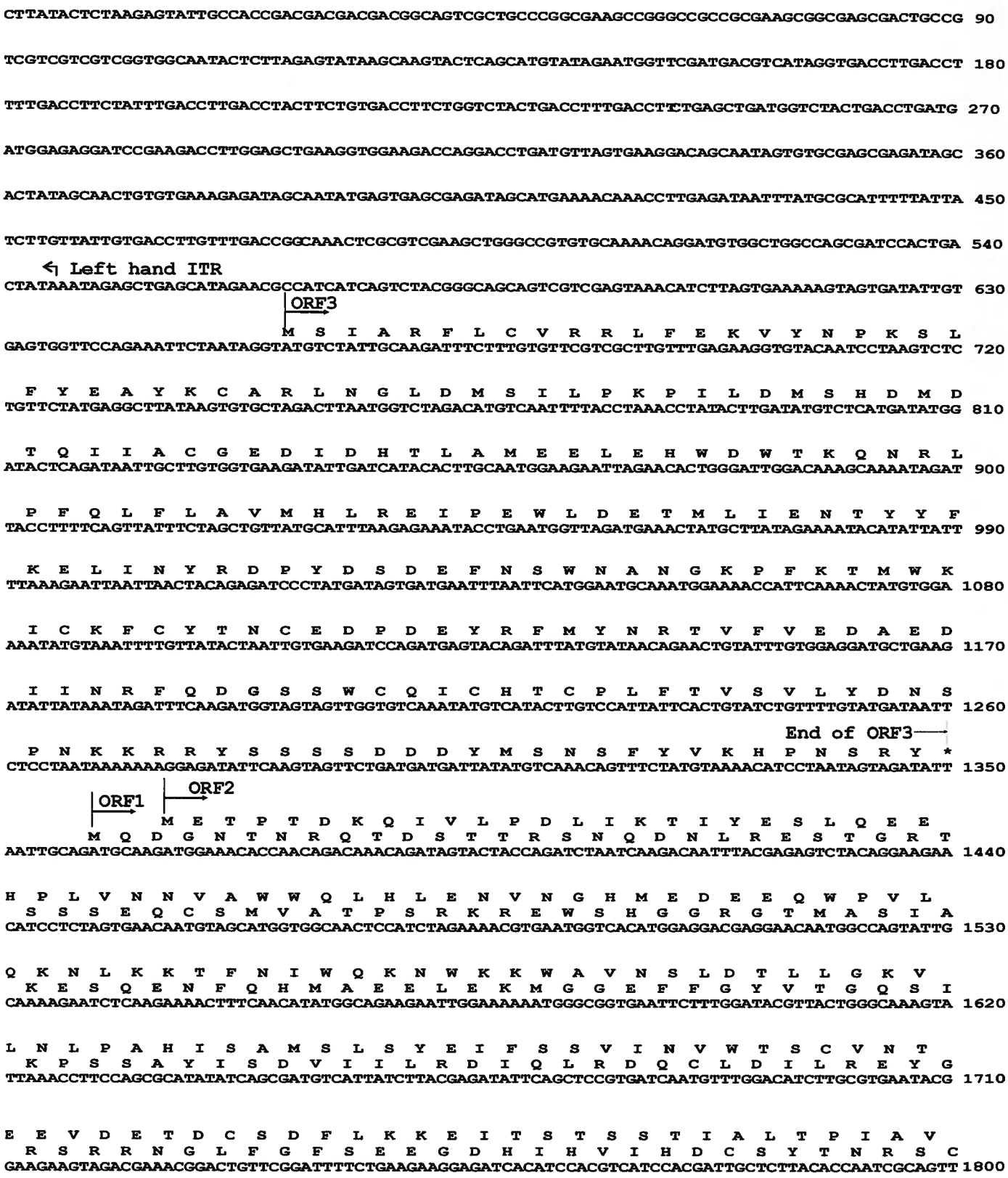
Sequence analysis

The HaDNV genome contains typical ITRs of the densovirus genus (classical densoviruses) with a length of 545 nts. The terminal hairpin of 126 nts is completely

conserved between HaDNV and MlDNV. However, sequence differences start to accumulate downstream of the hairpin with 13 nts changes and 2 extra nucleotide inserts in the ITRs of the HaDNV genome (Fig. 14). The overall sequence is 90% identical to MlDNV and 86% identical to GmDNV. Interestingly, MlDNV and GmDNV share 90% of sequence identity. The same degree of identity exists also between GmDNV and JcDNV as well as between MlDNV and JcDNV.

Using the NCBI ORF-Finder program, four large ORFs were identified (Fig. 15A). The large ORF1 on the forward strand has a capacity to code for the densovirus NS1 with a putative a.a. number of 547. ORF2 corresponds to NS2 with a capacity of 275 a.a. and ORF3 corresponds to NS3 with 231 a.a. On the complementary strand, a large ORF with a potential coding region of a 811 a.a. corresponds well to that of the VP structural proteins of related densoviruses. The distribution of the putative coding sequences of HaDNV on the two viral strands thus implied an ambisense organization.

Looking for conserved domains within the putative NS1 showed that the virus contains the highly conserved initiator (replicator) motifs with sequence identity and location similar to the other densoviruses as well as vertebrate parvoviruses (Fig. 15B). The second conserved motif is the NTP-ase NS-1 helicase superfamily III that is located between a.a. 404 and 499 (Fig. 15C).


CTTATACTCTAAGAGTATTGCCACCGACGACGACGACGGCAGTCGCTGCCCGGCGAAGCCGGGCCGCCGCGAAGCGGCGAGCGACTGCCG 90
TCGTCGTCGTCGGTGGCAATACTCTTAGAGTATAAGCAAGTACTCAGCATGTATAGAATGGTTCGATGACGTCATAGGTGACCTTGACCT 180
TTTGACCTTCTATTTGACCTTGACCTACTTCTGTGACCTTCTGGTCTACTGACCTTTGACCTTTGAGCTGATGGTCTACTGACCTGATG 270
ATGGAGAGGATCCGAAGACCTTGGAGCTGAAGGTGGAAGACCAGGACCTGATGTTAGTGAAGGACAGCAATAGTGTGCGAGCGAGATAGC 360
ACTATAGCAACTGTGTGAAAGAGATAGCAATATGAGTGAGCGAGATAGCATGAAAACAAACCTTGAGATAATTTATGCGCATTTTTATTA 450
TCTTGTTATTGTGACCTTGTTTGACCGGCAAACTCGCGTCGAAGCTGGGCCGTGTGCAAAACAGGATGTGGCTGGCCAGCGATCCACTGA 540
Left hand ITR
CTATAAATAGAGCTGAGCATAGAACGCCATCATCAGTCTACGGGCAGCAGTCGTCGAGTAAACATCTTAGTGAAAAAGTAGTGATATTGT 630
ORF3
M S I A R F L C V R R L F E K V Y N P K S L
GAGTGGTTCCAGAAATTCTAATAGGTATGTCTATTGCAAGATTTCTTTGTGTTCGTCGCTTGTTTGAGAAGGTGTACAATCCTAAGTCTC 720
F Y E A Y K C A R L N G L D M S I L P K P I L D M S H D M D
TGTTCTATGAGGCTTATAAGTGTGCTAGACTTAATGGTCTAGACATGTCAATTTTACCTAAACCTATACTTGATATGTCTCATGATATGG 810
T Q I I A C G E D I D H T L A M E E L E H W D W T K Q N R L
ATACTCAGATAATTGCTTGTGGTGAAGATATTGATCATACACTTGCAATGGAAGAATTAGAACACTGGGATTGGACAAAGCAAAATAGAT 900
P F Q L F L A V M H L R E I P E W L D E T M L I E N T Y Y F
TACCTTTTCAGTTATTTCTAGCTGTTATGCATTTAAGAGAAATACCTGAATGGTTAGATGAAACTATGCTTATAGAAAATACATATTATT 990
K E L I N Y R D P Y D S D E F N S W N A N G K P F K T M W K
TTAAAGAATTAATTAACTACAGAGATCCCTATGATAGTGATGAATTTAATTCATGGAATGCAAATGGAAAACCATTCAAAACTATGTGGA 1080
I C K F C Y T N C E D P D E Y R F M Y N R T V F V E D A E D
AAATATGTAAATTTTGTTATACTAATTGTGAAGATCCAGATGAGTACAGATTTATGTATAACAGAACTGTATTTGTGGAGGATGCTGAAG 1170
I I N R F Q D G S S W C Q I C H T C P L F T V S V L Y D N S
ATATTATAAATAGATTTCAAGATGGTAGTAGTTGGTGTCAAATATGTCATACTTGTCCATTATTCACTGTATCTGTTTTGTATGATAATT 1260
End of ORF3
P N K K R R Y S S S S S D D D Y M S N S F Y V K H P N S R Y *
CTCCTAATAAAAAAAGGAGATATTCAAGTAGTTCTGATGATGATTATATGTCAAACAGTTTCTATGTAAAACATCCTAATAGTAGATATT 1350
ORF1
ORF2
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M Q D G N T N R Q T D S T T R S N Q D N L R E S T G R T
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S S S E Q C S M V A T P S R K R E W S H G G R G T M A S I A
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Q K N L K K T F N I W Q K N W K K W A V N S L D T L L G K V
K E S Q E N F Q H M A E E L E K M G G E F F G Y V T G Q S I
CAAAAGAATCTCAAGAAAACTTTCAACATATGGCAGAAGAATTGGAAAAAATGGGCGGTGAATTCTTTGGATACGTTACTGGGCAAAGTA 1620
L N L P A H I S A M S L S Y E I F S S V I N V W T S C V N T
K P S S A Y I S D V I I L R D I Q L R D Q C L D I L R E Y G
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E E V D E T D C S D F L K K E I T S T S S T I A L T P I A V
R S R R N G L F G F S E E G D H I H V I H D C S Y T N R S C
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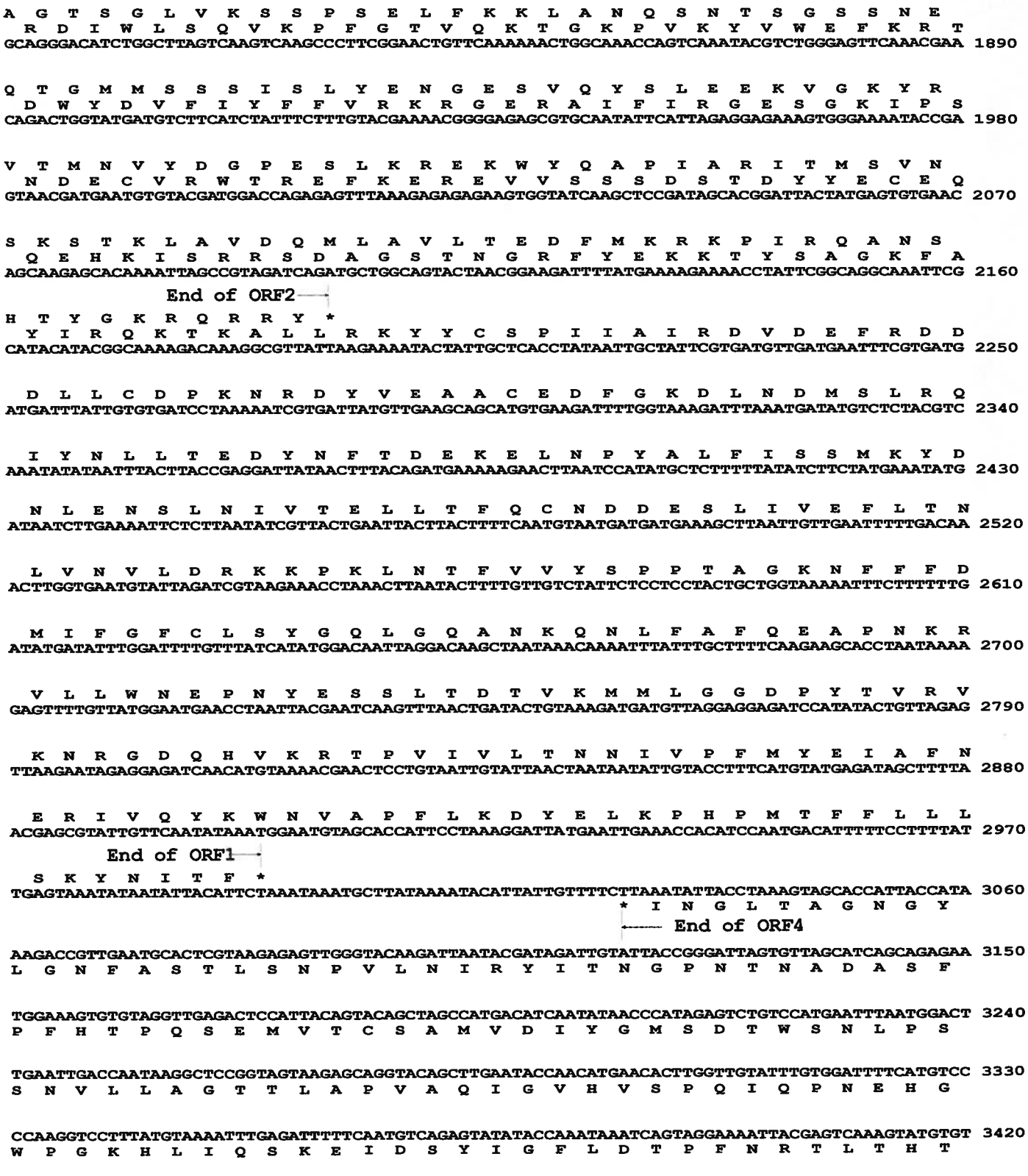
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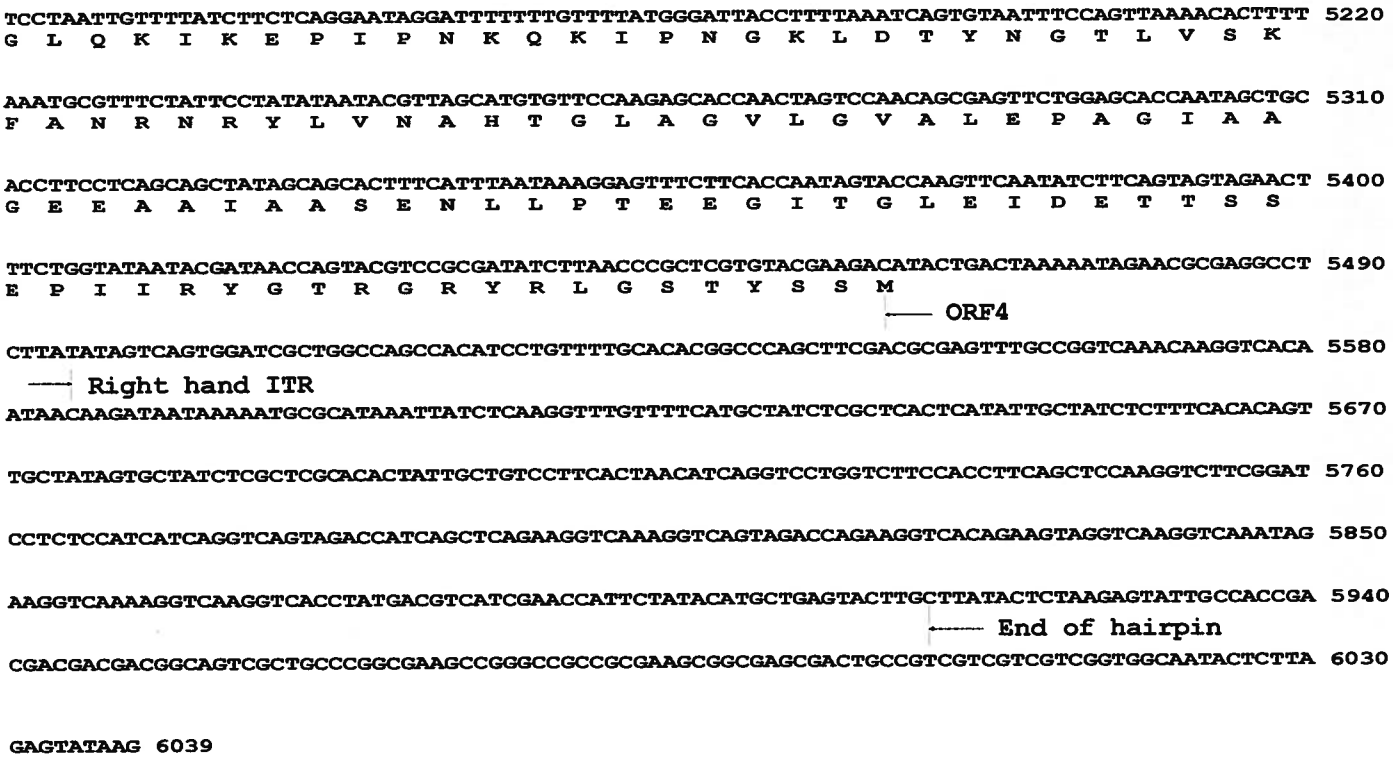


Fig. 13. HaDNV sequence. The viral DNA has two large ITRs of 545 nts. Large ORFs that correspond to NS and VP coding sequences in classical densoviruses are indicated on the figure. ORFs are predicted by ORF finder (NCBI utilities).

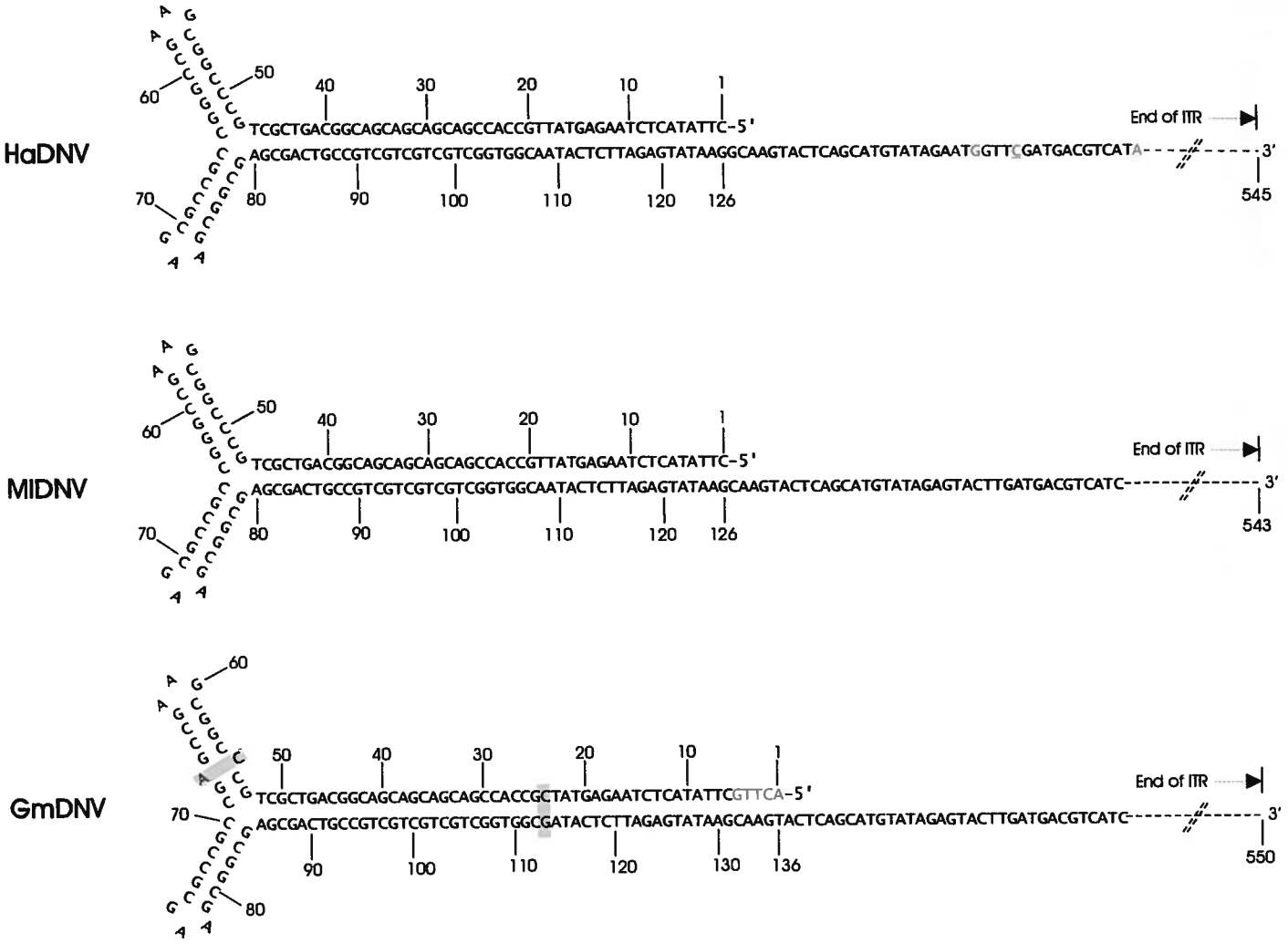


Fig. 14. Comparison between HaDNV hairpin and that of MIDNV and GmDNV. The terminal hairpin of 126 nts is fully conserved between HaDNV and MIDNV. Differences between the two virus genomes started to accumulate downwards from the hairpin (coloured in red in HaDNV). GmDNV has a longer hairpin of 136 nts, 10 nts longer than HaDNV and MIDNV, with two nts differences, one of them reside within the stem no 1 (in blue rectangle).

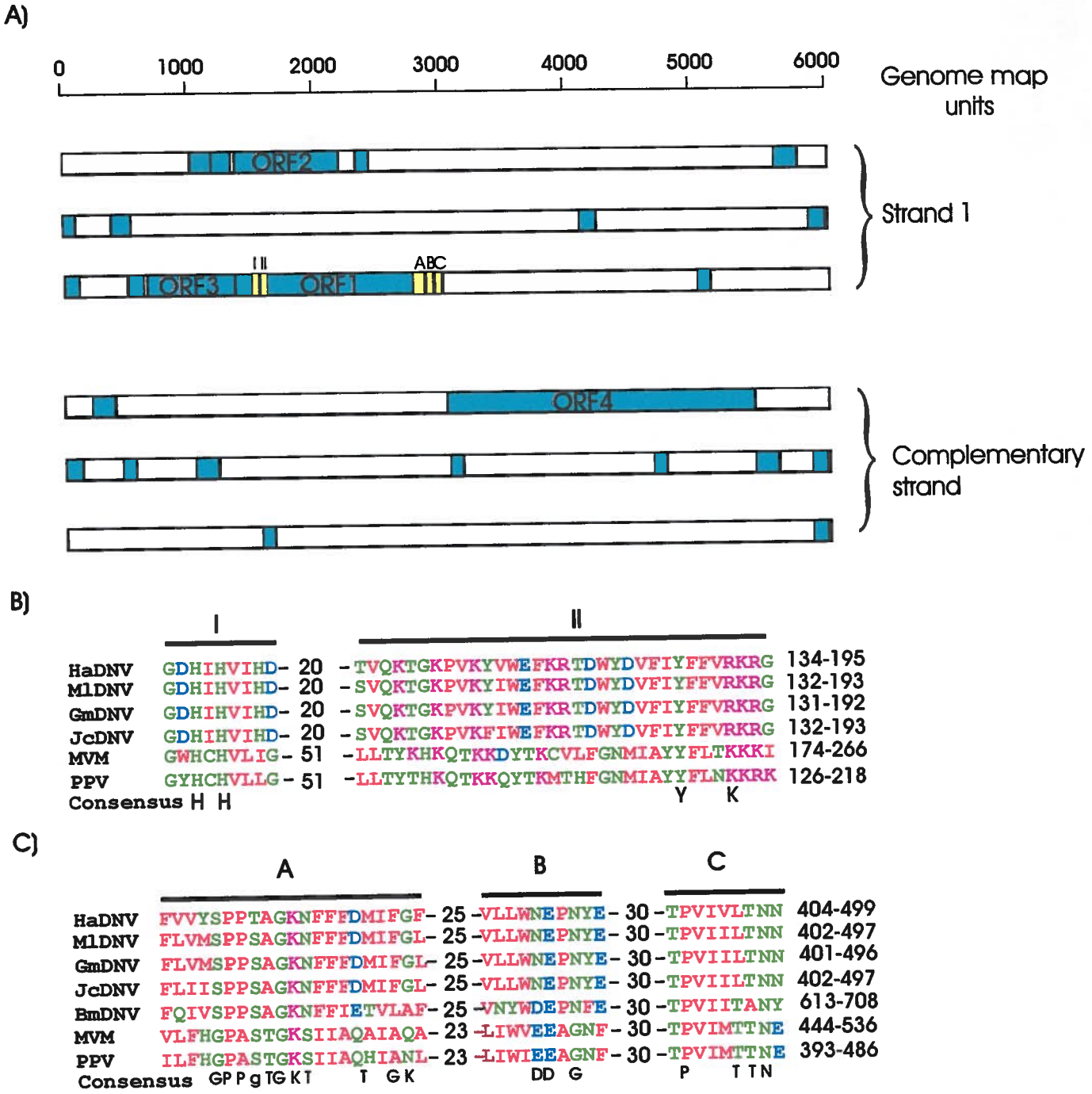


Fig. 15. HaDNV genome organization and conserved domains. A: HaDNV large ORFs as depicted by ORF-finder program. B: Conserved initiator (replicator) protein motifs found on putative NS1 sequence. C: Conserved NS-1 helicase superfamily III motifs.

DISCUSSION

The present work aimed to clone and to sequence the complete genome of the newly isolated densovirus HaDNV. As with other parvoviruses, which have single-stranded DNA genomes with complete terminal hairpins, cloning of complete genome is known to be difficult. For instance, after initial attempts to obtain an infectious clone of B19 parvovirus (Shade *et al.*, 1986) it took 18 years to obtain such a clone due to the instability of the terminal hairpins (Zhi *et al.*, 2004). The HaDNV viral plus and minus strands were annealed under high salt conditions then blunt-ended and cloned. Nevertheless, cloning remains difficult as the termini contain flip/flop orientation sequences that prevent perfect annealing. However, we overcame this difficulty by combining two methods of partial cloning. The partial clones were used to reconstitute the whole virus genome in one clone. Infectivity of this clone was confirmed by transfection of LD652 cells (Jozsef Szelei, personal communication).

The sequence of HaDNV DNA showed that it has a genome of 6039 nts, which is equal in size to that of GmDNV, the longest genome size among all parvoviruses. The genome analysis showed a high sequence identity to GmDNV, MlDNV and JcDNV of around 90 %. Interestingly, such a high degree of sequence homology exists only within members of the classical densoviruses (subgroup A), isolated from lepidopteran hosts. In contrast, other densoviruses isolated from dipteran, orthopteran, dictyopteran or hemipteran insects have a low level of sequence identity ($< 20\%$), which may due to their adaptation to evolutionarily distant hosts (Tijssen *et al.*, 2004). HaDNV shares with the classical densoviruses the ambisense genomic organization, which is typical for this group of viruses (Dumas *et al.*, 1992). This could be deduced from the presence of a set of three ORFs at the 5' half of one strand and only one large ORF at the 5' half on the complementary strand. The NS cassette can be recognized by the NTPase domain that

is present within the NS1 of all parvoviruses (Tijssen and Bergoin, 1995). The NTPase domain contains sequences homologous to the phylogenically conserved Walker A- and B-site motifs which are major components of the helicase superfamily III (Gorbalenya, 1989; Gorbalenya and Koonin, 1989; Koonin, 1993). Although densoviruses have divergent NTPase sequences, the helicase activity of this domain has been shown for JcDNV NS1 (Ding *et al.*, 2002). Interestingly, the sequence of HaDNV demonstrated that the NTPase domain is highly conserved, both in location and a.a. sequence, among classical or lepidopteran densoviruses.

On the complementary strand of HaDNV, a large ORF extends from the right-hand 5' end, downstream from the ITR, to the middle of the viral genome. This ORF contains the phospholipase A2 motif, similar to other densoviruses as well as vertebrate parvoviruses (Zádori *et al.*, 2001; Li *et al.*, 2001; Fédière *et al.*, 2002). The PLA2s form a superfamily of key enzymes involved in physiological and pathological processes such as lipid mediator release, cell proliferation and migration, signal transduction pathways, inflammation, acute hypersensitivity and degenerative diseases (Dennis, 1997; Kramer and Sharp, 1997; Balsinde *et al.*, 1999). In parvoviruses, the PLA2 is involved in the successful infectious cycle during the transfer of the viral nucleic acid to the nucleus. Its presence in both arthropod and vertebrate parvoviruses implies a common biological property as well as a strong evolutionary relationship among this group of viruses.

Genomic termini of parvoviruses contain GC-rich palindromic sequences that fold back on themselves to create secondary structures, called hairpins, which take different forms; T, J, I or Y shapes. These hairpins are contained in larger sequences that may exist in symmetrical (ITRs) or asymmetrical 5' and 3' telomeres (Berns, 1996; Dumas *et al.*, 1992; Mukha and Schal, 2003; Li *et al.*, 2001; Fédière *et al.*, 2002). The hairpins play a major role in the replication process of parvovirus DNA as inboard cis-acting primers

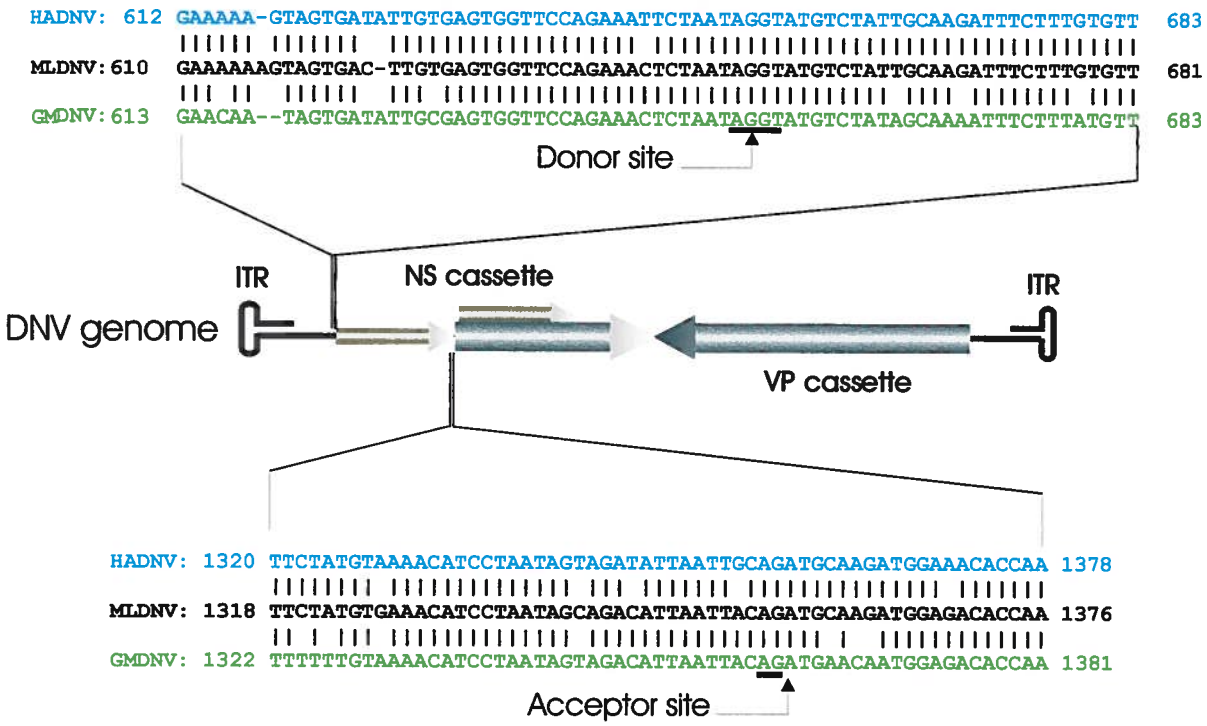
for genome extension by cellular DNA polymerase(s). They have also important signals which are necessary for the resolution of monomeric genome units during replication as well as liberation of the cloned genome during experimental transfections (Tattersall and Ward, 1976; Lusby, *et al.*, 1980; Bando *et al.*, 1990; Jourdan *et al.*, 1990; Berns, 1996).

The HaDNV genome sequence showed that it has long ITRs of 545 nts from which the distal 126 nts formed a typical Y-shaped hairpin, identical to that of MlDNV. However, the rest of the ITR contained a small number of insertions and nucleotide substitutions compared to MlDNV as well to GmDNV ITRs. The Y-shaped form of hairpins are thus conserved among classical densoviruses. The two arms of the Y-shaped hairpins can exist in either of two forms, flip or its reverse complement flop, a dimorphism which is the outcome of the replication process through what is called the hairpin transfer (Bando *et al.*, 1990; Bergoin and Tijssen, 2000). In the current work, we presented the sequence obtained from HaDNV using the primer-walking method on one of the two complementary strands of only one clone containing the complete viral ITR. So, the flip/flop dimorphism of the viral hairpins is still to be confirmed for HaDNV.

As in the case of MlDNV, HaDNV ITRs terminate in the middle of the TATA box of the putative promoter of both NS and VP gene cassettes. This indicates that all the upstream promoter elements are identical for NS and VP genes and hence, potential regulation of transcription of NS versus VP genes is controlled downstream of the TATA-box by other factors. Transcription mapping of HaDNV is still to be determined. However, similar sites for splicing, transcription start sites as well as polyadenylation sites to that of MlDNV and GmDNV could be found (Fig. 16). On the NS strand, the AG dinucleotide, which is required for exact positioning of the spliceosome prior to the splicing process, is present at the 3' end of the putative donor site at nt 653-654, followed by a GT dinucleotide at 655-656, which implies the presence of a 5' of a splicing site at

nt 654. A second AG can be found at nt 1357-1358, directly proceeding the 5' end of NS1/NS2 coding sequence. This AG can allocate a 3' splicing site at nt 1359. Splicing between nucleotides 654 and 1359 would then remove the coding sequence of NS3 to yield a smaller mRNA that serves for the production of NS1 and NS2 proteins. The NS1 potential ATG is separated by only 4 nts, CAAG, from NS2 ATG which fits exactly to MlDNV and GmDNV transcription mapping strategy. Thus, HaDNV sequence homology, genomic organization and putative transcription signal similarities to other classical densoviruses highly suggest its classification as a new member of the *Densovirus* genus. Yet, the virus expression strategy, host tropism and its potential to be used in biological control programs, are still aspects that need to be addressed.

(A)



(B)

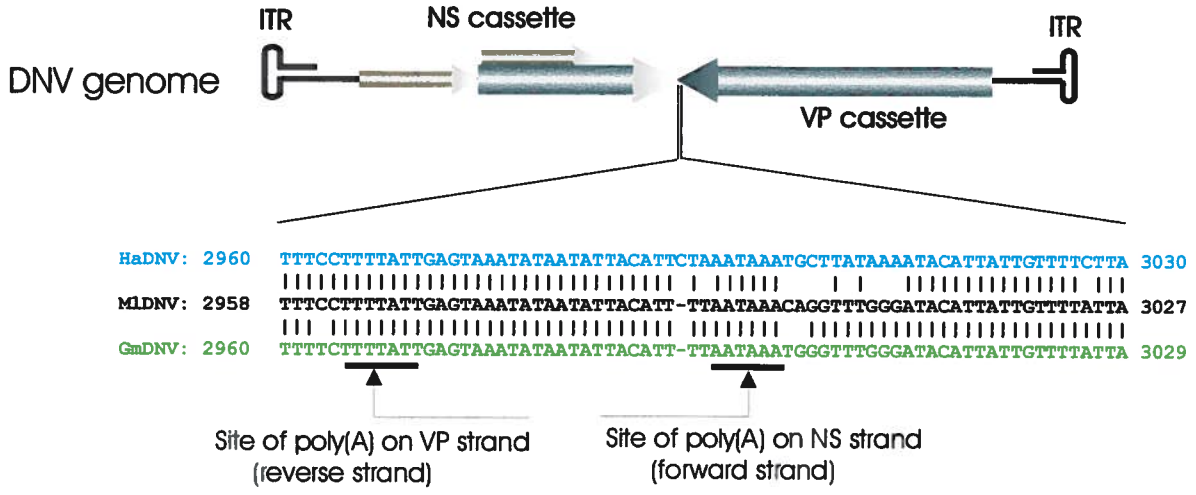


Fig. 16. Splicing and poly(A) sites on DNV genome. Comparison between splicing sites within the NS region of HaDNV, MlDNV and GmDNV (A) and poly(A) sites for NS and VP genes (B).

CHAPTER VI : RESULTS

DENSOVIRUS TROPISM

Introduction

The tropism determinants for several vertebrate parvoviruses have been attributed to a few amino acids residing on the viral capsid. Experimental interchange of these amino acids between closely related viruses, that differ in their host preference, results in switching in their tropism (Bergeron *et al.*, 1996; Mckenna *et al.*, 1999; Hueffer and Parrish, 2003). In densoviruses, the sequence homology between the members of the same group is lower and large differences exist within their host range. The current study aimed to localize determinants that control the tropism of two closely-related densoviruses, GmDNV and MlDNV. GmDNV is a monospecific virus restricted to its original host of isolation *G. mellonella*, despite the fact that it is polytropic, infecting almost all the insect tissues (Amargier *et al.*, 1965; Kurstak and Vago, 1967; Garzon and Kurstak, 1968). However, the polytropic MlDNV extends its host range to a broad spectrum of hosts, infecting, in addition to its host *M. loreyi*, several lepidopteran insects: *G. mellonella*, *Chilo agamemnon*, *Ostrinia nubilalis*, *Sesamia cretica*, *Pectiniphora gossypiella* and *Spodoptera littoralis* (Fédière, 1996).

The entire genome of both viruses has been cloned into plasmid vectors and the complete sequence was determined (data presented in chapter III and IV). The two viruses share about 90% sequence identity, where the 10% differences are equally distributed over the coding and non-coding regions. The availability of these two clones permitted us to swap restriction fragments that may carry the potential allotropic differences between the two virus genomes.

In vitro tropism of GmDNV and MlDNV

Tropism in the context of the work discussed here is defined as the differential permissivity of the cell lines to the different densoviruses. Using the infectious clones, pGm1 for GmDNV and pMl28 for MlDNV, we first investigated the *in vitro* tropism of the two viruses by scanning different insect cell lines. The data presented in Table 2 show that MlDNV could readily infect four cell types: Sf9, LD652, T-ni and Sl52. However, GmDNV replicated only in LD652 cells to a comparable titer to that of MlDNV, yielding between 12-15% infected cells, after transfection, as determined by immunofluorescence. Despite the permissiveness of the three other cell lines to MlDNV, the virus titer was very low, e.g. about 2% of infected cells as estimated by the FACS analysis, on Sl52 (Fig. 17). The infectivity of the progeny virus, produced after transfection of the different cell lines, was confirmed both *in vitro*, by one extra passage on the same cell line, as well as *in vivo*, by infecting the 4th instar larvae of *G. mellonella*.

Several attempts were made to sub-clone any of the low-titer virus-producing cell lines in order to obtain a cell population that supports a higher level of virus production. As a first attempt, we used the limiting dilution method to separate individual cells in the well units of the 96 well plates. However, single cells could not survive the dilution method and no further replication occurred. We then proceeded towards a logarithmic dilution from a starter concentration of 10^5 cells per ml. Only the T-ni cell line responded well to this method, except that the minimum cell number in a single well at the beginning of the experiment was limited to around 50 cells. This way, we could separate sub-populations of T-ni cells of which one was named T-ni03, yielding a high titer of MlDNV after infection which could reach about 20% of infected cells (estimated by immunofluorescence), but that was still refractory to GmDNV infection (Fig. 18). The combination of the two cell lines, LD652 and T-ni03, provided us with good *in vitro*

	MIDNV		GmDNV	
	Transfection	Infection*	Transfection	Infection*
Sl52	+ +	+	-	-
T-ni	+ +	+	-	-
Sf9	+ +	+	-	-
LD652	+++	++	+++	++

Table 2: *In vitro* tropism of MIDNV and GmDNV. Viral infection was measured by the number of positive fluorescent cells. * Infection was carried on a new passage of cells using the virus which was produced after transfection. The same virus was injected *in vivo* to the 4th instar larvae of *Galleria mellonella*.
+ signals signify the level of infection as measured by fluorescent cells:
+++: about 15%.
++ : about 5%.
+ : significantly less than 1%.

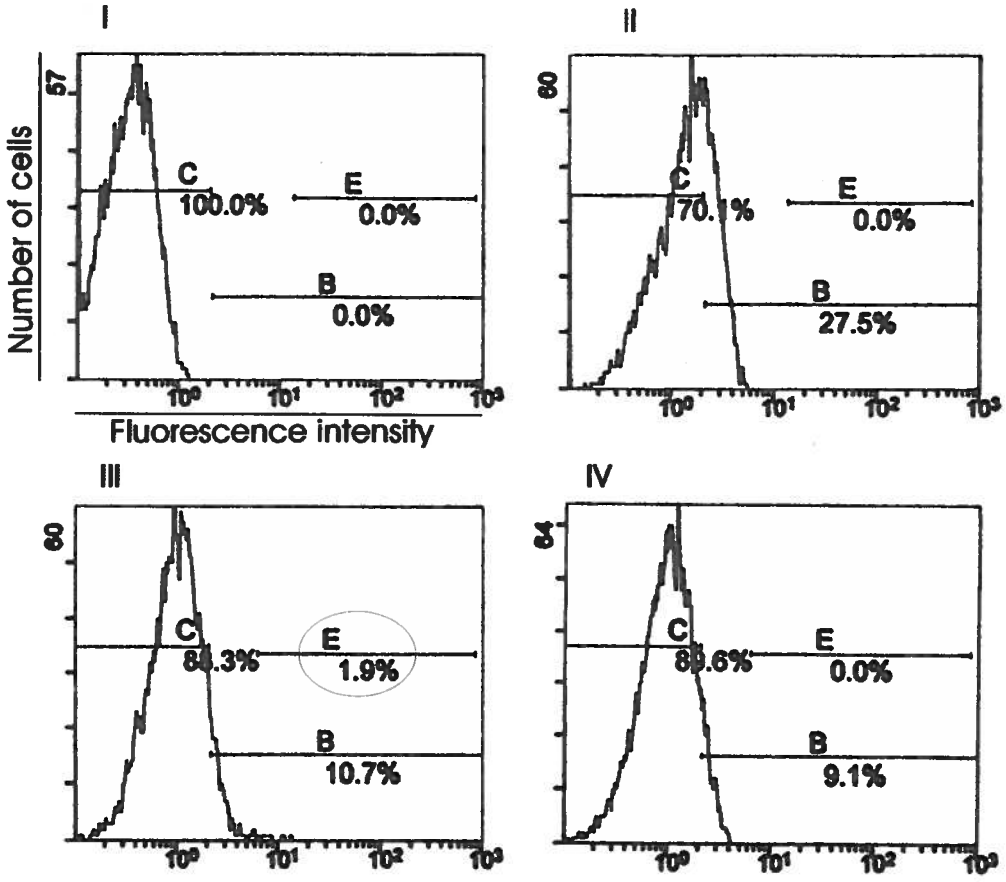


Fig. 17. Quantification of the infection level on SI52 cells by the FACS analysis method. C: natural fluorescence, B: specific and nonspecific binding, E: specific binding alone.

I : Control cells fixed with 3% formaldehyde

II : Control cells exposed to first and secondary antibodies

III : Cells transfected with MIDNV, the circle indicates the level of infection

IV : Cells transfected with GmDNV

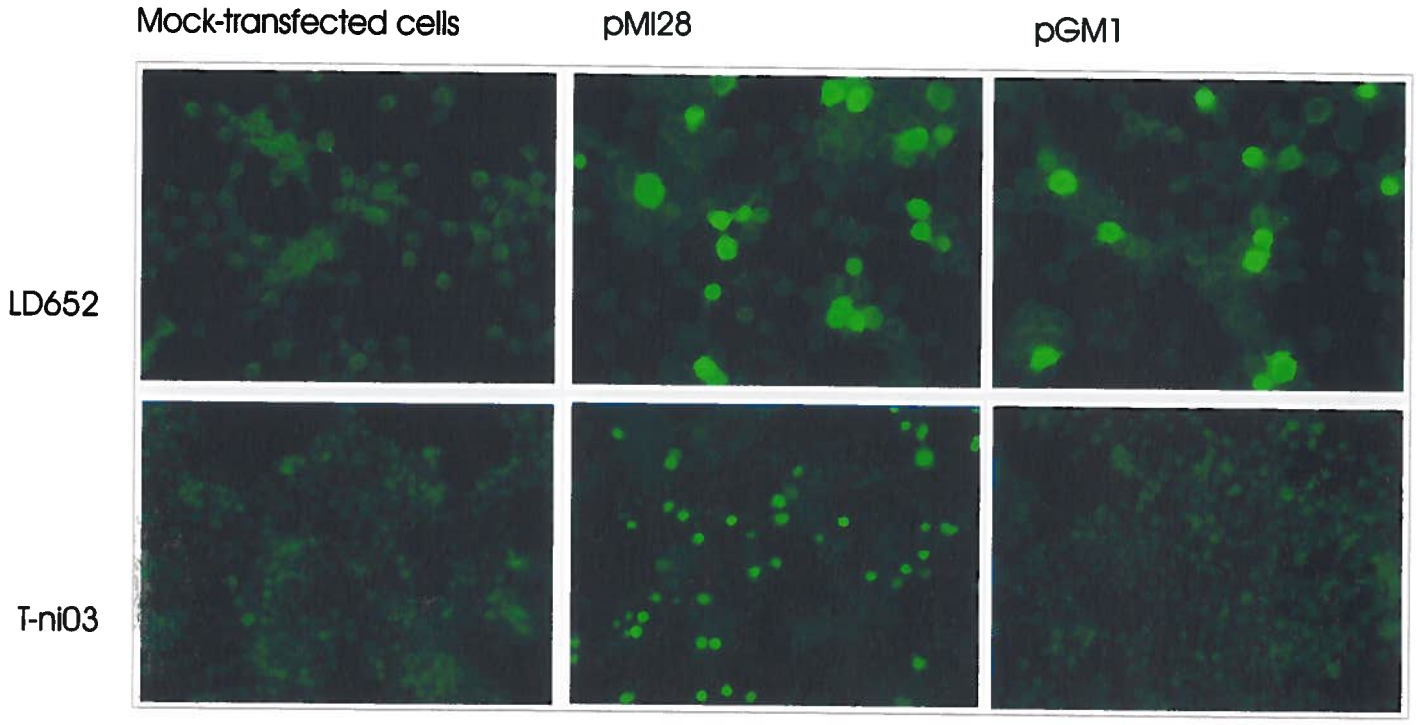


Fig. 18. Phenotypes of MIDNV and GmDNV on LD652 and T-ni03 cells. MIDNV is readily detectable by immunofluorescence on both cell types, however, GmDNV could be detected only on LD652 cells. Note that insect cells have a high natural fluorescence background. The number of LD652 transfected cells is superior to that of T-ni03. T-ni03 cells are smaller than LD652 in size.

marker systems that allowed for the differentiation between the phenotype of MlDNV and GmDNV infections.

Construction of the chimeras

The blockage in GmDNV infection in T-ni or its sub-clone T-ni03, Sf9 and Sl52 cells could not be overcome by transfection using the double-stranded infectious clone DNA. This strongly suggested that the blockage in the infection is controlled by intracellular events following the virus entry and conversion of the ssDNA into dsDNA, but prior to the virus transcription and replication. In order to localize the virus sequences that contain the allotropic determinants, we created several chimeras between GmDNV and MlDNV. The interchange of the coding sequences between MlDNV and GmDNV was done by homologous swapping of different restriction fragments between their cloned genomes. The transfer locations depended on conserved restriction sites and were chosen in a way that would maintain the integrity of the ORFs. This led to the creation of 10 chimeric clones as shown in Fig. 19 (sequence numbering is related to MlDNV genome).

pMl-GmBB and pGm-MlBB chimeras. The first couple of clones consisted of the homologous interchange of the total coding sequence, 5.5 Kb, contained within the two *Bam*HI sites, between the distal 280 nts of both ITRs. As shown in chapter I and II, the hairpins of GmDNV and MlDNV, which are the putative origins of replication, are contained within the distal 136 and 126 nts, respectively. To do this transfer, we first modified the vector sequence of pEMBL19^{+}, in which the MlDNV is cloned. The original vector contains a *Bam*HI site, 10 nts from the right-hand end of the cloned genome, hence *Bam*HI digestion would abolish the hairpin at this site. As shown in Fig. 20, pMl28 DNA was first digested with *Xba*I that has a recognition site 4 nts from the

viral end, treated by Klenow enzyme to create a blunt-ended DNA and finally super-digested by *Nco*I that has a unique site within the viral sequence at position 4105. A second sample of pMl28 DNA was double digested by *Sma*I, cutting the vector 17 nts to the viral end, and *Nco*I. Within the last digested vector, the *Sma*I-*Nco*I (fragment contains the *Bam*HI site of the vector) was then replaced by the *Xba*I-*Nco*I fragment, recovered from the former digestion. The resulting modified clone, therefore facilitated the transfer of GmDNV *BamH*I large fragment into MlDNV background, thus creating the pMl-GmBB recombinant clone (BB = *Bam*HI-*Bam*HI fragment). Whereas, the opposite transfer gave rise to the pGm-MlBB clone.

In vitro assays of the two recombinant *BamH*I clones permitted us to exclude a possible role for the terminal hairpins in controlling the viral tropism. As shown in Fig.21, the MlDNV coding sequence inserted between the *Bam*HI sites of GmDNV hairpins did not alter its tropism to T-ni03 cells. At the same time, the opposite clone, did not extend the tropism of GmDNV to these cells.

pGm-MlNS chimera. This chimera was constructed by use of the *Bam*HI-*Bsm*I small fragment of MlDNV that covers the whole NS coding sequence except for the distal 93 nts from the C-terminal. The opposite transfer was not successful, as the GmDNV has an additional recognition site for *Bsm*I, despite several assays by partial digestion. The phenotype of the new clone, pGm-MlNS, was then tested on the cell system and showed no significant change in GmDNV tropism to T-ni03 cells. However, very low numbers of fluorescent cells could be observed (Fig. 22). This result suggests that tropism determinants are located to the VP region.

pMl-GmVPc chimera. The stop codons of the two large ORFs, NS1 and VP cassettes, of both MlDNV and GmDNV are separated by only 29 nts on the

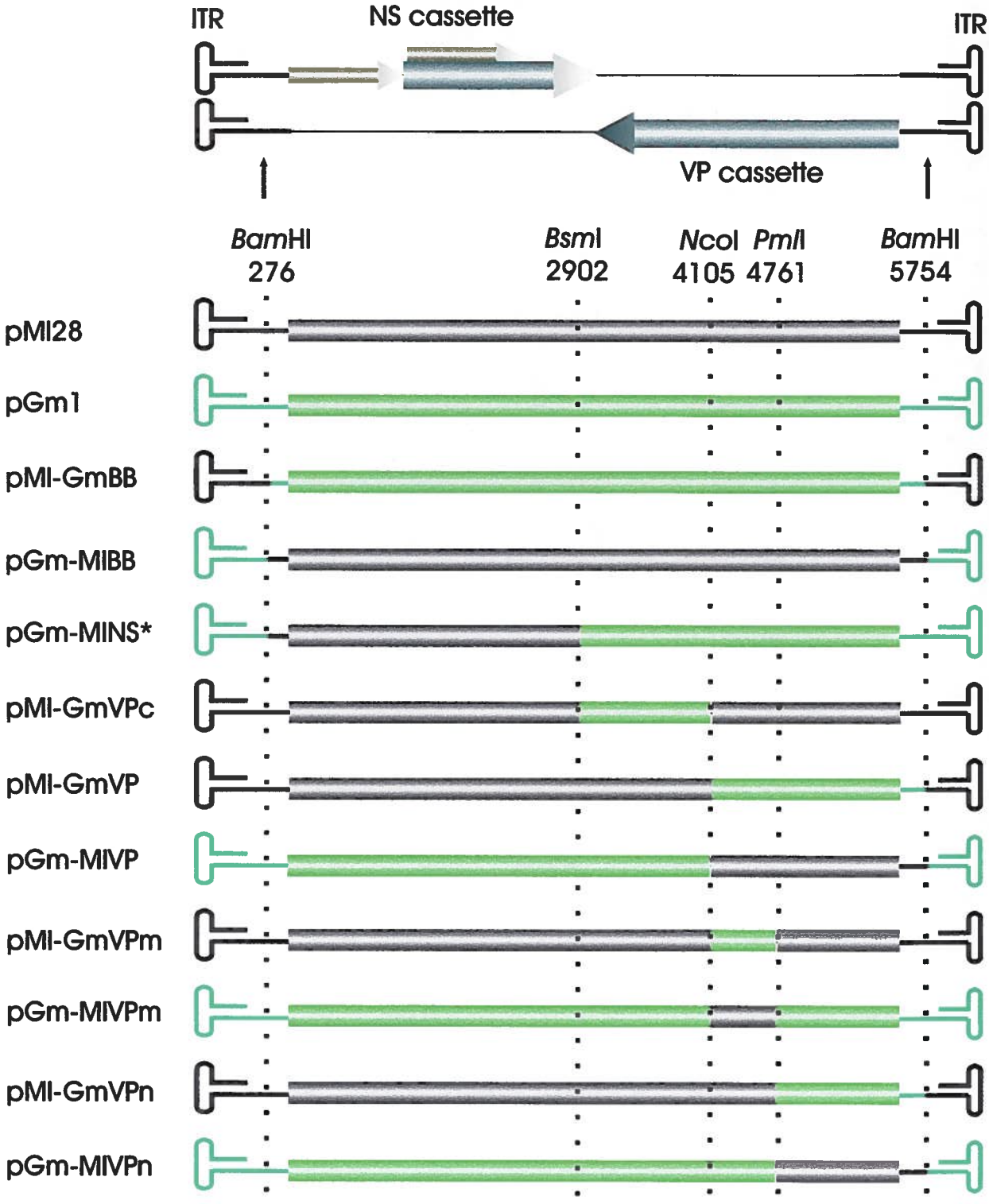


Fig. 19. Schematic representation for the different chimeras. These chimeras were created by homologous interchange of restriction fragments between MIDNV and GmDNV infectious clones, pMI28 and pGm1, respectively. * Indicates a unidirectional transfer, a GmDNV-based clone carrying the MIDNV NS within the entire left-hand ITR.

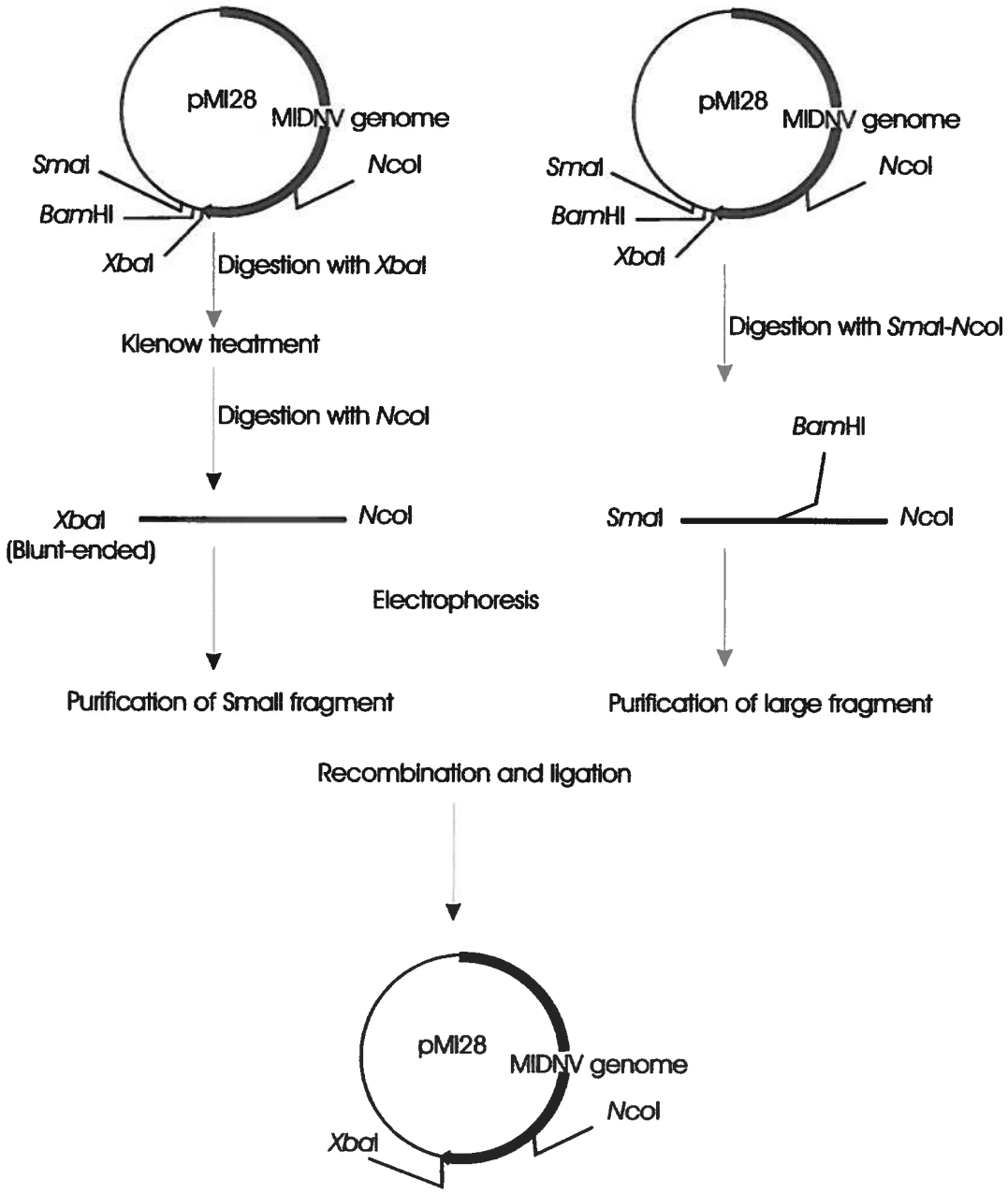


Fig. 20. Modifying the sequence of the cloning vector. A series of digestions and ligation to remove the vector *Bam*HI site.

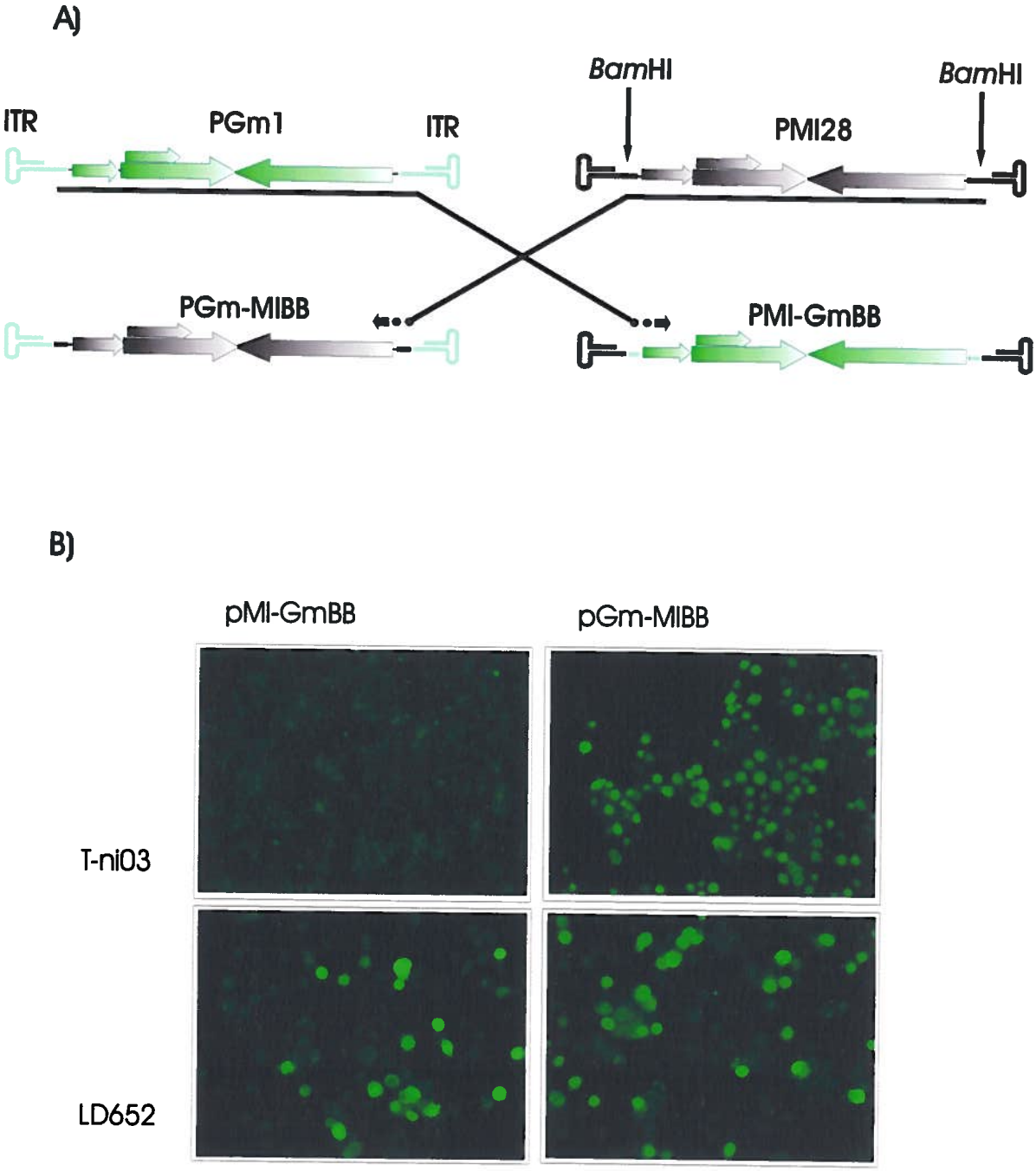


Fig. 21. Phenotypic effect of the *Bam*HI-*Bam*HI fragment interchange. A) Schematic representation for the fragment interchange. B) LD652 and T-ni03 cells transfected with pMI-GmBB and pGm-MIBB.

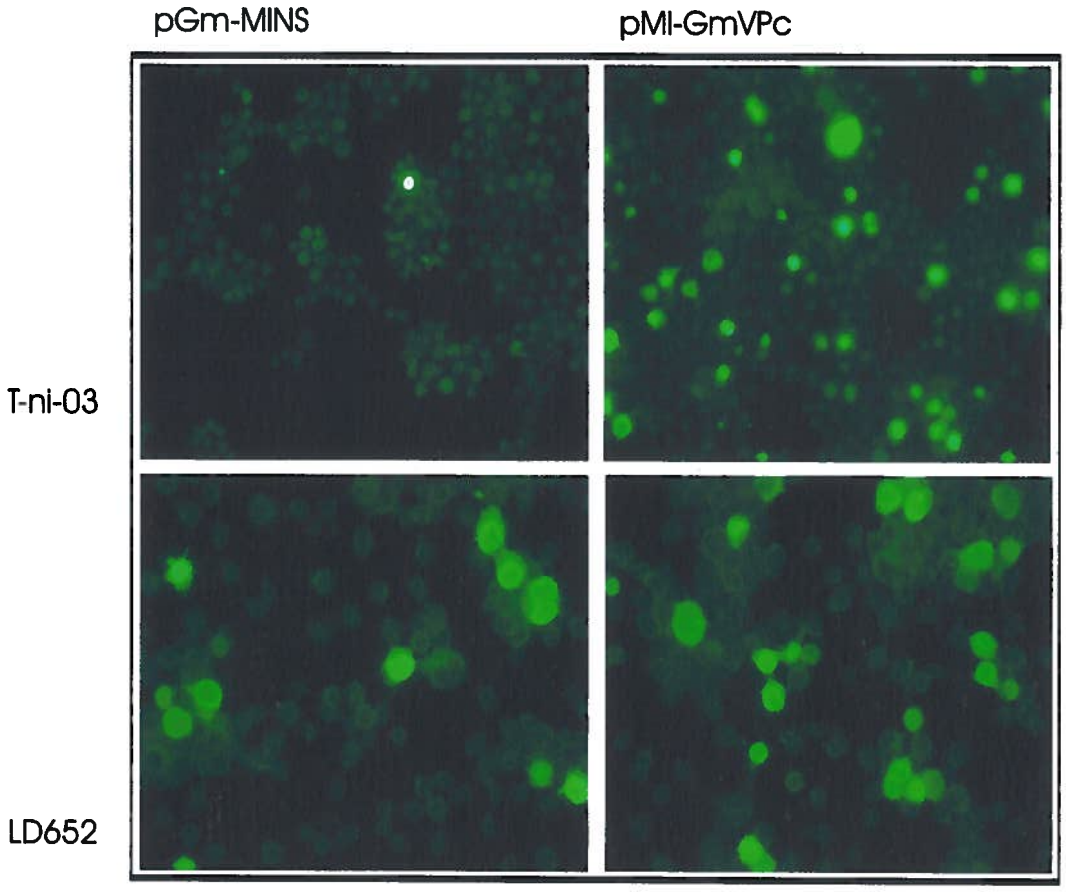


Fig. 22. Phenotypic effect of pGm-MINS and pMI-GmVPc. Transferring MIDNV NS into GmDNV background, and GmDNV VP C-terminal into MIDNV background. The two transfers did not change the virus tropism. Although rarely a fluorescent T-ni03 cell was seen after pGm-MINS transfection.

complementary strands. Transcription mapping showed that the VP and NS transcripts overlapped by 57 nts in the case of MlDNV and 59 nts for GmDNV. This overlapping region was transferred from GmDNV into MlDNV by the restriction fragment *Bam*I-*Nco*I. The transferred fragment thus also contained the C-terminal of the NS (the distal 93 nts), the C-terminal of VP cassette (the last 1080 nts) as well as the overlapping transcription termination region. This clone, pMl-GmVPc was still infectious for both LD652 and T-ni03 as clearly shown in Fig. 22. Therefore, tropism determinants are likely to be controlled by sequences located within the proximal half of the VP region (VP coding sequence as well as VP promoter sequence).

pMl-GmVP and pGm-MlVP chimeras. The rest of the VP coding sequence from the *Nco*I site up to the right-hand *Bam*HI site, was interchanged between the two viruses by the *Nco*I-*Bam*HI small fragments of 1.6 kb. This fragment thus carried the four potential ATGs of the viral VPs as well as putative promoter elements localized within the right-hand ITR. First, two sub-clones of MlDNV and GmDNV, created by *Eco*RI-*Kpn*I large fragments and cloned into the pBluescript vector, were created in order to use the *Bam*HI site without disrupting the left hand ITR (NS site). After the interchange of *Nco*I-*Bam*HI between the subclones, the recombinant fragments were cloned back into the two infectious clones by *Nco*I-*Kpn*I. This created two new clones, pMl-GmVP and pGm-MlVP. As indicated in Fig. 23, the VP recombinant clones were both expressed in LD652 cells. However, the pMl-GmVP tropism into T-ni03 was dramatically decreased, whereas the pGm-MlVP extended its tropism into these cells. Thus, the proximal half of MlDNV VP region clearly contains sequences, which are responsible to extend the GmDNV tropism to T-ni03 cells. Interestingly, pGm-MlVP expression in T-ni03 cells was very localized and very distinct from the wild type MlDNV expression (this phenomenon is clearer by confocal microscopy, fig. 28).

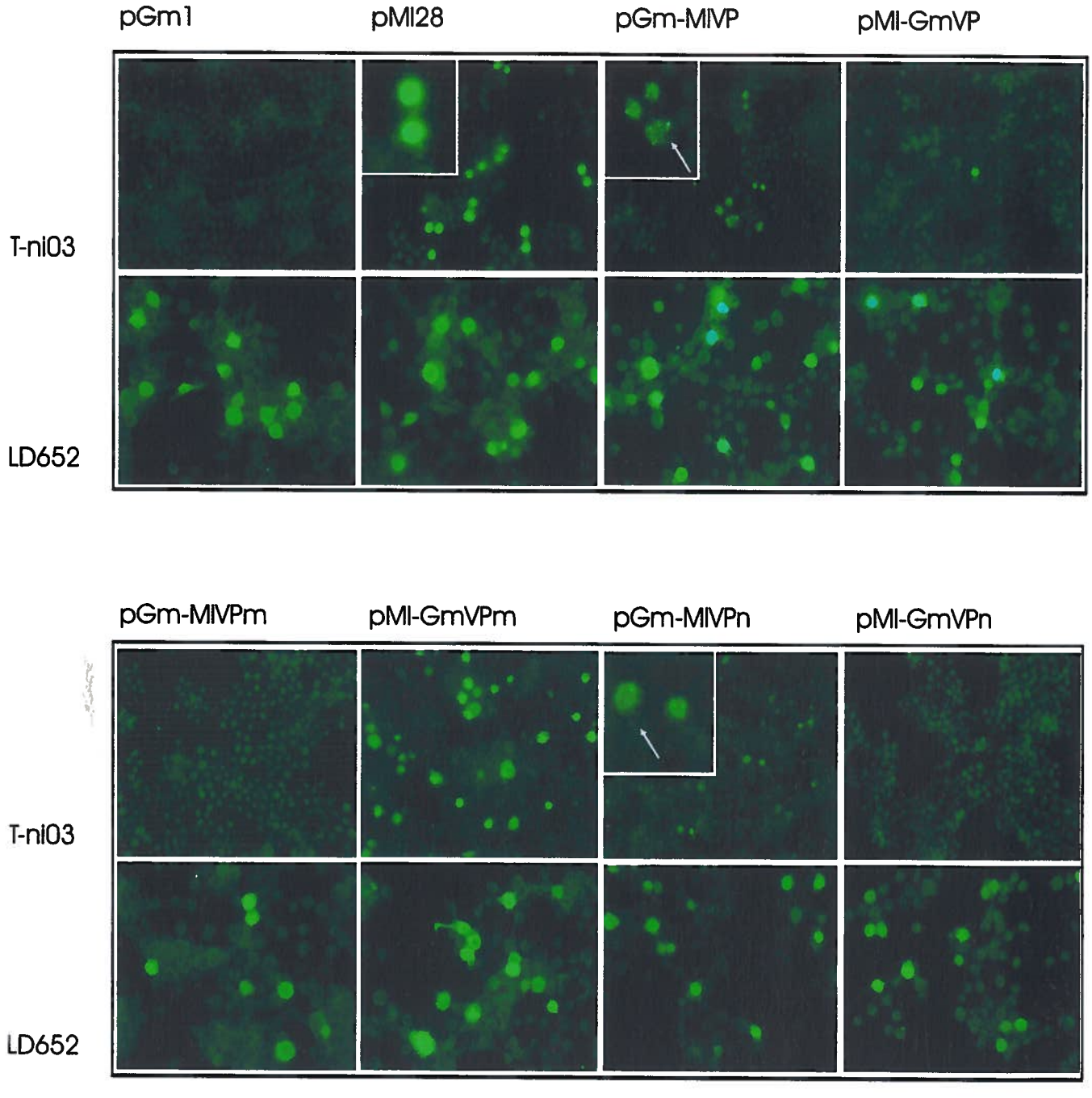


Fig. 23. Phenotypic effect of the homologous transfer within the VP region of GmDNV and MIDNV. The VP and VPn transfers caused the virus to change its tropism to the parent of the transferred region. In both cases, the recombinant virus expression was much lower than the regular MIDNV and its expression was very localised within the cell (indicated by the white arrow).

pMl-GmVPm, pGm-MlVPm, pMl-GmVPn and pGm-MlVPn chimeras. In order to define the minimal sequence of the VP cassette that conferred the tropism, the sequence contained within the *Nco*I-*Bam*HI fragment was divided into two distinct interchangeable regions: (I) *Nco*I at 4105 to *Pml*I at 4761 giving rise to two clones pMl-GmVPm and pGm-MlVPm and (II) *Pml*I to *Bam*HI at 5754, two new clones, pMl-GmVPn and pGm-MlVPn (Fig. 19). No change in the tropism was found by the former two clones, pMl-GmVPm and pGm-MlVPm. However, pGm-MlVPn, carrying the N-terminal part of MlDNV VP gene as well as upstream promoter elements in the GmDNV background, was clearly expressed in T-ni03 cells. At the same time, the tropism of MlDNV recombinant clone, pMl-GmVPn, to T-ni03 cells decreased drastically (Fig.23).

The role of the viral promoters in controlling the virus tropism

Studying the transcriptional activity of MlDNV and GmDNV in T-ni03 cells showed that the former virus was actively transcribed. Both the NS and VP transcripts were readily detected by RT-PCR, carried out on the total RNA isolated from transfected cells. Yet, in the case of GmDNV-transfected T-ni03 cells, neither the NS nor the VP transcripts could be detected (Fig. 24). This result, in addition to the data obtained from the phenotypic effect of the different chimeras, strongly suggested a blockage in GmDNV transcription in T-ni03 cells. In order to establish this hypothesis, the VP promoter elements of MlDNV were transferred into GmDNV background (Fig. 25). This was carried out by recombinant PCR method using two sets of primers (Fig. 26). The first set consisted of a forward primer DV547 (MlDNV-based sequence) which is complementary to the region containing the VP transcription start site in both MlDNV and GmDNV. This region is identical in both viruses with only one nucleotide mismatch of a G in MlDNV at +4 (counting from transcription start site) but a C in GmDNV. The second primer is DV580 complementary to a conserved region, 15 nts

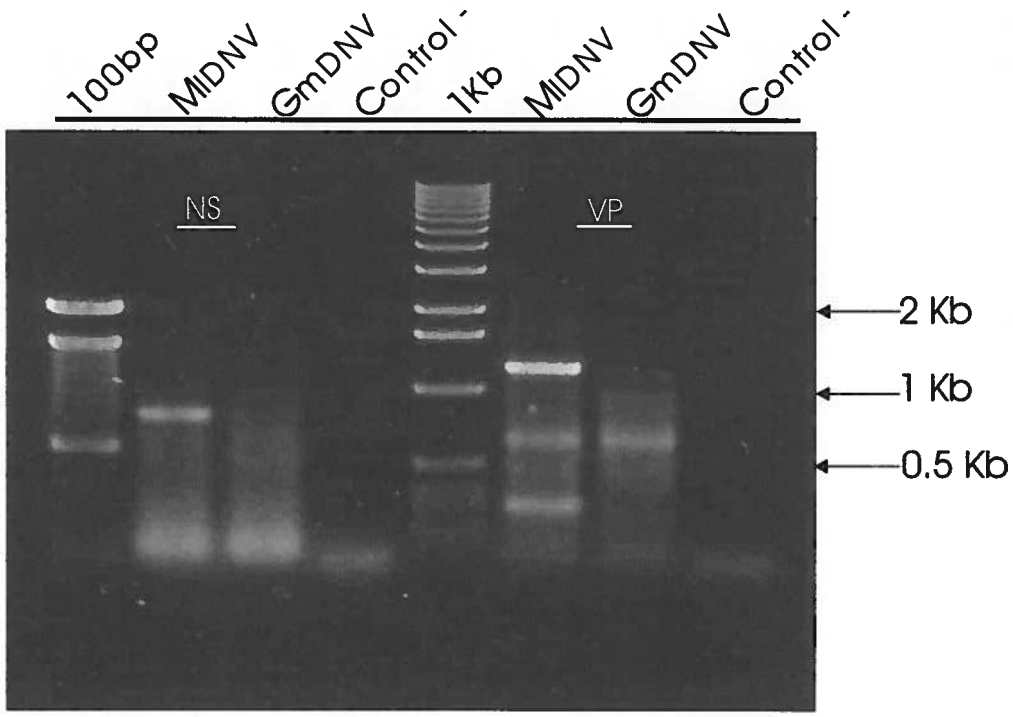


Fig. 24. RT PCR on total RNA isolated from T-ni03 cells transfected with MIDNV or GmDNV infectious clones. NS and VP transcripts are both detected in the case of MIDNV but not in GmDNV transfections. NS transcript, 0.7 Kb, was amplified using primers, DV225A+poly(dT). VP transcript, 1.2 Kb, was amplified by DV420+poly(dT).

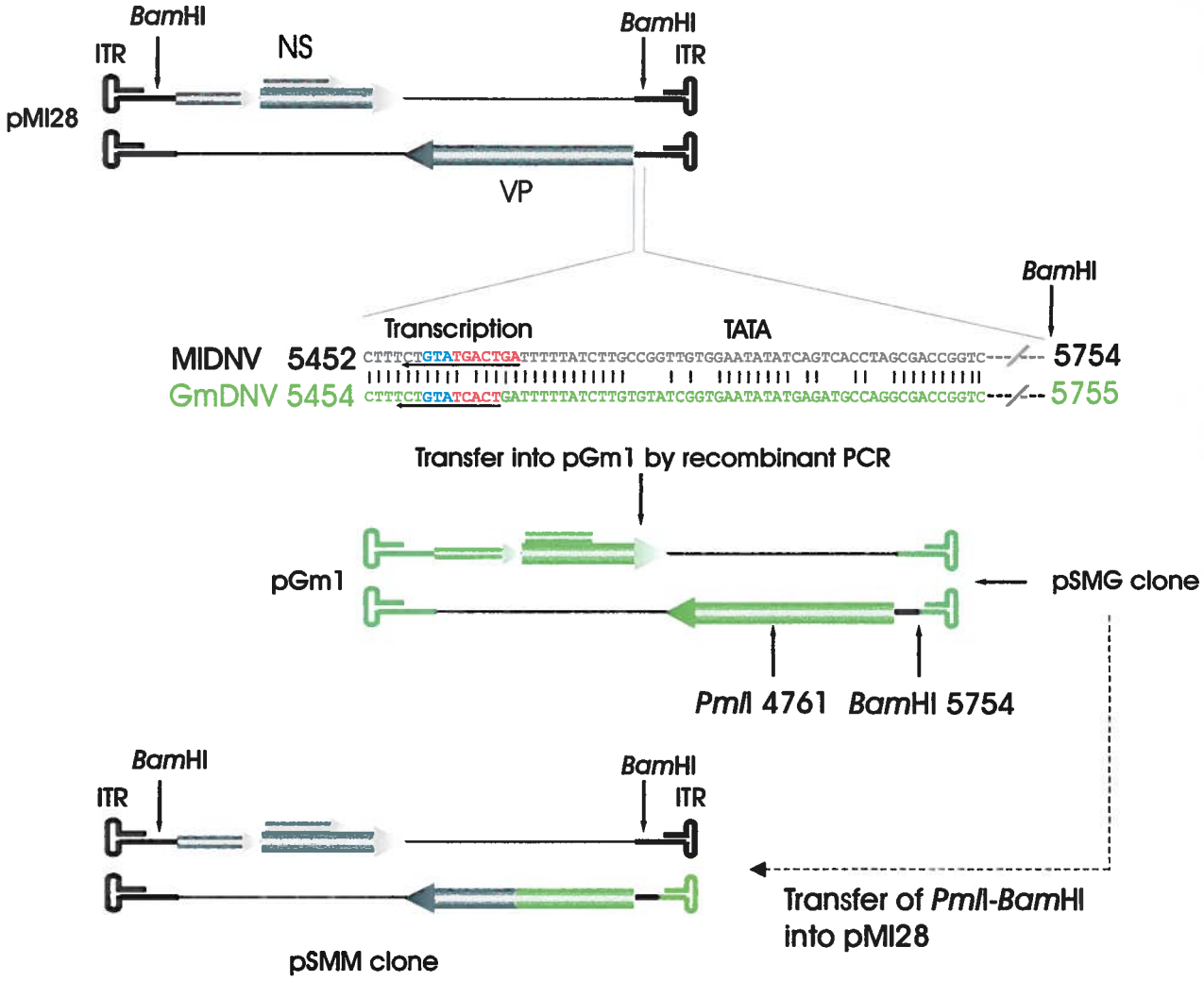


Fig. 25. Transfer of MIDNV VP promoter into GmDNV by Recombinant PCR method. Schematic representation for the sites of transfer. pSMG clone is used to create pSMM, a clone carrying a hybrid of GmDNV VP N-terminal and MIDNV VP C-terminal under the control of MIDNV VP promoter.
pSMG= MIDNV VP promoter inserted into GmDNV background.
pSMM= GmDNV VP N-terminal inserted into MIDNV background.

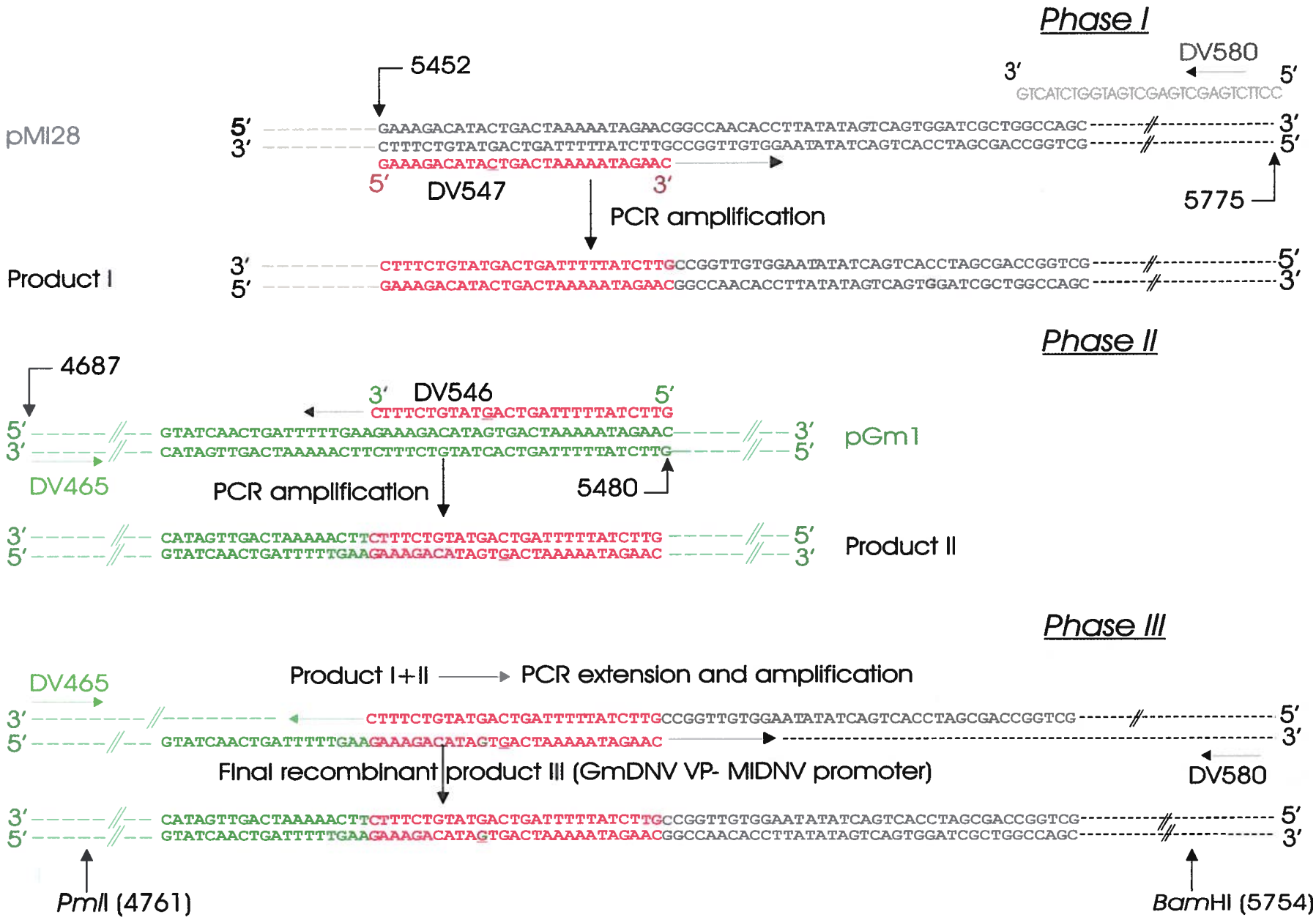


Fig. 26. Schematic representation for the three phases of the recombinant PCR. Primers are represented in their actual sequences.

downstream from the *BamH*I site in the ITR. A PCR reaction on MlDNV using the two primers gave a fragment of about 0.3 Kb carrying the upstream promoter elements of the VP cassette. The second set consisted of DV546 (exact complement to DV547) and DV465 (5' GTTCCAATTCCAATAGCTCC 3', a conserved sequence in MlDNV and GmDNV) that is complementary to the sequence upstream from the *Pml*I site. Using GmDNV as a template, these two primers amplified a fragment of 0.8 Kb that contained the GmDNV VP N-terminus. The two products (0.3 and 0.8) were then hybridized by PCR resulting in 1.1 Kb recombinant fragment carrying the GmDNV VP N-terminal and MlDNV upstream promoter elements (Fig. 27). This recombinant DNA was then transferred into the GmDNV background via a *BamH*I-*Pml*I restriction fragment, thus creating the pSMG clone. Indeed, this transfer was sufficient to extend the tropism of GmDNV into T-ni03 as indicated in fig. 28. However, the expression of pSMG VP proteins was localized and different from that of the wild type MlDNV in this cell line.

Our previous results on the pMl-GmVPn clone that carries GmDNV VP N-terminus under the control of its native promoter, showed that this recombination was sufficient to abolish MlDNV tropism in T-ni03 cells. Here we used the new clone pSMG to transfer, once again, the GmDNV VP N-terminus but this time under the control of the MlDNV promoter sequence to a MlDNV background. This transfer created the pSMM clone. Indeed, the phenotype of this clone in T-ni03 cells was identical to that of the wild-type MlDNV as shown by confocal microscopy (Fig. 28). This result confirms the major role of the VP promoter in controlling the *in vitro* tropism. The difference between the phenotypes of pSMG and pSMM thus suggests also a role for the autologous NS protein, which is readily expressed in pSMM. A possible role for the C-terminal region of MlDNV VP in the wild-type expression can not be excluded. However, a virus that carries GmDNV VP C-terminal on MlDNV background (pMl-Gm VPc chimera) has a phenotype similar to that of the wild-type.

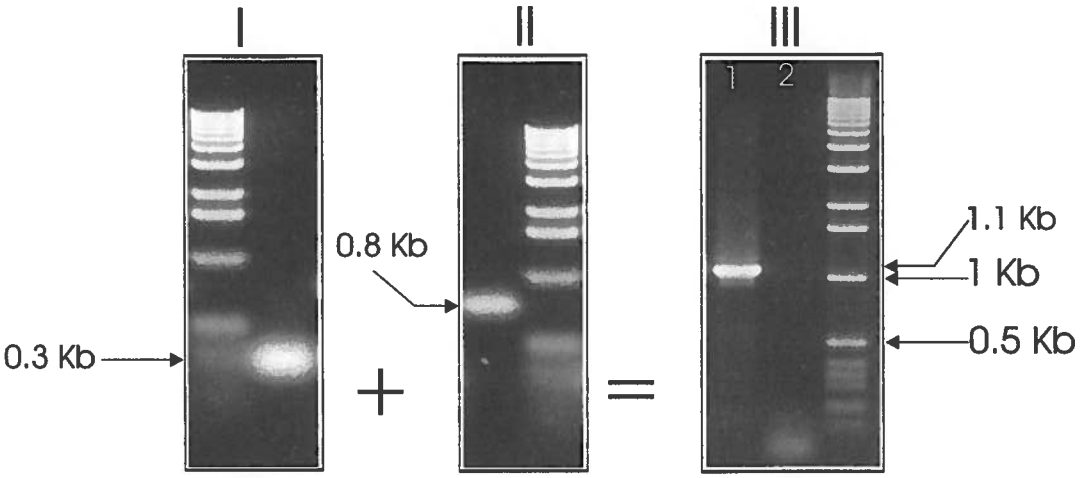


Fig. 27. Recombinant PCR products from phases I, II and III. The fragment sizes are 0.3, 0.8 and 1.1 Kb for I, II and III, respectively. The transfer was carried out by digesting the product III by *Pml*I-*Bam*HI. Ladder 1 Kb is used in the three panels. Lane 2 in panel III represents the PCR negative control.

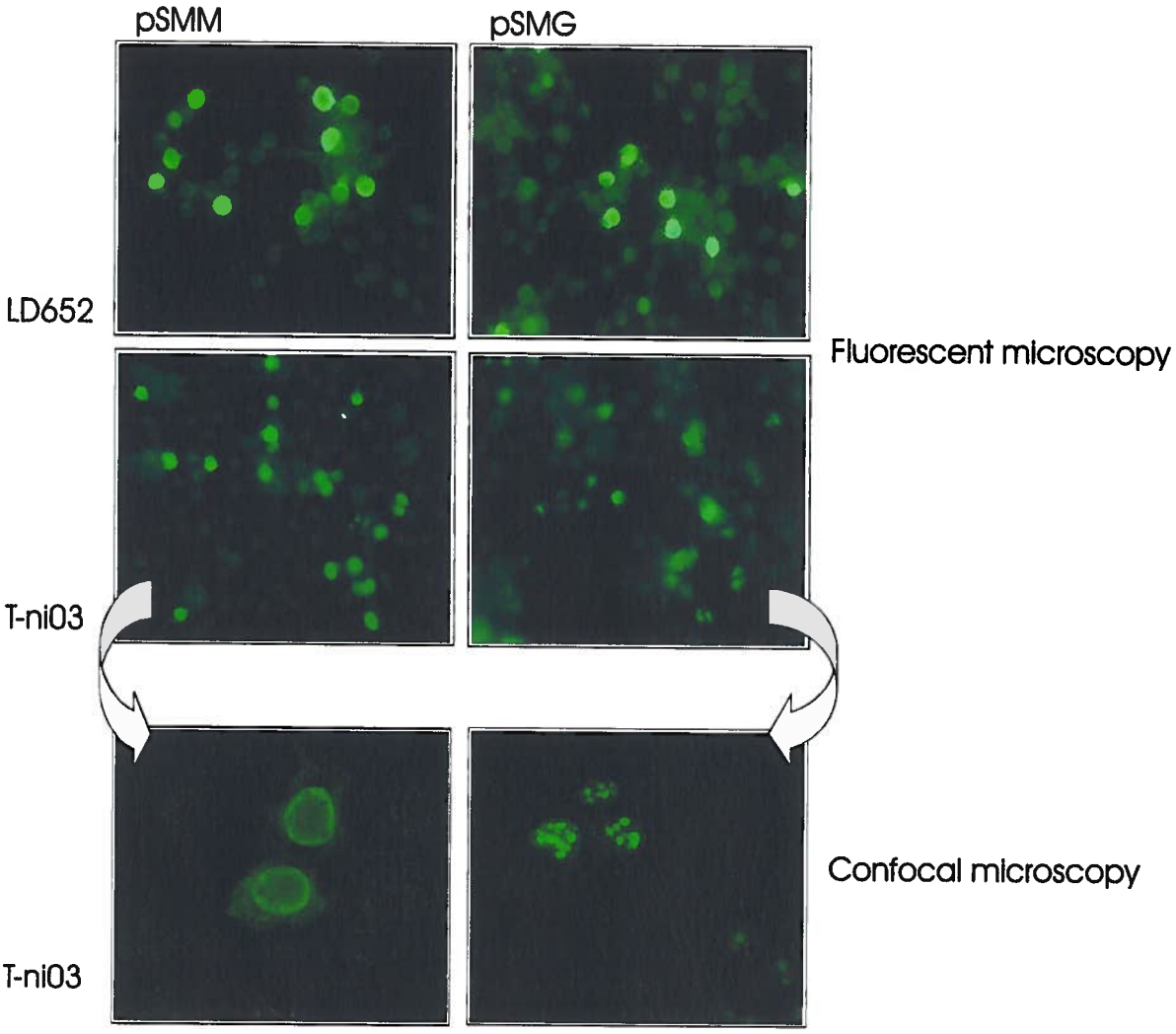


Fig. 28. Phenotypes of pSMG and pSMM on LD652 and T-ni03 cells. In T-ni03, pSMG has a limited and localized expression compared to the distributed and fully expressed pSMM, as shown by fluorescent and confocal microscopy.

MlDNV and GmDNV VP promoter activity in LD652 and T-ni03 cells

The previous results suggested that the GmDNV VP promoter is not active in T-ni03 cells. In order to support this hypothesis, we cloned the VP promoter sequence of GmDNV as well as MlDNV upstream of the chloramphenicol acetyl transferase (*CAT*) gene. This was carried out by PCR recovery of the VP promoter region using primers, MlCATF with the sequence 5'- TTT***AAGCTT***ACTGACTAAAAATAGAACGGCCA (sequence in the bold italic is the added recognition site for *Hind*III REN) and DVCATR with the sequence 5'- TTT***TCTAGA***GAGGATCCGAAGACCTTGGAGCTG (*Xba*I recognition site in bold italic) for MlDNV promoter and GmCATF, 5' TTT***AAGCTT***AGTGACTAAAAATAGAACACATA and DVCATR for GmDNV VP promoter. The PCR fragments were digested and cloned into the pKS-CAT vector by the two RENs, *Hin*dIII and *Xba*I (Fig. 29). The cloned promoter sequences included the short non-translated leader sequence of the VP transcript (AGTCAGT in MlDNV and TCACT in GmDNV) up to the *Bam*HI site in the middle of the ITR at nt 5754 and 5755, MlDNV and GmDNV, respectively. The two new expression vectors, pMlVP-CAT and pGmVP-CAT were then introduced to LD652 and T-ni03 cells by transfection. Incubation time of the *CAT* constructs with the transfected cells was three days in order to mimic the optimal time observed for densovirus infection in these cells (the time at which a maximum number of infected cells could be obtained). Activity of either of the two promoters in the two cell lines was then analyzed by the level of the acetylated ^{14}C-chloramphenicol which depends on the *CAT* level in the cellular extract. As expected, the MlDNV VP promoter drove the expression of the *CAT* gene in both cell types, even though in a two fold lower extent in T-ni03 than in LD652. In contrast, GmDNV VP promoter was as active as that of MlDNV in LD652 but was 13 times less active in T-ni03 compared to MlDNV (Fig. 29B, left panel). In a second set of experiments, we introduced the pGmVP-CAT construct to the two cell types combined with a co-

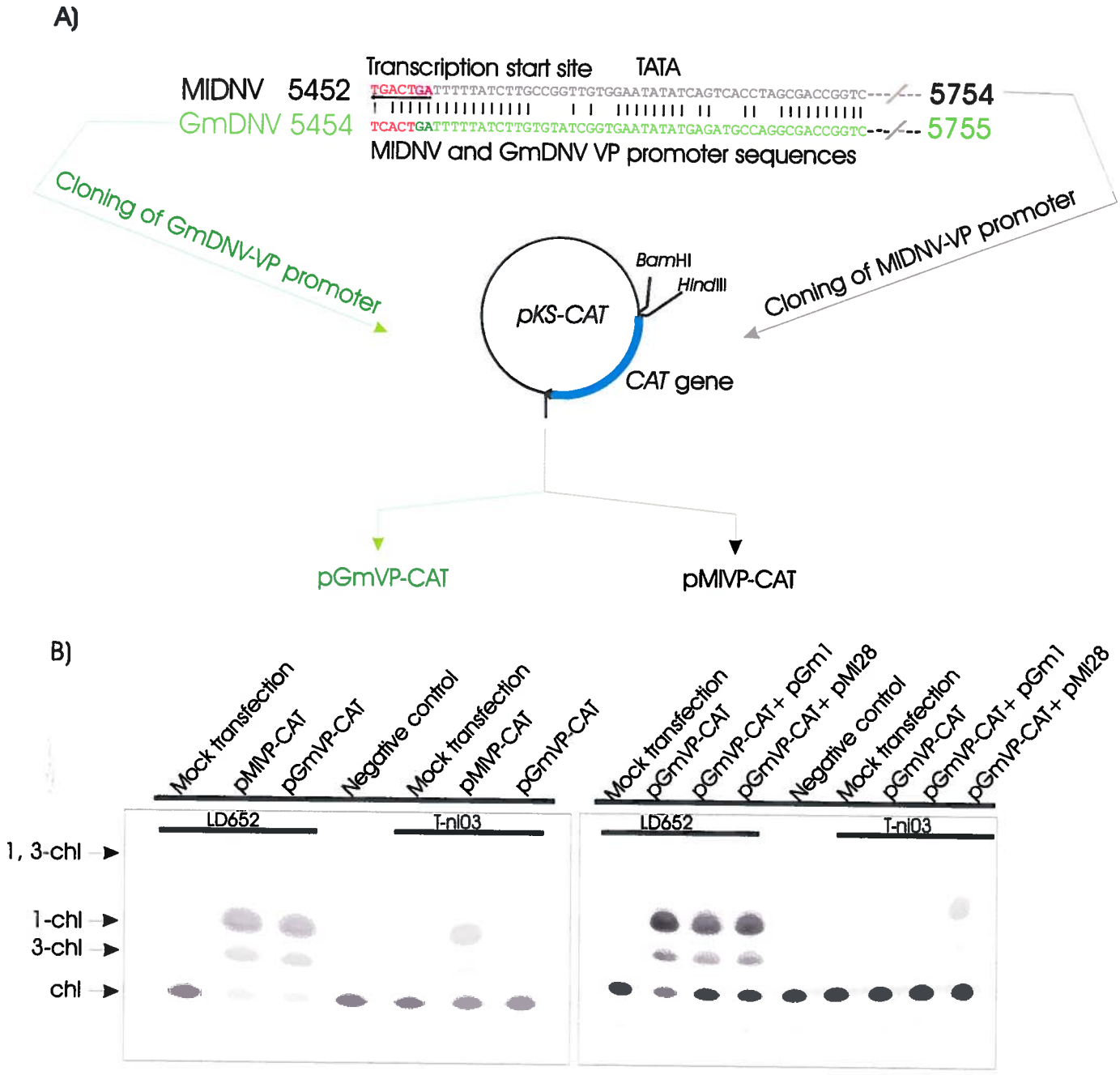


Fig. 29. The expression capacity of MIDNV and GmDNV VP promoters in LD652 and T-ni03 cells as measured by the *CAT* reporter gene. A) Schematic representation for the construction of pMIVP-CAT and pGmVP-CAT. B) In the left panel, pMIVP-CAT is actively expressed in the two cell types, whereas pGmVP-CAT could only be expressed in LD652 with comparable level to pMIVP-CAT. In the right panel, activating the expression of pGmVP-CAT in the two cell lines by the co-transfection with pMI28 and pGm1. pMI28 moderately activates pGmVP-CAT in T-ni03 cell. The positions of the unreacted chloramphenicol (chl), chloramphenicol acetylated on 1 position (1-chl), 3 position (3-chl) or the 1,3 position (1,3-chl) are shown.

transfection by pMl28 or pGm1 that express MlDNV NS and GmDNV NS proteins, respectively. These co-transfections showed that MlDNV had a moderate trans-activation effect on the GmDNV VP promoter in T-ni03 cells where it increased the *CAT* level to 6 fold, most probably due to the expression of NS protein (Fig. 29B, right panel). However, in LD652 cells the expression level of *CAT* gene driven by GmDNV VP promoter was not changed by either of the co-transfections.

Cellular factors and a possible role in controlling densovirus tropism

The difference in expression activity of GmDNV and MlDNV promoters in T-ni03 cells suggests an interaction between these sequences and cellular transcription factors. In order to study this possible interaction, we targeted three sets of prominent sequence differences surrounding the TATA boxes in GmDNV and MlDNV as indicated in figs. 30 and 31. Synthetic oligomers mimicking these three regions were synthesized (Table 3). Radioactive labelling of the mimic oligomers and preparation of cellular extracts from LD652 and T-ni03 are detailed in the Materials and Methods. The supershift assay using these oligomers, surprisingly, showed that the chosen GmDNV promoter sequences behaved similarly in permissive and non-permissive cell types (Fig. 30 and 31). The specificity of the interaction between the promoter regions and the cellular extracts were confirmed by competing the radiolabelled oligomers by cold or non-labelled ones from either GmDNV or MlDNV origins. However, the level of cellular proteins recruited by regions P1 and P2 differed in the two cell types. GmDNV P1 and P2 recruited more protein from LD652 than from T-ni03 cells. Nevertheless, P3 had a very similar profile with MlDNV and GmDNV sequences.

	5' 3'
GmP1F	AATAGGATGTGACTGGCCAGCGGACCGTAGAGTAT
GmP1R	ATACTCTACGGTCCGCTGGCCAGTCACATCCTATT
GmP2F	ATAAGTGGCTATGTGTTCTATTTTTAGTCACTATG
GmP2R	CATAGTGACTAAAAATAGAACACATAGCCACTTAT
GmP3F	CTCGCGTCGAAGCTAAACCGTGTGCAAAACAGGAT
GmP3R	ATCCTGTTTTGCACACGGTTTAGCTTCGACGCGAG
MIP3F	CTCGCGTCGAAGCTGGGCCGTGTGCAAAACAGGAT
MIP3R	ATCCTGTTTTGCACACGGCCCAGCTTCGACGCGAG

Table 3. Sequence of the oligomers mimicking the GmDNV targeted promoter regions. F and R signify forward and reverse, respectively. Labeling reactions for P1 and P2 regions required a mixture of F and R oligomers.

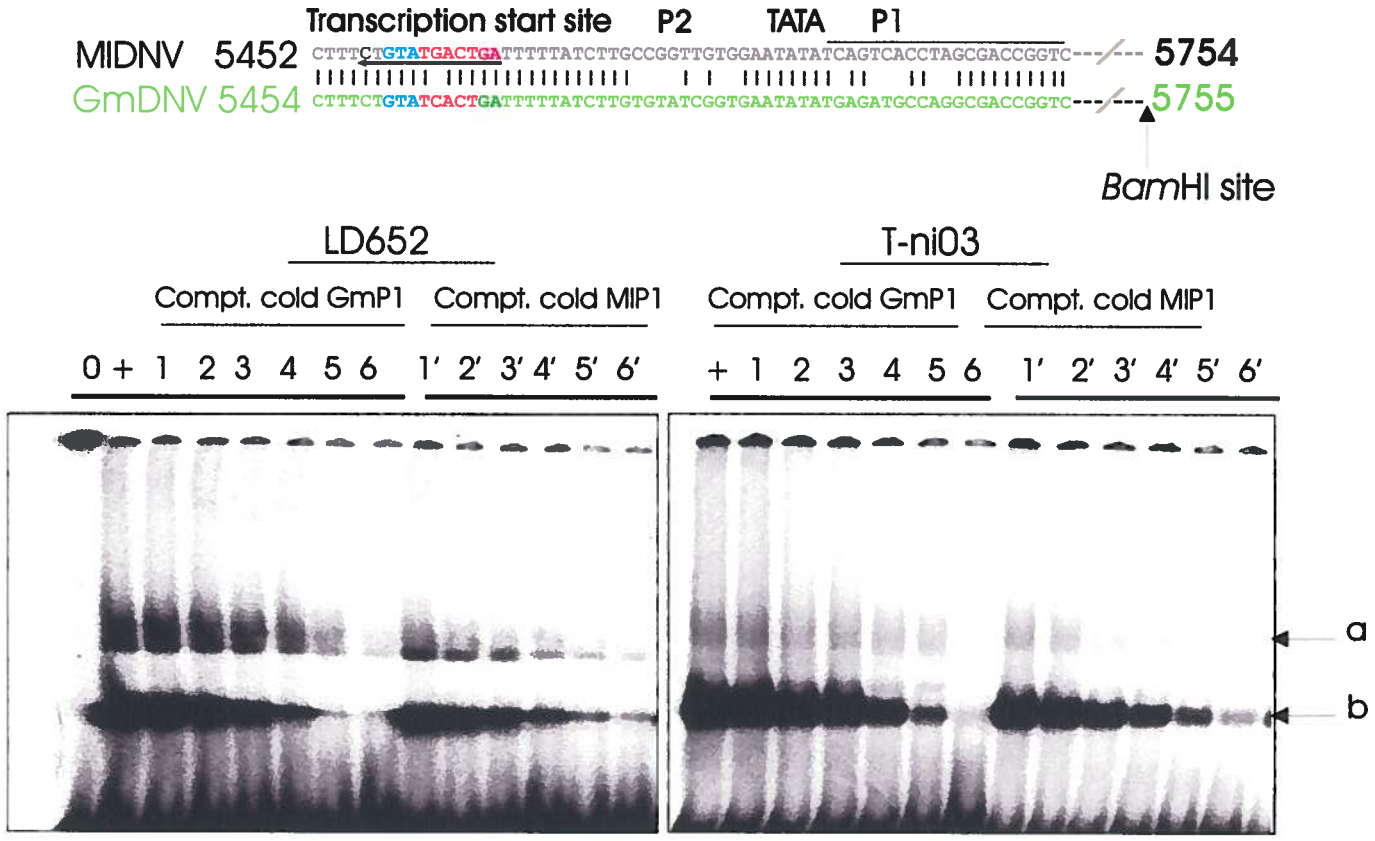


Fig. 30. EMSA after the interaction between the cellular extracts and the viral sequences. Cellular extracts from LD652 and T-ni03 cells recovered at 300 mM of elution buffer reacting to GmDNV VP promoter region P1 (indicated above). This region behaved equally in both cell types. However, some cellular proteins, in a, were present at a higher level in LD652 than in T-ni03. The level of protein b was the same in the two cell types. Specificity of the interaction was confirmed by competing the radiolabelled primers with non-radiolabelled or cold primers from GmDNV- as well as MIDNV-based sequences.

0 : No added cellular extract
+: Cellular extract with radiolabelled P1

1&1' to 6&6' are competitor concentrations, 1X, 2X, 4X, 8X and 30X, respectively, of non-labelled P1, where X refers to the used concentration of labelled P1

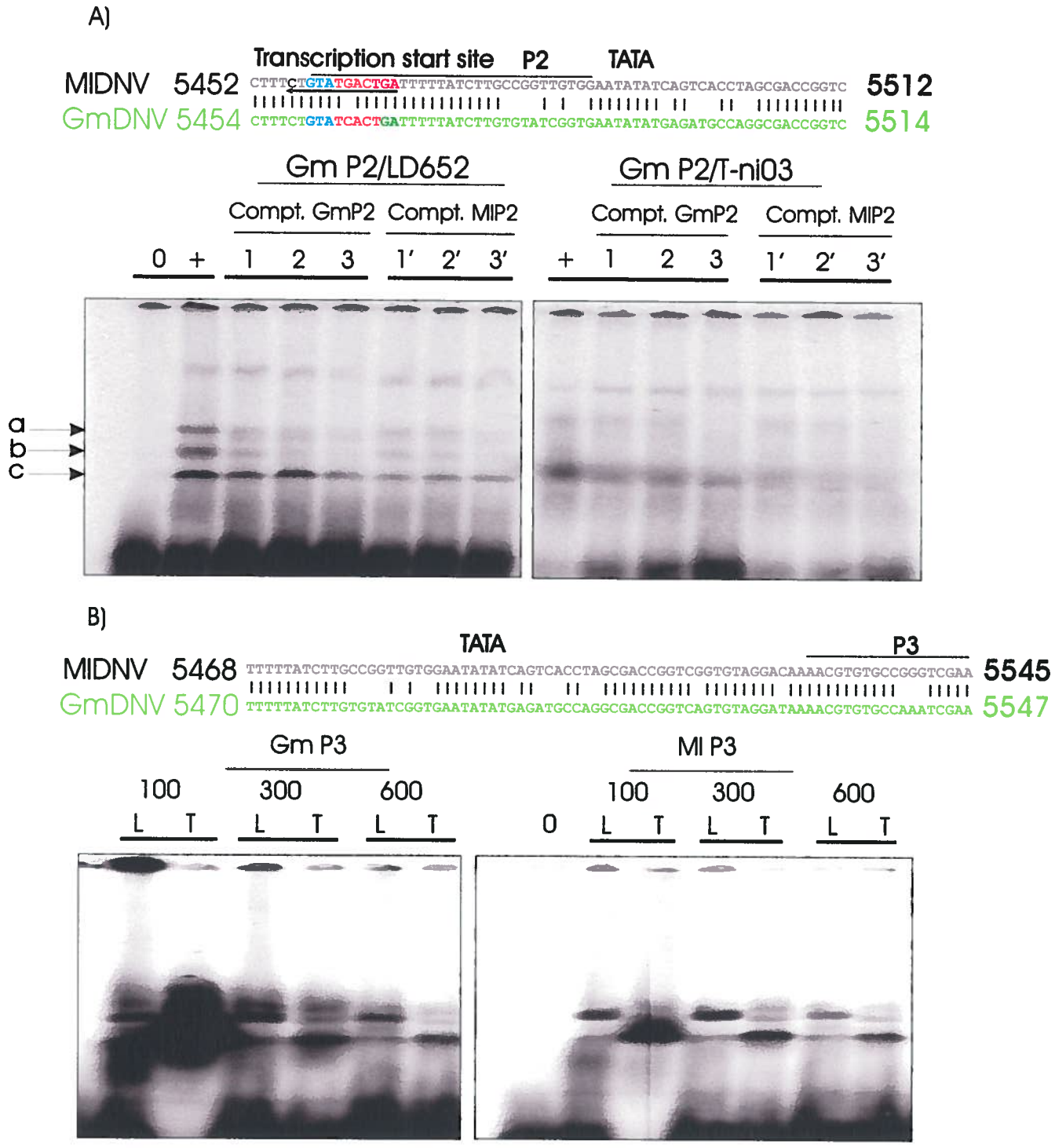


Fig. 31. EMSA for the promoter regions P2, of GmDNV, and P3, of GmDNV and MIDNV. A) Behaviour of GmDNV P2 in LD652 cells (to the left) and T-ni03 cells (to the right). Cellular extracts were recovered at 300 mM. 0: no added cellular extract, +: cellular extract, 1, 2 & 3 and 1', 2' & 3' are the EMSA profile with competition by 1X, 2X and 3X of cold P2 of GmDNV and MIDNV, respectively (where X signifies the original concentration of the radiolabelled P2. Protein bands a and b are more present in LD652 than in T-ni03 cells, whereas protein c seems non-specific since it did not disappear after competition. B) To the left, behaviour of GmDNV P3 in LD652 cells (L) and T-ni03 (T) with three different cellular fractions recovered at 100, 300 and 600 mM. To the right, MIDNV P3 behaviour at the same conditions.

DISCUSSION

The virus infectious cycle begins with attachment to cellular receptors followed by internalization and uncoating. In order to initiate infection, the virus has to undergo several intracellular interactions that lead to its expression, replication and maturation, which all require the fitness of virus genetics to cellular biological machinery. Parvoviruses represent a very useful tool to study such intracellular interactions since they have very compact genomes with a limited number of genes. Consequently, these viruses depend largely on the cell machinery and on certain functions that are expressed during the S phase of the cell cycle (Tennant *et al.*, 1969; Tattersall, 1972; Bashir *et al.*, 2000). The present work represents the first model to study the allotropic determinants that control an insect parvovirus tropism *in vitro*. Our model consisted of two closely related densoviruses, *G. mellonella* and *M. loreyi* DNVs, GmDNV and MlDNV, respectively. The data represented in chapters III and IV described the cloning of the complete genome of both viruses and showed that these clones are indeed infectious, both *in vivo* and *in vitro*. GmDNV was the first member of the invertebrate parvoviruses to be recognized in insects. The virus has a restricted host range, infecting only its original host *G. mellonella* (Amargier *et al.*, 1965; Kurstatk and Vago, 1967; Garzon and Kurstak, 1968). MlDNV was recently isolated from the maize worm *M. loreyi* in Egypt and was shown to be polytropic, like GmDNV, but has a relatively large host range (Fédière, 1996).

The objective of this work was then to locate the allotropic determinants that control the tropism of MlDNV and GmDNV. We first studied the *in vitro* tropism of the two viruses on several established insect cell lines. MlDNV could infect and successfully replicated in four cell types from which the LD652 cells were the most permissive, giving the highest virus titer. The T-ni cell line was also permissive to MlDNV but to a much

lower extent. GmDNV could replicate to a similar titer as MlDNV in LD652 cells, but not in any of the other cell types. By limiting dilutions, we isolated a sub-population of T-ni cells, T-ni03, which yielded high levels of MlDNV titers and was still refractory to GmDNV infection.

The 10% of sequence differences between the MlDNV and GmDNV are distributed throughout the whole genome, coding and non-coding and ITRs sequences. In order to determine which of these sequence differences is responsible for the tropism, we created several chimeras that cover the whole genomes. Our results on the sequence comparison between MlDNV and GmDNV (chapters III and IV) showed that the major difference between the hairpins of the two viruses existed in the asymmetrical arms, where the base pair #50/62 in MlDNV is not complementary in GmDNV, thus forming a bubble. Interestingly, it is absent in the case of the closely related JcDNV. This makes the hairpin an interesting region to investigate for its role in the virus tropism. In parvoviruses, the terminal hairpins serve for DNA replication as inboard cis-acting primers for genome extension by cellular DNA polymerase(s) using oneself as a template (Tattersall and Ward, 1976; Lusby, *et al.*, 1980; Bando *et al.*, 1990; Berns, 1996). The conversion of ssDNA to dsDNA is a prerequisite for viral transcription. The hairpins also play a major role in the rescue of the cloned genome during the experimental transfections, both *in vitro* and *in vivo* (Jourdan *et al.*, 1990). Both, the replication and rescue of the cloned genome are NS1-dependent and site specific. The NS1 binds specifically to a site of (GAC)4 located on the A-A' of the densovirus hairpin, catalysing the cleavage of single-stranded DNA within the putative origin of replication, primarily at two sites in the motif 5'-G°TAT°TG-3' (Ding *et al.* 2002). The genome of GmDNV as well as MlDNV has a *Bam*HI site in the middle of the two ITRs. Therefore, digesting the cloned genome by this REN resulted in the recovery of the whole coding sequence in addition to the internal halves of both ITRs, leaving the distal halves (280 nts) of the

ITRs attached to the cloning vector. This permitted the interchange of the complete coding sequences of either virus to the origin of replication of the other one. Our data on this homologous interchange showed no effect for the genome hairpins, origin of replication, on the viral tropism. Yet, the significance of the sequence difference contained in the hairpins of the two viruses remains to be established.

The difference in the cellular tropism between GmDNV and MlDNV could not be overcome by introducing the virus genome in a double-stranded form (infectious clone) into the cultured cells through transfection. In contrast, in vertebrate parvoviruses, i.g. MVM, the cellular barrier to the tropism of the two closely related strains MVMp and MVMi could be overcome by transfection, and viral transcripts could be readily detected (Antonietti *et al.*, 1988). This may lead to the conclusion that, abortive virus infection in the non-permissive T-ni03 cells to GmDNV is most probably due to intracellular interactions after the conversion of the viral ssDNA to dsDNA and prior to translation rather than cell receptor interactions and entry. The parvovirus NS proteins play complex roles during the virus life cycle. The NS1 is a multifunctional protein and exhibits site-specific DNA binding, ATPase, helicase and nickase activities which are all required during viral DNA replication (Cotmore and Tattersall, 1995). In vertebrate parvoviruses, the NS1 has been shown to be involved in the regulation of the P4 and transactivation of the P38 promoters which control the expression of nonstructural and capsid proteins, respectively (Cotmore and Tattersall, 1987; Doerig *et al.*, 1988; Doerig *et al.*, 1990; Vanacker *et al.*, 1996). MVM parvovirus NS1 seems to contribute to the virus tropism by recruiting certain cellular factors in a cell-type dependant manner (Rubio *et al.*, 2001). The NS2 protein is needed for efficient nuclear egress of progeny virions through interaction with nuclear Crm1, and may, therefore, participate in virus spread (Eichwald *et al.*, 2002). In densoviruses, the NS1 plays a central role in the virus DNA replication and rescue of the cloned genome from the

cloning vector (Ding *et al.*, 2002). NS3 was also shown to be critically needed for the densovirus DNA replication (Abd-Alla *et al.*, 2004). Together, these data show that blockage or altering mutations within the NS genes would then be harmful for the parvovirus replication and expression. In order to investigate the role of densovirus NS proteins in controlling the virus tropism, we transferred the NS cassette of MlDNV under its proper promoter into the GmDNV background. However, this transfer did not confer GmDNV tropism into T-ni03 cells, although, MlDNV NS genes are functional in this cell type. Only a few fluorescent cells could be observed by UV microscopy after transfection. This may be due to a limited activation of GmDNV VP promoter by the MlDNV NS protein. However, this recombinant GmDNV virus was still infectious in LD652 cells. Thus, it seems plausible that in the case of GmDNV and MlDNV tropism, the NS protein alone does not control the tropism and hence other factors are involved.

Allotropic determinants of vertebrate parvoviruses have been localized to the viral capsid proteins for several members of this group of viruses (Bergeron *et al.*, 1996; Mckenna *et al.*, 1999; Simpson *et al.*, 2002; Hueffer and Parrish, 2003). A few amino acid residues, usually on or near the three-fold spikes, control the virus tropism (Gardiner and Tattersall, 1988; Bloom *et al.*, 1993; Agbandje-McKenna *et al.*, 1998; Bloom *et al.*, 1998; Mckenna *et al.*, 1999). Our results on the recombinant MlDNV/GmDNV initial chimeras showed that the VP and/or the VP promoter are involved in controlling the densovirus tropism. A series of chimeras have been obtained within the VP coding sequence of GmDNV by transferring homologous regions from MlDNV VP gene. Indeed, none of the VP coding sequences could change the virus tropism. Moreover, when GmDNV was transfected into T-ni03 cells, no virus transcripts could be detected, which implies a blockage in the virus transcription. The transfer of MlDNV VP promoter elements, including the non-translated region of the VP transcript, the TATA and the upstream sequences up to the *BamH*I site, into GmDNV extended its tropism to these cells. As

indicated in chapter III, GmDNV upstream promoter elements as well as the TATA boxes are completely embedded within the identical viral ITRs. Therefore, if the TATA upstream elements of GmDNV VP promoter are not active in T-ni03 cells, then the symmetrical NS promoter elements are also non-functional. This may explain the absence both viral transcripts in the non-permissive cells.

The difference in capacity of MlDNV and GmDNV VP promoters to drive the virus expression in either LD652 or T-ni03 cells could be studied by the *CAT* reporter gene. The entire upstream promoter elements of MlDNV VP promoter, including the 7 nts nontranslated-leader region, were inserted upstream of the *CAT* gene. This construct, pMlVP-CAT, could successfully drive the expression of the *CAT* gene in LD652 as well as T-ni03 cells. Nevertheless, the expression level was two fold higher in LD652 than in T-ni03 cells. This difference could be a result of one of two possibilities; the first is that the two cell lines responded differently to the employed transfection methods. The second possibility involves the nature of the cultured cells populations. Insect cells in culture are usually difficult to clone, and in many cases, they are a mixture of different sub-populations. These sub-populations may consequently have different kinds or levels of the intracellular factors that are required for the optimal transcription of MlDNV VP promoter. At the same time, GmDNV VP promoter, in pGmVP-CAT construct, actively drove the expression of the *CAT* gene in LD652 cells to a comparable level to that of MlDNV, but failed to do so in T-ni03 cells. The difference between the *CAT* activities driven by GmDNV VP promoter in the two cell lines was up to 13 fold. However, cotransfecting T-ni03 cells with pGmVP-CAT and MlDNV original clone pMl28, that actively expresses the NS proteins in this cell line, increased the pGmVP-CAT activity by 6 fold. Nonstructural proteins of vertebrate parvoviruses autoregulate the NS genes and transactivate the VP gene promoter (Cotmore and Tattersall, 1987; Doerig *et al.*, 1988; Doerig *et al.*, 1990; Vanacker *et al.*, 1996). On the other hand, similar functions

have been assigned to densovirus nonstructural proteins (Tijssen and Bergoin, 1995). This may explain the few number of fluorescent T-ni03 cells observed after transfection of the recombinant clone pGm-MlNS, the GmDNV clone that carries MlDNV NS cassette. It may also explain the localized expression of the pSMG chimeric virus that carries the VP promoter of MlDNV on GmDNV background. This clone has the original GmDNV NS cassette which is not active in T-ni03 cells and when replaced by MlDNV NS cassette in the new chimeric virus pSMM, a phenotype similar to the wt could be observed. In other terms, the NS proteins are then plausibly required to some extent for the activation of the virus expression to the optimal levels. But, there are still other factors that keep the GmDNV initial expression at a very low level in the T-ni03 cells.

The limited activity of GmDNV VP promoter in T-ni03 cells, measured by the *CAT* reporter gene, in addition to the absence of the virus transcripts, strongly suggested that cellular transcription factors determined the virus tropism. So, it was interesting to look for these factors by DNA/protein interaction methods such as the EMSA technique that we employed here. The MlDNV VP promoter has 31 nts differences and one nucleotide insertion between the *Bam*HI site and the transcription start site comparing to that of GmDNV. We targeted three regions surrounding the putative TATA-box in the GmDNV VP promoter unit and which contained some prominent sequence differences with MlDNV. These regions contained 50 % of the promoter sequence differences between the two viruses. Each of the individual regions, P1, preceding the TATA box, P2, preceding the transcription start site and P3, which has a of GC box-like sequence in MlDNV but a stretch of Adenine in GmDNV upstream of the TATA, behaved equally in the two cell types. However, the amounts of cellular protein factors attached to regions P1 and P2 from T-ni03 cell extracts were much lower than that of their counterparts from LD652. The interaction specificity was confirmed by the

competition by either GmDNV or MlDNV "cold" (non-labelled) primers. If these regions are implicated in the virus tropism, then it would be plausible that the transcription factors, necessary for GmDNV expression, are present in the two cell types. But, in T-ni03 cells, GmDNV VP promoter sequences are less efficient in recruiting these transcription factors than their counterparts of MlDNV promoters and the amount recruited may be insufficient to initiate the virus transcription. The competence of GmDNV sequence itself in the two cell types was not similar, which may indicate the presence of alternative transcription factors. In vertebrate parvovirus MVM, activation of the P4 promoter is a prerequisite for a successful infection. This activation is controlled by the host cell transcription factors that vary among the different cell types. Consequently, the MVM P4 promoter plays an important role in the virus tropism (Davis *et al.*, 2003).

The EMSA experiments should be done with MlDNV VP promoter sequences P2 and P3 in order to compare the levels of the recruited proteins in the two cell types. Also, it is necessary to carry out a number of site-directed mutations on these regions, using the VP-CAT reporter constructs, and to measure the effect of these mutations on the expression level. This could be followed by the back-transfer of interesting mutants to the wt virus in order to study their effect on the phenotype and hence the virus tropism. It is also important to test the DNA/protein interaction profile of the rest of the VP promoter sequences, which were not yet included in these experiments.

The possibility that some cellular repressor factors may interact with GmDNV promoters and block its tropism in T-ni03 cells cannot be excluded either. However, if it is the case, the P1, P2 and P3 regions are unlikely to be involved, since they have similar profiles of DNA/protein interaction in T-ni03 and LD652 cells. Studying the interaction profile of the rest of the VP promoter sequences also remains to be done.

CHAPTER VII : RESULTS
ARTICLE III

LACK OF INFECTIVITY OF VERTEBRATE CELLS BY DENSOVIRUS FROM THE MAIZE WüRM *MYTHIMNA LOREY!* (MLDNV)

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

El-Far M, Li Y, Fédière G, Abol-Ela S, Tijssen P. Lack of infection of vertebrate cells by the densovirus from the maize worm Mythimna loreyi (MlDNV).
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PREFACE

The innocuity of the densovirus has been debated for a long rime since earlier reports claimed that these viroses could infect and transform mammalian cells in culture. A major part of my study was to verify the validity of these reports, we choose the MlDNV as a relatively polyspecific virus but we have also used the GmDNV. The availability of the infectious clones of both viroses provided us with more powerful molecular tools to study both mammalian as well as insect cell systems. The experiments were carried out by myself under the direction of Dr.Tijssen and the help of Dr. Li. Dr. Gilles Fédière and Dr. Said Aboi-Ela from the Faculty of Agriculture, Cairo University, have provided us with the original virus MlDNV preparations, as well by sorne other materials.

General Discussion and Perspectives

Densoviruses are known by their high virulence to their insect hosts (Meynadier *et al.*, 1964; Vago *et al.*, 1964; Shimuzu, 1975). These viruses show a high resistance to harsh environmental conditions such as high temperatures and as suspensions in a wide range of pH. They also maintain their infectivity for prolonged periods in non-purified forms (Boemare *et al.*, 1970; Suto, 1979; Siegl *et al.*, 1985). Another advantage of densoviruses concerns their isolation form a wide variety of insects which are economically and medically important pests. These characteristics make the densoviruses important candidates for biological control.

In the current work we characterized two new members of the classical or Lepidopteran densoviruses, MlDNV and HaDNV and compared their sequence to that of GmDNV. These viruses were isolated from agriculturally important pests. The study was concentrated on the biology of densoviruses, their expression strategies, the mechanism that control their tropism and their ability to infect mammalian cells.

Our results on the sequence analysis of GmDNV, MlDNV and HaDNV showed that they possess the longest genome size among all parvoviruses. HaDNV, like GmDNV, has a genome of 6039 nts and is only 4 nts longer than that of MlDNV. An identity of around 90 % was found among the sequences of the three viruses. This is the highest level of identity among all densoviruses. The three viruses shared also the same ambisense genomic organization. They have ITRs of about 550 nts, the longest in all parvoviruses, and that contained the typical terminal hairpins that are made up of 120-140 nts. The hairpin dimorphism of flip/flop could be seen in MlDNV and GmDNV, but not in HaDNV since we obtained and sequenced only one clone that carries the hairpin. The hairpin size, of 126 nts, as well as the sequence were highly conserved between MlDNV and HaDNV. However, they slightly differed with the hairpin of GmDNV which has a one mismatch in the asymmetrical arms. Whether or not this difference has

a role to play is not yet clear.

The sequence analysis showed that the TATA box of GmDNV VP and NS promoters was completely embedded within the viral ITRs. However, in MlDNV as well as HaDNV the ITRs terminated in the middle of TATA sequence. In both cases, all of the upstream promoter elements are then identical for the two viral promoters which opens the way to question the manner by which these viruses regulate their expression. A small difference between the VP and NS promoters of the densovirus prototype JcDNV was previously shown (Dumas *et al.* 1992). However, it seems that it is of little consequence.

The expression strategies employed by both MlDNV and GmDNV for their protein production were very similar. The two viruses use splicing and leaky scanning mechanisms to produce three NS proteins. NS3 is translated from a non-spliced/full length mRNA of 2.5 Kb, whereas, the NS1 and NS2 are translated from a spliced mRNA of 1.7 Kb through leaky scanning mechanism. The ATG of NS2 succeeded that of NS1 by a spacer of only 4 nts. Interestingly, influenza B virus uses a leaky scanning mechanism to produce two proteins separated also by exactly 4 nts (Shaw *et al.*, 1983; Williams and Lamb, 1989). The use of alternative spliced and no-spliced forms to produce the virus structural proteins is also employed for vertebrate parvoviruses.

For the VP expression, only one population of mRNA with a molecular mass of 2.6 Kb with no splicing could be found. The two viruses then employed a leaky scanning mechanism for the expression of the different VPs as confirmed by the baculovirus expression system. In MlDNV, as in the case of GmDNV, cloning of the putative ATGs of the four capsid proteins led to the production of wild-type-similar proteins. These proteins auto-assembled into virus-like particles and were indistinguishable in size and

antigenicity to the wt virus proteins. The leaky scanning mechanism was previously proposed for densoviruses of the *Iteravirus* genus (Li *et al.*, 2001; Fédière *et al.*, 2002). However, in non-classical ambisense densoviruses, such as PfDNV, the expression strategy is different. After transcription, the two ORFs of PfDNV VP are connected by splicing and reorganized by frame-shifting in order to produce the largest VP protein (Yamagishi *et al.*, 1999). PfDNV has also different termination sites for the VP transcripts, by alternative polyadenylation. It is important to mention that leaky scanning is not known in vertebrate parvovirus expression and thus, densoviruses may be distinct in this respect. It is not yet clear whether or not densoviruses do regulate the transcription of their VP and NS genes. Recent studies showed a constant expression level of the VP and NS proteins during the densovirus infection (Abd-Alla, 2003). In the case of MlDNV, we observed an increase in the spliced form of the NS mRNA, that serves to produce the NS1 and NS2, to the unspliced form, coding for NS3. It is not known whether it reflects a higher concentration of NS1/NS2 or if it is just a matter of faster degradation of the unspliced mRNA.

Little is known about the genetic basis of the resistance manifested by insects and their cultured cells to virus infections. During the past three decades, viruses have gained much attention as biological control agents as well as potential candidates for use in insect transgenesis (Corsini *et al.*, 1996; Royer *et al.*, 2001). Hence, it is imperative to identify factors that control viral resistance in order to understand the nature of the interaction between the insect and virus. The only known case for insect resistance to parvoviruses is that of *B. mori* strains to BmDNV1 and BmDNV2.The molecular basis that govern densovirus tropism in these strains was found to be controlled by host genes. Resistance to BmDNV1 infection is controlled by two insect recessive genes: Nonsusceptibility to BmDNV1, *nsd-1* (Watanabe and Maeda, 1981) and Non-infection to densovirus gene, *Nid-1* (Eguchi *et al.*, 1986). Resistance to BmDNV2 is controlled by

a third gene, *nsd-2* (Seki, 1984). *Nsd-1* was located on chromosome 21 (Eguchi *et al.*, 1991), whereas, *Nid-1* was located to chromosome 17 (cited by Ogoyi *et al.*, 2003). However, the molecular mechanism that controls this resistance is not yet clear. It is noteworthy to mention that, BmDNV2 has a bipartite single-stranded DNA genome and hence differs dramatically from parvoviruses. BmDNV2 is no longer regarded as a densovirus or parvovirus.

In the case of GmDNV and MlDNV, despite the large degree of identity between their genomes and the use of similar expression mechanisms, the two viruses have a very different host preference. Several studies proved the involvement of the parvovirus capsid proteins in controlling the virus tropism (Gardiner and Tattersall, 1988a; Bloom *et al.*, 1993; Bergeron *et al.*, 1996; Parker and Parrish, 1997; Bloom *et al.*, 1998; Hauck and Xiao, 2003). A limited number of reports raised the importance of the intracellular factors in determining the permissiveness to the virus infection (Ridpath and Mengeling, 1988; Oraveerakul *et al.*, 1992).

Our results on the tropism determinants of MlDNV and GmDNV showed that the virus VP promoter plays an important role in regulating the host preference. The VP promoter of GmDNV actively drove the expression of the *CAT* reporter gene in cells permissive to the virus infection, LD652 cells. The same construct was not efficient in the non-permissive T-ni03 cells. At the same time MlDNV VP promoter was active in the two cell types as measured by the reporter gene expression. Large differences have been found within the promoter sequence of the two viruses. Most of these differences accumulated around the TATA box. Therefore, the VP promoter of MlDNV is distinct from that of GmDNV and may differ in the recruitment of the transcription factors. The involvement of the VP promoter in controlling the virus tropism does not exclude other factors since we studied such tropism on the cellular system LD652/T-ni03.

Studying the safety of the densoviruses towards mammalian cells showed that MlDNV as well as GmDNV were not able to infect, replicate or even transcribe their genes in these cells. This study was carried out by infection as well as transfection on different mammalian cell types. Our results on the virus tropism also showed that the densovirus host range is controlled by the highly specific interaction between the virus promoters and the host cell proteins. Moreover, both viral promoters are needed for a successful infection, a prerequisite that makes the permissiveness, of a certain cell type, more complicated. Several studies showed significant differences between the vertebrate and invertebrate transcription machinery (Courey and Tjian, 1988; Burke and Kadonaga, 1996; Kutach and Kadonaga, 2000). The high dependence of densovirus expression on the intracellular environment and the difference between vertebrate and invertebrate expression accessories may then explain the inefficacy of these densoviruses toward the mammalian cells. Further studies may can be done by introducing coding sequences of densovirus under the control of a vertebrate parvovirus promoters and ITRs. This may help to verify whether densovirus can replicate in mammalian cells under these artificial conditions. Collectively, our results, thus contradict the findings of Kurstak *et al.* (1969 a and b) and suggested that the homologation process requiring *in vivo* tests on the densovirus safety will likely be successful.

General Conclusions

Adding a virus to the biological control arsenal is a multi-step process. This includes the isolation of the virus from its natural hosts, molecular characterization, studying its pathogenicity and its safety towards non target organisms. In the current thesis we studied three members of the *Densovirus* genus, GmDNV, MlDNV and HaDNV. The three viruses belong to the subgroup A, which includes viruses that are isolated exclusively from lepidopteran insects. They have the longest genome size as well as the longest ITRs among all members of the *Parvoviridae*. They also shared a high sequence identity of over 90% with a very close genomic organization.

The expression strategies employed by GmDNV and MlDNV have been determined. They use splicing and leaky scanning mechanisms to produce a set of three NS proteins. However, the four viral capsid proteins were produced by leaky scanning from one, non-spliced, population of mRNA. The splicing sites within the NS sequence of both MlDNV and GmDNV were also conserved in HaDNV genome, which suggests a similar mechanism. However, the expression strategy of HaDNV is still to be determined.

MlDNV and GmDNV differ dramatically in their host preference despite the high level of sequence identity. In order to find out their allotropic determinants, we used their infectious clones to create chimeric genomes. Conserved restriction sites permitted swapping of corresponding regions between the two viruses to study the impact on tropism. A cell line, T-ni03, was established that permits replication and passaging of MlDNV (a first for densoviruses). This cell line does not support the replication of GmDNV. However, both MlDNV and GmDNV could readily infect the LD652 cells. Therefore, T-ni03 and LD652 cells were found to be able to distinguish the tropism phenotypes of MlDNV and GmDNV. The VP promoter was found to be responsible for the phenotypic difference in this system. The critical role of the VP promoter was

confirmed using the *CAT*-reporter system. EMSA experiments were employed to investigate binding of the cellular transcription factors to the VP promoter region. Quantitative differences in binding of the transcription factors from the two cell types, LD652 and T-ni03 to GmDNV VP promoter have been shown. These results raised the importance of the virus promoters to control its host tropism. This is in contrast to most parvoviruses where the tropism is controlled by a few amino acid residues located on the viral capsid proteins.

The *in vitro* safety of MlDNV and GmDNV was also studied in the current work. Both viruses could not infect, replicate or transcribe their genes in mammalian cells, in contrast to susceptible insect cells. The densovirus genome integrated into the mammalian cellular DNA after transfection by the infectious clone but not after infection. However, neither toxicity nor cytopathic effects could be observed on clones carrying this integrated viral DNA. These clones were still refractory to the superinfection by densoviruses.

Together, our results show that densoviruses have a unique biology that is highly dependent on the host cell type. They do not have tropism in mammalian cells. In insect cells, their tropism is controlled by intracellular factors that interact with the virus promoters. Thus, densoviruses are suitable candidate for biological control.

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APPENDIX A

PUBLICATIONS

El-Far, M., Li, Y., Fédière, G., Abol-Ela, S. and Tijssen, P. 2004. Lack of infection of vertebrate cells by the densovirus from the maize worm *Mythimna loreyi* (MlDNV). *Virus Res.* **99**, 17-24.

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APPENDIX B

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SOMMAIRE (FRENCH SUMMARY)

INTRODUCTION

La famille des *Parvoviridae* se devise en deux sous-familles: les *Densovirinae* et les *Parvovirinae*. Les membres des *Densovirinae*, habituellement appellés densovirus, sont des pathogènes isolés exclusivement chez certains invertébrés soient les insectes et les crevettes. Pour leur part, les membres des *Parvovirinae* se trouvent chez les animaux à sang chaud. Plusieurs études ont démontré que les densovirus sont des pathogènes ubiquitaires associés à leurs hôtes, sous une forme aigüe ou latente, afin de les contrôler. La mortalité, l'effet aigüe, survient quelques jours suite à l'infection dépendemment de la concentration de l'inoculum viral et du stade larvaire. Un des effets les plus marquants de l'infection virale est la perte d'appétit qui prend place tôt après l'infection; un symptôme fort recherché dans la mise en place de programmes de lutte biologique.

La recherche concernant la biologie des densovirus est peu avancée. Cependant, au cours de la dernière décennie, un nombre grandissant de publications décrivant le clonage et la détermination de la séquence complète des membres des différents genres des *Densovirinae* a fait son apparition. Les génomes clonés ont la faculté d'initier une infection typique lorsque transfectés, autant *in vitro* qu'*in vivo*. Cette dernière caractéristique est d'un intérêt particulier puisqu'elle facilite grandement la recherche sur la biologie de ces virus in vitro. Les densovirus partagent avec les autres *Parvoviridae* la propriété de posséder un génome constitué d'ADN linéaire, simple brin de polarité positive ou négative, et encapsidé individuellement. Les télomères de ce génome comportent des séquences se présentant sous la forme d'épingle à cheveux qui sont impliquées dans la réplication de l'ADN. Ces épingles à cheveux sont incluses, soit dans les séquences terminales répétées inverses (ITR), soit dans les séquences uniques. Les membres du genre *Densovirus* sont uniques parmi tous les parvovirus par leur organisation génomique ambisens. Dans cette organisation, les deux brins complémentaires ont la

capacité de coder pour des protéines virales. Les stratégies d'expression de ces densovirus n'ont pas encore été clarifiées. Cependant, quelques recherches indiquent l'implication d'un mécanisme de balayage ribosomal (leaky scanning) pour produire un ensemble de protéines structurales.

Jusqu'à ce jour, les facteurs contrôlant le tropisme des densovirus n'ont pas encore été étudiés. Chez les parvovirus de vertébrés, le tropisme est contrôlé par un nombre restreint d'acides aminés, retrouvés habituellement sur ou près de la protubérance de l'axe de symétrie d'ordre 3 (three-fold spike) des protéines de la capside virale. Cependant, très peu de rapports ont soulevé l'importance de l'environnement intracellulaire dans la détermination de la permissivité à l'infection par les parvovirus.

Voici donc la structure de la présente thèse: tout d'abord, le premier chapitre résume l'ensemble de la littérature concernant l'histoire, la biologie et l'utilisation potentielle des densovirus dans le contrôle de la lutte biologique, ainsi que son utilisation en tant que vecteur d'expression. En plusieurs reprises, la comparaison a été faite entre les parvovirus de vertébrés et les densovirus puisque ceux-ci partagent de nombreuses caractéristiques de base. Le second chapitre mentionne les matériaux et les méthodes utilisés, afin de réaliser nos recherches, mis à part ceux déjà indiqués dans la section publiée du projet. Les chapitres suivants introduisent les résultats qui ont été obtenus au cours de notre projet de recherche et qui ont donné suite à trois articles publiés. Deux de ces articles (chapitres III et IV) résument le travail qui a été fait sur la biologie des virus GmDNV et MlDNV, des candidats pour la lutte biologique. Une section séparée (chapitre V) a été consacrée au séquençage et à la détermination de l'organisation génomique d'un nouveau densovirus, HaDNV, un nouveau membre des densovirus classiques. Le chapitre suivant (chapitre VI) détaille nos résultats sur le tropisme des densovirus qui ont été réalisés sur GmDNV et MlDNV suite à la construction de

plusieurs chimères. Le chapitre VII contient notre troisième publication qui décrit le travail qui a été fait sur la sécurité des densovirus envers les cellules de mammifères in vitro. Enfin, le dernier chapitre est constitué de la discussion générale de l'ensemble de nos résultats en fonction de récents rapports. Il traite également de l'impact de nos résultats dans la recherche sur les densovirus, ainsi que sur les perspectives qui ont émergées de notre travail.

CONNAISSANCES GÉNÉRALES

Histoire et importance économique des densovirus

Les densovirus constituent un groupe de virus qui sont hautement pathogènes et fatals envers leurs hôtes. Ils ont été rapportés, pour la première fois, en 1964 lorsque Meynadier et ses collègues ont observé une forte mortalité à l'intérieur de l'unité d'élevage d'insectes chez les larves du lépidoptère *G. mellonella* dans leur laboratoire de recherche à St-Christol, Montpellier (France). Ils ont observé une épidémie causée par un nouveau virus qui, plus tard, a été désigné comme un virus de la densonucléose (DNV) dû à la présence d'un symptôme cytopathologique, soit des noyaux denses en électrons (Meynadier *et al.*, 1964; Vago *et al.*, 1964). Depuis, un nombre grandissant de densovirus ont été isolés de différents ordres d'insectes, spécialement Lepidoptera, ordre qui regroupe des papillons et des larves importants économiquement. En raison de leur forte virulence et de leur efficacité à mettre fin à l'alimentation des insectes tôt suite à l'infection, les densovirus sont hautement intéressants pour le contrôle des insectes ravageurs (Belloncik, 1990; Bergoin et Tijssen, 1998).

Dans les champs d'huile de palmier de la Colombie et de la Côte d'Ivoire, infestés par *Silbine fusca* et *Casphalia extranea* respectivement, l'application d'aussi peu que 50

larves infectées par hectare a suffit à assurer 90 % de protection (Genty et Mariau, 1975; Belloncik, 1990). Des essais sur le terrain d'un densovirus isolé chez l'insecte *Aedes aegypti*, dans le but de contrôler différentes espèces d'insectes ayant une importance médicale, ont démontré que le virus avait une efficacité significative et pouvait être utilisé avec succès pour le biocontrôle de ces insectes (Buchatsky *et al.*, 1987). Plusieurs autres densovirus possèdent un grand potentiel et pourraient également être intégrés à l'arsenal de la lutte biologique. Cependant, des tests d'innocuité sont préalablement nécessaires (Belloncik, 1990; Bergoin et Tijssen, 1998; Fédière, 2000).

Le développement de la recherche sur les densovirus en tant qu'agents dans la lutte biologique a été mis de côté durant une longue période suite à des rapports non confirmés clamant l'infectivité des densovirus envers des cellules de mammifères. Kurstak *et al.* (1969 a et b) ont publié le premier rapport controversé au sujet d'un parvovirus d'insecte, isolé du lépidoptère *G. mellonella*, en tant que pathogène pour les cellules de mammifères. Ce groupe de recherche a dénoté que le virus peut infecter et transformer les cellules L de souris en culture. Comme outil diagnostique, ils ont utilisé l'immunofluorescence indirecte, afin de détecter les antigènes viraux dans les cellules infectées à partir d'un inoculum de virus purifié. Cependant, la mise en évidence de différences entre les cellules d'invertébrés et de vertébrés, en ce qui a trait aux éléments génétiques tels que les promoteurs et les séquences initiatrices de transcription, a été démontrée (Tijssen et Bergoin, 1995; Kutach et Kadonaga, 2000). Ainsi, l'infectivité des densovirus d'insectes envers les cellules de mammifères n'est pas anticipée. Par ailleurs, plusieurs tentatives infructueuses ont été réalisées *in vitro* afin de reproduire les résultats de Kurstak et ses collègues, soit par GmDNV (Kawase *et al.*, 1990) ou par un autre type de densovirus isolé des cellules d'insectes C6/36 dérivées de *Aedes albopictus*, AalDNV (Jousset *et al.*, 1993).

Propriétés biophysiques

Les densovirus sont de petits virus (20-25 nm) non enveloppés, de forme icosahédrale contenant un génome d'ADN linéaire simple brin (Tijssen et Bergoin, 1995). Il existe environ une trentaine de densovirus divisée en trois genres, *Densovirus*, *Iteravirus* et *Brevidensovirus* (van Regenmortel *et al.*, 2000). Chacun d'eux contient un génome linéaire simple brin où les brins " + " et "-" sont encapsidés dans des virions séparés. Ces deux brins viraux (+ /-) se lient au moment de l'extraction dans des conditions élevées en sels (50-100 mM NaCl) pour former des molécules d'ADN double brins (Barwise et Walker, 1970; Kurstak *et al.*, 1971; Kelly *et al.*, 1977). La proportion entre les brins positif et négatif d'ADN encapsidés varie selon le genre. Chez les genres *Densovirus* et *Iteravirus*, les deux brins sont encapsidés dans une proportion équimolaire partageant ainsi cette caractéristique avec les dependovirus de vertébrés (AAVs) (Kelly *et al.*, 1977; Berns et Adler, 1972). Par contre, chez les *Brevidensovirus*, AaeDNV et AalDNV, le brin négatif est préférentiellement encapsidé (90 %) tout comme chez les parvovirus de vertébrés tels que MVM et PPV (Tijssen et Bergoin, 1995; Berns, 1996).

Le génome des densovirus, comme tous les parvovirus, possède des terminaisons palindromiques (épingle à cheveux) qui agissent comme amorces pour la réplication de l'ADN. Ces épingles à cheveux pourraient être retrouvées à l'intérieur des ITRs ou des séquences uniques. La structure secondaire, aussi bien que la longueur de ces séquences terminales, varient selon les différents membres. Les membres du genre *Densovirus* possèdent des génomes de 5,5 à 6 Kb avec une organisation ambisens qui est unique à l'intérieur de la famille des *Parvoviridae*. Les densovirus classiques isolés de lépidoptères (papillons) tels que GmDNV et JcDNV, possédant des génomes de 6 Kb et partagent une forte identité de séquence qui atteint environ 90 %. Chacun de leurs brins d'ADN complémentaires a la capacité de coder pour des protéines, selon un nombre limité de

grands cadres de lecture ouverts (ORFs), distribués sur la moitié 5' des brins complémentaires. Un des brins code pour les protéines structurales, habituellement 4 protéines de capside, VP1-VP4 (Tijssen *et al.*, 1976) et le brin complémentaire code pour trois protéines non structurales ou protéines Rep, NS1-NS3. Ce groupe de virus est très distinct par la présence des séquences terminales répétées inverses (ITR) les plus longues parmi tous les parvovirus, soit plus de 500 nucléotides (nts) (Tijssen, 1990; Dumas *et al.*, 1992).

Les deux autres genres de parvovirus d'insectes, *Brevidensovirus* et *Iteravirus*, se présentent sous une organisation génomique monosens. Ils possèdent une cassette de deux gènes NS au lieu de trois dans le cas des *Densovirus* et une seconde cassette pour les protéines VP, chacune contrôlée apparemment par seulement un promoteur fonctionnel (Bergoin et Tijssen, 1998). Cependant, ces deux genres diffèrent par la longueur de leur génome, avec 5 Kb pour les membres d'*Iteravirus*, mais seulement 4 Kb pour ceux de *Brevidensovirus*. Ils se différencient également par la structure de leurs terminaisons, où les iteravirus (BmDNV et CeDNV) ont des ITRs en forme de J de 230 nts (Li *et al.*, 2001; Fédière *et al.*, 2002), tandis que les brevidensovirus (Aedes densovirus) ont, pour leur part, des structures palindromiques en forme de T à leurs deux extrémités (Afanasiev *et al.*, 1991; Boublik *et al.*, 1994; Shike *et al.*, 2000).

La capside des densovirus est assemblée par 60 unités d'une protéine structurale essentielle. Cette protéine peut avoir différentes extensions N-terminales (des isoformes) tel que montré par le séquençage du peptide (Tijssen *et al.*, 1976; Tijssen et Kurstak, 1981) ou encore par crystallographie par rayon X (Simpson *et al.*, 1998). Cependant, le rôle de ces extensions N-terminales demeure inconnu jusqu'à ce jour. Deux des cinq isoformes des VPs, lesquelles ont habituellement des masses moléculaires de 40 à 100 KDa, peuvent être détectés chez les densovirus. La production de ces isoformes

N-terminales des protéines virales est une stratégie employée par les densovirus, afin d'exploiter la capacité codante de leur génome limité de 4 à 6 Kb. Environ la moitié du génome est dédiée à produire les différentes protéines structurales à partir d'un seul gène.

Les densovirus, aussi bien que les parvovirus de vertébrés, ont un nombre limité de gènes codants puisqu'ils possèdent un génome petit et compact, et donc, un nombre limité de protéines produites. Ceci implique, en partie, une organisation de gènes chevauchants, afin de maximiser l'utilisation du matériel génétique limité et, d'autre part, implique la production de protéines multifonctionnelles. Il a précédemment été rapporté que la protéine virale VP1 des parvovirus possède une région conservée, à l'intérieur d'un segment de 39 a.a., appellée le motif PGY, de 70-98% d'identité (Dumas *et al.*, 1992; Tijssen et Bergoin, 1998). Il a été démontré, par la suite, que ce domaine possède une activité enzymatique, phospholipase A2 (PLA2). Cette PLA2 est située à l'intérieur de la région N-terminale de la VP1 (VP1up) de presque tous les parvovirus connus et est requise à l'atteinte d'une infection efficace (Zádori *et al.*, 2001). Lors de l'entrée du virus, la VP1 N-terminale s'expose à l'extérieur de la capside sans la désassembler, tel que démontré chez quelques parvovirus (Cotmore *et al.*, 1999). Cette protéine pourrait être nécessaire pour le transport des virions des endosomes tardifs/lysosomes jusqu'au noyau (Zádori *et al.*, 2001). Les PLA2s forment une superfamille d'enzymes clés impliqués dans les processus physiologiques et pathologiques tels que le métabolisme des lipides membranaires, les signaux transductionnels, l'inflammation, l'hypersensibilité aigüe et les maladies dégénératives (Dennis, 1997; Kramer et Sharp, 1997; Balsinde *et al.*, 1999). La boucle de liaison au Ca^{+2} (GPGN) ainsi que le site catalytique de PLA2 (DxxAxxHDxxY) sont conservés autant chez les parvovirus de vertébrés que d'invertébrés. Cependant, l'activité spécifique de la PLA2, des membres des *Densovirus*, est signativement plus faible que celle des membres d'*Iteravirus* ou des parvovirus de vertébrés (Zádori *et al.*, 2001; Li *et al.*, 2001; Fédière *et al.*, 2002). L'altération de la

protéine de capside de JcDNV en délétant la VP1 N-terminale, abolie l'infection virale, probablement dû à l'absence de la PLA2. Par contre, la production de particules contenant l'ADN a été observée suite à une transfection par ce virus altéré (Abd-Alla, 2003). Il est à noter que les virus du troisième genre, *Brevidensovirus*, ne possèdent pas une telle activité enzymatique sur leur protéine VP, ce qui pourrait résulter d'une divergence durant l'évolution depuis qu'ils infectent des moustiques et des crevettes.

Comme dans le cas des protéines VPs, les densovirus ont un nombre très limité de protéines non structurales, habituellement 2 protéines NS. Cependant, les densovirus classiques ambisens sont uniques parmi les parvovirus d'invertébrés aussi bien que de vertébrés, en possédant trois protéines NS. Celles-ci sont produites par trois ORFs distribués sur la moitié 5' d'un des deux brins complémentaires et qui sont contrôlés par un promoteur situé à l'intérieur du grand ITR (Tijssen et Bergoin, 1995). Ces protéines sont probablement impliquées dans la réplication de l'ADN viral, mais peut-être aussi dans d'autres fonctions. Chez les parvovirus de vertébrés, il est bien connu que les protéines NS (NS1 et NS2) sont impliquées dans la réplication de l'ADN viral et dans la régulation de l'expression génique (Cotmore et Tattersall, 1995; Berns, 1996). NS1 est une protéine multifonctionnelle qui exhibe un site spécifique pour la liaison de l'ADN et des activités ATPase, hélicase et nickase qui sont toutes nécessaires à la réplication de l'ADN (Cotmore et Tattersall, 1995). Elle est aussi impliquée dans la régulation du promoteur P4 et la transactivation du promoteur P38 qui contrôlent l'expression des protéines non structurales et de capside, respectivement (Cotmore et Tattersall, 1987; Doerig *et al.*, 1988; Doerig *et al.*, 1990; Vanacker *et al.*, 1996). Cependant, il a été démontré que la protéine NS2 est requise pour la sortie des virions par une interaction avec le Crm1 nucléaire, et peut, alors, participer dans la propagation virale (Eichwald *et al.*, 2002).

OBJECTIFS DE TRAVAIL

Le premier objectif de la thèse sera d'étudier la biologie des densovirus. Trois membres seront exclusivement utilisés dans ce travail: les densovirus de *Mythimna loreyi*, *G. mellonella* et *Helicoverpa armigera* (MlDNV, GmDNV et HaDNV, respectivement). Ces virus ont été isolés de lépidoptères qui sont des insectes ravageurs, ayant un impact économique important dans le domaine de l'agriculture. Cloner le génome complet, étudier l'organisation génomique ainsi que la stratégie d'expression de ces virus, sont tous des sujets majeurs à l'investigation.

Le second objectif est de localiser les déterminants allotropiques qui contrôlent le tropisme de GmDNV et MlDNV. Les deux virus partagent plus de 90 % d'identité de séquence, mais diffèrent grandement quant à leur préférence d'hôte. MlDNV est polyspécifique, infectant plusieurs insectes lépidoptères. Cependant, GmDNV est restreint à son hôte *G. mellonella*. Chez les virus de vertébrés, les déterminants allotropiques ont été identifiés sur les protéines de la capside virale. Dans le but de vérifier cette possibilité chez les densovirus, une série de chimères devra être construits utilisant les génomes de MlDNV et GmDNV. Les effets phénotypiques de ces chimères seront ensuite observés dans un système cellulaire discriminant, ce qui sélectionnera un des deux virus.

Le troisième objectif sera d'étudier la sécurité des densovirus MlDNV et GmDNV *in vitro* comme première étape, avant de procéder à des tests *in vivo* extrêmement coûteux. Dans le but de réaliser cette partie du travail, des infections et des transfections par de l'ADN de densovirus devront être effectuées dans différentes cellules de mammifères.

RÉSULTATS

Nous avons cloné et séquencé les génomes complets de MlDNV et HaDNV. La taille du génome de MlDNV est de 6034 nts (GenBank AY61507) et celui de HaDNV est de 6039 nts, la même taille que GmDNV. Le génome de MlDNV contient un ITR de 534 nucléotides à l'une de ses extrémités et de 439 nts à l'autre extrémité. La centaine de nucléotides manquants à cette dernière extrémité (comparé à l'ADN viral), qui aurait facilité le clonage, a été réparée en utilisant la partie complète de l'ITR de l'autre extrémité. Les régions distales, de 126 nts, des ITRs peuvent être repliées en des terminaisons en épingle à cheveux sous une forme typique en Y telle qu'observée chez plusieurs parvovirus. Les premiers 47 nts de la tige sont parfaitement complémentaires aux nucléotides 80-126, dont les nucléotides 48-79 dans les bras asymétriques sont riches en GC. Un dimorphisme de l'épingle à cheveux, nommé flip/flop, a pu être observé dans le génome de MlDNV.

Le génome de HaDNV contient également des ITRs typiques du genre *Densovirus* (les densovirus classiques) de 545 nucléotides. L'épingle à cheveux terminale, de 126 nts, a été complètement conservée entre HaDNV et MlDNV sans aucune insertion ou délétion. Cependant, des différences de séquence commencent à s'accumuler en aval de l'épingle à cheveux avec des changements de 13 nts et une insertion supplémentaire de 2 nts chez HaDNV par rapport à MlDNV. L'ensemble de la séquence est identique à 90% à celle de MlDNV et de 86% à celle de GmDNV. Il est intéressant de noter que MlDNV et GmDNV partagent 90% d'homologie de séquence. Le même niveau d'identité existe aussi entre GmDNV et JcDNV aussi bien qu'entre MlDNV et JcDNV. Le dimorphisme de la séquence de l'épingle à cheveux chez le HaDNV n'a pu être étudié puisqu'un seul clone, contenant les séquences terminales, a été obtenu.

Les deux virus, MlDNV et HaDNV, possèdent une organisation génomique ambisens typique aux densovirus classiques. Chez MlDNV, le brin " + " contient trois ORFs (NS1-3) sur la moitié 5'. Le premier commence à 112 nts après l'ITR et est immédiatement suivi de deux ORFs chevauchants au niveau desquels les codons d'initiation ne sont séparés que de 4 nts. Le plus grand ORF (1635 nts) contient le domaine ATPase typique trouvé chez la protéine NS1 de tous les parvovirus. Le brin complémentaire "-" contient également un grand ORF de 2,5 Kb (ORF4) sur sa moitié 5'. L'ORF4, sur le brin "-", est séparé de la cassette de l'ORF1-3, qui se trouve sur le brin " + ", par 36 nts au milieu du génome. Cet ORF4 commence seulement à 32 nts en aval de l'ITR et contient le domaine phospholipase A2 qui est typiquement retrouvé chez les protéines structurales mineures des parvovirus (Zádori *et al.*, 2001). L'analyse de la capside virale par SDS-PAGE a démontré que les virions de MlDNV contiennent 4 protéines structurales avec une stoechiométrie et une masse moléculaire similaire aux protéines de structure de GmDNV. L'ORF4 est long de 2463 nts et est ainsi le seul ORF assez long pour coder la plus grande protéine de structure VP1. Cette protéine possède une masse moléculaire d'environ 90 KDa, estimée par SDS-PAGE. En effet, la masse moléculaire de chacune des 4 protéines de structure de MlDNV, comme déterminée par SDS-PAGE, correspond à la masse prédite si la traduction est initiée aux quatre premiers codons d'initiation de l'ORF4 (ORF4a, b, c et d) et co-termine au codon stop. Le grand ORF1, sur le brin sens, a la capacité de coder pour la NS1 de densovirus et le nombre d'a.a. putatif qui résulte de cet ORF est de 547. L'ORF2 correspond à la protéine NS2 avec une capacité codante de 275 a.a. et l'ORF3 à NS3 avec 231 a.a. Sur le brin complémentaire, un grand ORF possédant une capacité codante de 811 a.a. s'apparente en tous points à celui des densovirus.

Le génome de HaDNV a également une organisation génomique similaire à celle de MlDNV. La recherche de domaines conservés à l'intérieur de sa NS1 putative a

démontré que le virus contient des motifs initiateurs (replicateurs) hautement conservés avec une identité de séquence et d'emplacement similaires aux autres densovirus ainsi qu'aux parvovirus de vertébrés. Le second motif conservé est la NTP-ase NS1 hélicase de la superfamille III qui est localisé entre les a.a. 404 et 499.

L'infectivité des clones MlDNV et GmDNV a été testée par transfection chez les larves de *Spodoptera littoralis* et chez les cellules LD652 en culture. L'inoculum contenait 10 µl de l'ADN viral cloné ou d'ADN viral comme contrôle, précipité avec du DEAE-dextran pour les larves, et avec DOTAP pour les transfections *in vitro*. Environ 80% de mortalité, 9 jours suite à la transfection, a été obtenue par le génome cloné de MlDNV, pMl28, ainsi que par l'ADN viral. Le virus ainsi obtenu a pu ensuite être utilisé pour des infections subséquentes. Par ailleurs, l'introduction de l'ADN cloné ainsi que l'ADN viral dans les cellules en culture a mené à la production de virus infectieux. Le surnageant obtenu, 4 jours suite à la transfection de cellules LD652 ou Sf9, était infectieux chez les larves. Des extraits de larves mortes soumises à la microscopie électronique ont montré la présence de hautes concentrations de virus. Le génome cloné de GmDNV était également infectieux autant *in vivo* qu'*in vitro*. Cependant, seules les cellules LD652 ont supporté la réplication du clone infectieux.

Les transcrits de MlDNV et GmDNV ont été séquencés, afin d'étudier la stratégie d'expression de ces virus. Dans les deux cas, l'immunobuvardage de type Northern a révélé deux transcrits pour les gènes NS, un de 2,5 Kb et un de 1,8 Kb, mais un seul transcrit pour la VP d'environ 2,6 Kb. Chez MlDNV, le séquençage des produits de RT-PCR du transcrit NS de 2,5 Kb a démontré qu'il débute 27 nts en aval de l'ITR et termine au nucléotide 3008. Il a également été démontré que le transcrit de 1,8 Kb subit un épissage entre les nucléotides 652 et 1357, ce qui engendre l'élimination complète du gène NS3 qui débute au nt 655 et qui se termine au nt 1347. Le tandem de codons

d'initiation pour NS1 et NS2 deviennent donc les premiers AUGs dans les transcrits épissés alternativement. La région 5' non traduite dans le cas de NS1 et NS2 est de 80 nts. Le transcrit de VP commence, pour sa part, 25 nts en aval de l'ITR et se termine au nt 2952. Ceci indique que ses éléments de promoteurs sont localisés entièrement dans l'ITR. Ainsi, les transcrits de NS et de VP possèdent 57 nucléotides antisens qui se chevauchent à leur extrémité 3'. Aucun épissage n'a été mis en évidence dans le transcrit de VP suggérant un mécanisme alternatif, tel qu'un balayage ribosomal, pour la traduction des quatre protéines de structure.

Chez GmDNV, il a été démontré que le transcrit de VP débute 23 nucléotides en amont de l'ITR, situé à l'extrémité 3', soit au nt 5467. Pour leur part, les transcrits des NS débutent au nt 573, soit à 23 nts en aval de l'ITR de l'extrémité 5'. Ceci suggère que la majorité des éléments du promoteur, situés en amont, seraient localisés à l'intérieur de l'ITR et seraient, en conséquence, identiques chez le VP et le NS. Les extrémités intérieures des ITRs (en amont des séquences codantes) contiennent des boîtes TATA potentielles. La séquence des sites d'initiation de transcription correspond, selon toute vraisemblance, à la séquence consensus des boîtes Inr (TCAGTG).

La séquence non traduite de l'extrémité 5' de l'ARNm de VP est de seulement 5 nts de longueur. Par contre, dans le cas des transcrits de NS, la séquence non traduite de l'extrémité 5' est de 82 nts pour le petit transcrit (1,8 Kb) et de 84 nts pour le grand transcrit (2,5 Kb). Les extrémités 3' des transcrits ont été identifiées au nt 2954 pour le transcrit de VP et au nt 3013 pour les transcrits de NS. Conséquemment, le transcrit de VP chevauche celui de NS par 60 nts. Il n'est pas encore déterminé si cet ARN antisens possède une fonction régulatrice. Aucun épissage n'a été observé chez les transcrits de VP. Cependant, le transcrit de NS de 1,8 Kb est retrouvé sous une forme épissée du transcrit de NS de 2,5 Kb non épissée. Comme estimé par l'immunobuvardage de type

Northern, environ 50-75% des transcrits de NS sont épissés. Le premier codon d'initiation dans le transcrit de NS de 2,5 Kb, au nt 657, est dans un contexte favorable à la traduction et pourrait potentiellement conduire à la production d'une protéine de 232 a.a. Cette séquence codante de NS3 a été complétement éliminée du transcrit épissé de 1,8 Kb. Un simple épissage de 707 nts a été observé entre le nt G (654), soit 2 nts en amont du codon AUG de NS3, et le nt A (1362) du codon d'initiation AUG de NS1, soit 7 nts en aval du codon d'arrêt de NS3. Les deux grands ORFs situés en aval de ce site d'épissage, pourraient ainsi être traduits par un mécanisme de balayage ribosomal.

Le GmDNV utilise un mécanisme de balayage ribosomal pour l'expression de ses protéines de structure, VPs. Pour confirmer ce mécanisme chez le MlDNV, les quatre gènes chevauchants des VPs ont été amplifiés par PCR, afin d'éliminer les séquences 5' qui précèdent les ATGs et qui pourraient porter des structures IRES (Internal Ribosome Entry Sites). Les amplicons ont ensuite été clonés dans un système d'expression baculoviral et les protéines produites ont été analysées par microscopie électronique ainsi que par immunobuvardage de type Western. Dans ce système, les quatre amplicons ont produits des protéines qui se sont auto-assemblées en particules semblables au virus sauvage (virus-like particles, VLPs). Ces VLPs ont réagi avec l'antisérum spécifique au virus sauvage confirmant que l'ORF4 code pour les protéines structurales de MlDNV. Leur taille est semblable à celle des protéines de structure des virus sauvages. Il est intéressant de noter que le grand amplicon a produit, en plus des protéines attendues, des protéines de plus petites masses moléculaires dans un ratio comparable. Par ailleurs, les amplicons de VP3 et VP4 ont produit des protéines de masse moléculaire inférieure à celle de VP4, en plus des protéines VP3 et VP4, respectivement. Ces protéines de petite masse moléculaire ont réagi positivement avec les anticorps spécifiques aux protéines virales de MlDNV. Il semble que le codon d'initiation de VP4 est devenu, en quelque sorte, moins favorable en l'absence des codons d'initiation de VP1 et VP2.

Les séquences déterminantes du tropisme de MlDNV et GmDNV ont été étudiées dans le présent travail. Les génomes complets des deux virus ont été clonés dans des vecteurs plasmidiques et les séquences ont été déterminées (résultats présentés dans les chapitres III et IV). Les deux virus partagent 90 % d'identité de séquence, où les 10 % de différences sont distribués autant sur les séquences codantes que non codantes. La disponibilité de ces génomes clonés nous ont permis de créer des chimères entre les deux virus en utilisant des fragments de restriction.

En utilisant les clones infectieux, pGm1 de GmDNV et pMl28 de MlDNV, nous avons étudié le tropisme des deux virus *in vitro* dans différentes lignées cellulaires. MlDNV s'est avéré infectieux chez ces lignées cellulaires: Sf9, LD652, T-ni et SL52. D'autre part, GmDNV peut seulement infecter la lignée cellulaire LD652 à un titre comparable à celui de MlDNV chez ces mêmes cellules, soit de 12-15 % d'infection déterminée par immunofluorescence. En dépit de la permissivité des différentes lignées cellulaires pour l'infection par MlDNV, le titre viral était faible chez les cellules Sf9, SL52 et T-ni tel qu'estimé par l'analyse FACS. Plusieurs essais ont été tentés pour sous-cloner ces types de cellules dans le but d'obtenir une population qui supporte un titre élevé de l'infection virale. Comme premier essai, nous avons utilisé la méthode de dilution limite pour séparer les cellules individuellement. Cependant, aucune de ces cellules n'a pu survivre à la dilution. Nous avons alors procédé par une dilution logarithmique d'une concentration mère de 10^5 cellules par ml. Seulement les cellules T-ni ont bien répondu à cette méthode, bien que le nombre minimum de cellules ait été de 50 cellules par puits au début de l'expérimentation. De cette façon, nous avons séparé une sous population de cellules T-ni appelée T-ni03 qui supporte un niveau plus élevé de l'infection virale, soit d'environ de 20 %. De façon intéressante, cette sous population est demeurée non permissive à l'infection par le GmDNV. La combinaison entre les deux

lignées cellulaires, LD652 et T-ni03, nous a fourni un système efficace nous permettant de différencier les phénotypes d'infection de MlDNV et GmDNV.

L'échange homogène des séquences codantes entre le MlDNV et GmDNV a été effectué par des fragments de restriction en utilisant le génome cloné. Les sites de transfert ont été choisis de façon à garder l'intégrité des différents ORFs. Ceci a mené à la création de 10 chimères (comme il est montré au chapitre VI). Les deux clones recombinants de *Bam*HI nous ont permis d'exclure un rôle possible dans le contrôle du tropisme par les séquences terminales (épingles à cheveux).

Il a été possible de transférer la cassette des gènes NS de MlDNV chez le GmDNV sans endommager ses trois ORFs. Ce transfert a été effectué par le petit fragment *Bam*HI-*Bsm*I de MlDNV qui couvre toute la région codante de NS sauf les derniers 93 nucléotides du côté C-terminal. Le transfert inverse, soit de la cassette NS de GmDNV vers MlDNV, n'a pas réussi en raison de la présence de deux sites de reconnaissance de *Bsm*I, et ceci malgré plusieurs essais de digestions partielles. Le phénotype du nouveau clone, soit le pGm-MlNS, a été étudié dans le système cellulaire LD652/T-ni03 par transfection. Aucun effet significatif sur le tropisme n'a été observé. Cependant, un nombre très limité de cellules T-ni03 étaient positives en fluorescence après la transfection.

Seulement le transfert de la région codante de MlDNV VP vers GmDNV a permis le changement de tropisme de GmDNV. Le transfert inverse a bloqué le tropisme de MlDNV chez les cellules T-ni03. Pour déteminer les séquences minimales qui sont responsables de ce changement de tropisme, la cassette VP a été divisée en trois régions interchangeables. Aucune des régions codantes de VP n'a permis le changement de tropisme des deux virus. Cependant, le transfert du promoteur des gènes VP de MlDNV

vers GmDNV, par PCR recombinant, a étendu le tropisme de GmDNV aux cellules T-ni03.

Les résultats précédents suggèrent que le promoteur des gènes VP du GmDNV n'est pas actif chez les cellules T-ni03. Afin de vérifier cette hypothèse, nous avons cloné le promoteur de VP de MlDNV, ainsi que celui de GmDNV, en amont du gène rapporteur de *CAT* (chloramphenicol acetyl transferase). La séquence clonée du promoteur inclus les courtes séquences N-terminales non traduites du transcrit de VP (TCAGT dans le cas de MlDNV et TCACT chez le GmDNV) jusqu'au site *Bam*HI au milieu de l'ITR. Les nouveaux clones d'expression, pMlVP-CAT et pGmVP-CAT ont été étudiés chez les cellules LD652 et T-ni03 par transfection. Le temps d'incubation de ces constructions chez les cellules a été fixé à trois jours pour imiter le temps optimal d'infection des densovirus dans ces cellules (le temps nécessaire pour retrouver le nombre maximal de cellules infectées). L'activité des deux promoteurs, dans les deux types cellulaires, a été analysée par le niveau de ^{14}C-chloramphénicol acétylé qui est, pour sa part, dépendant du niveau de *CAT* exprimé dans les cellules. Comme il était attendu, le promoteur de MlDNV VP a conduit à l'expression du gène *CAT* chez les deux types cellulaires, bien qu'un niveau d'expression plus faible a été détecté chez les cellules T-ni03 comparé aux cellules LD652. Cependant, le promoteur de GmDNV VP est fonctionnel chez les cellules LD652 avec une activité semblable à celle retrouvée dans le cas de MlDNV, mais 13 fois moins élevée dans les cellules T-ni03. Au cours d'expérimentations subséquentes, nous avons introduit, dans les cellules, la construction *CAT* de GmDNV accompagnée du clone infectieux de MlDNV ou celui de GmDNV. Ces expérimentations ont démontré que le MlDNV a activé modéremment la VP de GmDNV chez les cellules T-ni03, soit de 6 fois. Par contre, chez les cellules LD652, l'activité du promoteur de VP de GmDNV n'a pas subit de changement suite à la co-transfection.

La différence de l'activité d'expression des promoteurs de MlDNV et GmDNV chez les cellules T-ni03, suggère une interaction entre ces séquences et des facteurs de transcription cellulaire. Afin d'étudier ce type d'interaction, nous avons ciblé trois régions promotrices qui contiennent des différences majeures entre les promoteurs de MlDNV et GmDNV. Des oligos synthétiques qui imitent ces régions ont été créés et marqués avec la radioactivité. Ces oligos marqués ont été mis en contact avec des extraits cellulaires de LD652 et T-ni03. Les complexes formés, oligos/protéines, ont été utilisés en "gel shift assay". Les résultats de ce test ont montré que les séquences promotrices de GmDNV se comportent de façon similaire chez les cellules permissives aussi bien que chez les cellules non-permissives. Cependant, la quantité de protéines recrutées chez les cellules permissives a été plus élevée que chez les cellules non-permissives.

DISCUSSION

Les densovirus sont connus en raison de leur forte virulence envers leurs hôtes. La mortalité se manifeste quelques jours après l'infection selon la concentration virale et le stade larvaire (Meynadier *et al.*, 1964; Vago *et al.*, 1964; Shimuzu, 1975). Les densovirus sont résistants à des conditions extrêmes telles que la température élevée, 56°C pendant 60 minutes, et un large spectre de pH, 3 à 9 (en suspension). Ils sont également capables de maintenir leur infectivité pour des périodes prolongées, mais dans des formes non purifiées (Boemare *et al.*, 1970; Suto, 1979; Siegl *et al.*, 1985). Un autre avantage concernant les densovirus consiste en leur présence chez un grand nombre d'insectes ravageurs ayant un impact important tant sur le plan économique que médical. Ces caractéristiques font des densovirus des candidats intéressants pour la lutte biologique.

Dans le présent travail, nous avons caractérisé deux membres des densovirus classiques, MlDNV et HaDNV, et comparé leur séquence à celle de GmDNV. Ces virus ont été isolés chez des insectes ravageurs intéressants dans le domaine de l'agriculture. Notre but était d'étudier la biologie de ces densovirus, leurs modalités d'expression, leurs mécanismes de contrôle du tropisme et leur innocuité envers les cellules de mammifères.

Nos résultats sur l'analyse des séquences de GmDNV, MlDNV et HaDNV ont démontré qu'ils possèdent les plus grands génomes parmi tous les parvovirus. La taille du génome de HaDNV est identique à celui de GmDNV, 6039 nucléotides, soit 4 nts plus long que MlDNV. Une identité de 90% a été trouvée entre les séquences des 3 virus. Cette homologie est la plus élevée parmi tous les densovirus. Les trois virus ont la même organisation génomique, ambisens, qui est typique de ce groupe. Ils ont des ITRs d'environ 550 nts, soit les plus longs parmi tous les parvovirus, et contiennent des épingles à cheveux de 120 à 140 nts. Le dimorphisme des épingles à cheveux, flip/flop, a été observé chez le MlDNV, similaire à celui de GmDNV. Par contre, ce dimorphisme n'a pas été étudié chez le HaDNV étant donné qu'un seul clone comportant l'épingle à cheveux a été séquencé. La taille de l'épingle à cheveux, de 126 nts, aussi bien que sa séquence exacte, sont conservées entre le MlDNV et le HaDNV. Cependant, l'épingle à cheveux de GmDNV contient un seul nucléotide de différence. Il n'est pas encore clair si cette différence joue un rôle dans la biologie du virus.

L'analyse des séquences a montré que les boîtes TATA des promoteurs de GmDNV se trouvent entièrement dans les deux ITRs. Cependant, chez le MlDNV ainsi que le HaDNV, les ITRs se terminent au milieu des séquences TATA. Dans les deux cas, tous les éléments régulateurs de promoteurs se trouvent dans les ITRs. Étant donné que les séquences des ITRs sont identiques, les séquences régulatrices des promoteurs de VP et NS sont identiques entre elles. Ce fait a soulevé la question sur la manière dont

laquelle les densovirus régularisent leur expression. Une petite différence entre les séquences des promoteurs de VP et NS du densovirus JcDNV a été précédement démontrée (Dumas *et al.*, 1992). Par contre, il semble que cette différence est de faible conséquence.

Les stratégies d'expression employées par MlDNV et GmDNV sont très semblables. Les deux virus utilisent les mécanismes d'épissage et de balayage ribosomal pour produire trois types de protéines NS. Les protéines NS1 et NS2 sont traduites à partir d'un ARNm non épissé selon un mécanisme de balayage ribosomal. Le ATG de NS2 se trouve en aval de celui de NS1, séparé par 4 nts. Ce phénomène a été observé chez le virus B d'influenza qui utilise aussi un mécanisme de balayage ribosomal pour produire deux protéines dont les séquences codantes sont également séparées par 4 nts (Shaw *et al.*, 1983; Williams et Lamb, 1989). Les parvovirus de vertébrés utilisent également des formes épissées et non épissées d'ARNm pour produire leurs protéines non structurales.

Pour l'expression de protéines structurales (VP), le MlDNV et le GmDNV utilisent une seule population d'ARNm non épissés de 2,6 Kb de taille. Comme nous l'avons montré par le système d'expression baculoviral, les deux virus utilisent un mécanisme de balayage ribosomal pour produire les différentes VPs à partir de ce type d'ARNm non épissés. Le mécanisme de balayage ribosomal a déjà été proposé pour l'expression de protéines structurales chez le genre *Iteravirus* (Li *et al.*, 2001; Fédière *et al.*, 2002). Par contre, chez les densovirus non classiques, tels que PfDNV, la stratégie d'expression est différente. Après la transcription, les deux ORFs de VP de PfDNV s'attachent entre eux par l'épissage et se réorganisent par un mécanisme de changement de cadre (frame-shift), afin de produire la VP1 (Yamagishi *et al.*, 1999). Le PfDNV présente également une différence dans les sites de terminaison de transcrits de VP, car

ils contiennent des sites de poly-A alternatifs. Il est important de noter que le mécanisme de balayage ribosomal n'est pas connu chez les parvovirus de vertébrés, de sorte que les densovirus sont distincts dû à ce mécanisme.

Jusqu'à ce jour, il n'est pas encore clair si les densovirus ont un certain type de régulation de transcription. Cependant, des études récentes ont démontré un niveau d'expression constant pour les protéines VP et NS pendant l'infection virale (Abd-Alla, 2003). Dans le cas de MlDNV, nous avons observé une augmentation dans le rapport de l'ARNm épissé de NS qui sert à produire la NS1 et la NS2 versus l'ARNm non épissé, 72 hrs après l'infection. Par contre, il n'est toujours pas clair si cette augmentation signifie un niveau plus élevé de protéines NS1/NS2 ou simplement une dégradation de l'ARNm non épissé.

Malgré le grand degré d'homologie entre le GmDNV et le MlDNV, soit de 90 % d'identité de séquence, et leurs mécanismes d'expression similaires, les deux virus sont différents radicalement quant à leur tropisme. Plusieurs études ont démontré l'importance des protéines de la capside de parvovirus en contrôlant le tropisme viral (Gardiner et Tattersall, 1988a; Bloom *et al.*, 1993; Bergeron *et al.*, 1996; Parker et Parrish, 1997; Bloom *et al.*, 1998; Hauck et Xiao, 2003). Par contre, un nombre limité d'études ont soulevé l'importance des facteurs intracellulaires dans le contrôle de la permissivité des cellules pour l'infection virale (Ridpath et Mengeling, 1988; Oraveerakul *et al.*, 1992; Itah *et al.*, 2004).

Nos résultats sur le tropisme de MlDNV et GmDNV ont démontré que le promoteur viral de VP joue un rôle majeur dans le contrôle du spectre d'hôtes. Le promoteur de VP de GmDNV a activement conduit l'expression du gène rapporteur *CAT* dans les cellules permissives LD652. La même construction s'est avérée non efficace

dans les cellules non permissives, T-ni03. D'un autre côté, le promoteur de VP de MlDNV est actif dans les deux types cellulaires, tel qu'il a été démontré par l'expression du gène rapporteur. Des différences majeures ont été observées dans les séquences promotrices entre les deux virus. La majorité de ces différences sont regroupées autour de la boîte TATA.

Ces résultats confirment que le tropisme de GmDNV et MlDNV est contrôlé par le promoteur du gène de VP dans le système cellulaire LD652/T-ni03. Cependant, ceci n'exclut pas la possibilité que d'autres facteurs soient impliqués dans un autre système, soit *in vivo* ou *in vitro*.

L'étude de la sécurité des densovirus chez les cellules de mammifères a montré que ni le MlDNV, ni le GmDNV ont été capables d'infecter, se répliquer ou même de transcrire leurs gènes dans ce type de cellules. Cette étude a été effectuée par l'infection, mais aussi par la transfection, pour dépasser des barrières possibles de l'entrée virale chez les cellules de mammifères. Nos résultats sur le tropisme ont démontré que le spectre d'hôtes des densovirus est contrôlé par des interactions très spécifiques entre les promoteurs viraux et les protéines cellulaires. D'autre part, plusieurs études ont démontré des différences significatives entre la machinerie de transcription des vertébrés et des invertébrés (Courey et Tjian, 1988; Burke et Kadonaga, 1996; Kutach et Kadonaga, 2000). La forte dépendance de l'expression des densovirus à l'environnement intracellulaire ainsi que la différence entre les accessoires d'expression de cellules de vertébrés et d'invertébrés, pourraient, peut-être, expliquer l'inefficacité des densovirus chez les cellules de mammifères. Collectivement, nos résultats contredisent les rapports de Kurstak *et al.* (1969a et b) et suggèrent que le processus d'homologation nécessite des tests *in vivo* sur l'innocuité des densovirus. Ces tests doivent être réalisés avant de procéder à l'application des densovirus dans les champs.