BACULOVIRUS DNA REPLICATION

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BACULOVIRUS DNA REPLICATION

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Stellingen

1. Het passage-effekt bij seriële passage van baculovirussen in insektecelcultuur wordt veroorzaakt door interferentie van defekte virusdeeltjes met de replicatie van intakte virusdeeltjes.

   Dit proefschrift.

2. Herpesvirussen zijn mogelijk ontstaan uit baculovirussen.

   Dit proefschrift.

3. Interactie van het humaan cytomegalovirus (HCMV) DNA polymerase met het *Autographa californica* kernpolyedervirus LEF-2 eiwit kan de oorzaak zijn van de overeenkomstige activiteit van dit polymerase in met recombinant-baculovirus geïnfecteerde insektecellen en in met HCMV geïnfecteerde cellen.

   Dit proefschrift.
   Ertl, P.F., and Powell, K.L. (1992). *J. Virol.* **66**, 4126-4133.

4. De conclusie van Maeda *et al.* dat recombinatie van een 572 bp fragment vanuit het helicasegen van *Bombyx mori* kernpolyedervirus naar het helicasegen van *Autographa californica* kernpolyedervirus het gastheerbereik van laatstgenoemd virus uitbreidt is onjuist.

   Maeda, S. *et al.* (1993). *J. Virol.* **67**, 6234-6238.

5. De veronderstelling van DeAngelis *et al.* dat de voedingskwaliteit van met *Impatiens* vlekkenvirus geïnfecteerd bladmateriaal geen invloed heeft op de ontwikkeling en reproduktie van de trips *Frankliniella occidentalis* is niet gerechtvaardigd.

   DeAngelis, J.D. *et al.* (1993). *Environ. Entomol.* **22**, 1308-1312.

6. Het veelvoud aan methodes en computerprogramma's voor het vergelijken van aminozuurvolgorden maakt het vinden van geringe homologie tussen eiwitten niet eenvoudiger.

7. Het gegeven dat vrouwen beter zijn in het onthouden van verjaardagen dan mannen wordt verklaard door het feit dat verjaardagskalenders meestal aan de binnenzijde van de toiletdeur worden opgehangen.

8. De gewoonte om in Nederland een bioscoopfilm te onderbreken voor een pauze verhoogt niet het kijkgenot maar wel de omzet van de bioscoop.

9. Het feit dat rokers meer en meer gedwongen worden om tijdens pauzes buiten te roken komt het ziekteverzuim onder rokers niet ten goede.

10. Wetenschappers moeten eens wat vaker hun nek durven uit te steken.

11. Bij verkiezingen wordt bij het presenteren van uitkomsten van opiniepeilingen te weinig ingegaan op de onzekerheden en marges van deze peilingen.

12. Soms overtreft de biologie de wiskunde, omdat in de biologie vermenigvuldigen ook een vorm van delen is.

Stellingen behorend bij het proefschrift:

Baculovirus DNA replication

Wageningen, 17 juni 1994 Marcel Kool

VOORWOORD

Zondagmiddag 24 april 1994. Het proefschrift is bijna af. Alleen het voorwoord nog. En inderdaad, dat blijkt niet het makkelijkste deel. Zittend achter mijn PC kijk ik terug op de afgelopen vijf jaar. Jaren van wisselend succes. De eerste jaren zeer moeizaam, de laatste jaren zeer succesvol. Wie had toen kunnen denken dat het proefschrift er uiteindelijk zo uit zou zien. Ik zeker niet. Velen zullen zich nog wel weten te herinneren dat toen na 2-3 jaar het onderzoek volledig vast zat, of ik het om andere redenen niet meer zag zitten, ik wel eens gedacht heb om er maar mee te stoppen. Maar door de voortdurende steun van familie en goede vrienden, die ik daar bij deze voor wil bedanken, en met name Henk Schaap en Frank van Poelwijk, heb ik gelukkig toch doorgezet en zie hier het resultaat.

Maar zo'n proefschrift schrijf je niet alleen. Allereerst wil ik Just Vlak en Rob Goldbach bedanken voor hun begeleiding en kritische houding tijdens dit onderzoek, tot de laatste komma aan toe. Verder wil ik alle baculovirologen bedanken voor hun bijdrage, de zeer gezellige samenwerking en de leuke tijd in en buiten het lab: John Martens, Monique van Oers, Bep van Strien, Rene Broer, Douwe Zuidema, Magda Usmany, Els Klinge-Roode, Hans Flipsen, Jacco Heldens, Ruud Mans, Peter Roelvink, Jan Roosien, Rose Zhi-Hong, Peter Kulczar, Primitivo Caballero, Miguel Medina, Fons Feldmann, Basil Arif en Jordi Cairo. Verder een woord van dank aan de (ex)-studenten Paul Koetsier, Pieter van den Berg, Petra van den Berg en Theo Voeten, voor hun niet geringe bijdrage aan dit onderzoek.

Daarnaast wil ik bedanken Jan van Lent en Joop Groenewegen van de EM-afdeling, Wout Rozeboom voor al die keren dat ik, tegen de regels in, toch maar weer mijn chemicaliën kreeg als ik weer eens "buiten kantoortijd" kwam, en de fotolocatie van de Binnenhaven.

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Tot slot wil ik naast alle bovengenoemde personen ook alle niet met name genoemde (ex)-medewerkers en (ex)-studenten van de Vakgroep Virologie bedanken voor hun bijdrage aan het feit dat ik met veel plezier terug kijk op deze afgelopen vijf jaar.

CONTENTS

CHAPTER 1

INTRODUCTION

About 15% of the losses in global agricultural production is caused by insects. Insect control usually includes the use of chemical insecticides. However, the rapid build up of resistance and the hazards associated with the introduction of insecticides in the environment have led to a continuous search for alternative strategies of insect control. One of the alternatives is the use of insect pathogens, such as baculoviruses, which cause a chronic or fatal disease in insects (WHO Report, 1973; Summers *et al.*, 1975).

Baculoviruses are found almost exclusively among insects and are usually specific for only a single or a few related insect species, mainly of the orders *Lepidoptera*, *Hymenoptera*, and *Diptera* (Martignoni and Iwai, 1986; Adams and McClintock, 1991). They are neither a threat to human health nor to the environment. Despite their long-time use and presence in the environment, not even a single casualty has been recorded giving these pathogens a perfect safety record. Insects develop no resistance against baculoviruses and they lack an immune system similar to that found in vertebrates. Baculoviruses are an integral part of the ecosystem and play an important role in the regulation of the size of insect populations. As such, baculoviruses are attractive biological agents for inoculative or innundative insect pest control (Granados and Federici, 1986).

There are, however, some limitations to the widespread application of baculoviruses as insect control agents. Firstly, they have a relative slow speed of action. Upon infection, insects stop feeding only after a few days, whereas an immediate insecticidal effect may often be required. Secondly, the specificity of these viruses, which is an asset from the environmental point of view, is not an attractive property for commercial development where

broad-spectrum agents are desired. Thirdly, the production of these viruses at large scale requires a variety of insect cultures, which often leads to a cumbersome, labour-intensive and uneconomical production process with limited scale up potential. Biotechnological approaches offer opportunities to eliminate some of these drawbacks, notably by increasing the speed of action of baculoviruses and by their production in insect-cell cultures as opposed to insects. In this thesis the initial emphasis is on the production of baculoviruses in large-scale cell cultures.

The first interest in baculoviruses was confined to their use in insect control. Several baculoviruses are (semi-)commercially used as bio-insecticides. Especially in forestry and horticulture, the use of baculoviruses is receiving much attention. Recently, a baculovirus of *Spodoptera exigua* has been registered for use in greenhouses in the Netherlands. A bottleneck in many applications is the cost-effective production of large quantities of virus. Production usually occurs in insect larvae (Shieh and Bohmfalk, 1980). On a large scale, production in insect-cell cultures may be a viable alternative when some difficulties with this system have been overcome (Tramper and Vlak, 1986).

When recombinant-DNA technology became available (1973), expectations were high regarding to large-scale protein production using genetically modified bacteria. The majority of commercially attractive proteins, however, is from higher animals and it was soon realized that bacteria have limitations in their capacity to express eukaryotic DNA into (near) authentic, biologically-active proteins. Lower eukaryotic organisms such as yeast and fungi lack the ability to adequately perform many of the post-translational modifications which are necessary to produce biologically-active proteins (Vlak and Keus, 1990). Hence animal cells are now more frequently used as substrates for the production of recombinant proteins.

Among animal cells, insect cells are of eminent importance for the production of recombinant proteins. A convenient and versatile viral expression vector system has been developed (reviewed in Luckow and Summers, 1988; Miller, 1989; Luckow, 1991). It is this application in particular which resulted in a renewed interest in baculoviruses since 1983. Expression of foreign genes via a baculovirus vector is usually high compared to recombinant protein expression in other systems (Vlak and Keus, 1990). Post-translational modification of proteins in insect cells is comparable to processing of proteins in mammalian cells, except for glycosylation (Kuroda *et al.*, 1989). Insect cell lines are easy to maintain: the cells grow at ambient temperatures and equally well in suspension as on surfaces, they are relatively

insensitive to environmental changes and they do not support growth of vertebrate viruses (Agathos *et al.*, 1990). A major impact on the field was the FDA approval to MicroGenSys Inc. to test a baculovirus-expressed gp160 envelope protein of human immunodeficiency virus type 1 as a possible AIDS vaccine in clinical trials (Van Brunt, 1987). In the meantime many baculovirus-produced recombinant proteins are being developed as vaccines or diagnostics for use in human and veterinary medicine.

Until now large-scale production of baculoviruses and baculovirus-expressed proteins in insect cells is limited to bioreactors up to about 10 dm^3 in repeated batch operations. For scale up, suspension cultures of insect cells offer the best possibility. Operational costs of recombinant protein production favour the use of continuous production systems. A fully continuous production process involving cell growth and baculovirus production has been designed and employed by Kompier *et al.* (1988). Two head-space-aerated stirred reactors with a working volume of 0.8 dm^3 were connected in series. Medium was continuously fed to the first reactor where *Spodoptera frugiperda* insect cells were cultured. The outlet of this reactor was connected to the second reactor where the infection of the cells took place. Virus c.q. protein production was maintained for about four weeks; then virus production dropped sharply (Kompier *et al.*, 1988; Van Lier *et al.*, 1992). A recombinant baculovirus expressing ß-galactosidase was used to describe (Van Lier, 1994) and model (De Gooijer, 1994) this production process in this reactor system.

The decrease of virus production in cell culture was provisionally ascribed to a phenomenon known as the passage effect (Tramper and Vlak, 1986). When virus is propagated *in vitro* and serially passaged, the infectivity decreases as evidenced by the production of fewer virus particles and polyhedra (FP phenotype) per cell and an increasing number of polyhedra with abnormal morphology (MacKinnon *et al.*, 1974; Potter *et al.*, 1976; Fraser and Hink, 1982). For *S. frugiperda* cells infected with *Autographa californica* multi-nucleocapsid nuclear polyhedrosis virus (AcMNPV) a decrease of extracellular virus was observed at the ninth passage (De Gooijer *et al.*, 1992). Faulkner (1981) concluded that the effects of prolonged serial passage become significant after 10 passages and severe after 25 passages.

The present study aimed at the analysis of baculoviruses produced in continuous bioractor systems, in particular towards the replication of the viral DNA. To assist in the interpretation of the data in this thesis first an overview of the structural and functional organization of

AcMNPV, the baculovirus which was used in this study, is given in Chapter 2. The analysis of virus obtained from continuous bioreactor systems showed that during the production in time deletion mutants became dominant and that the replication of these mutants appeared to be dependent on the presence of intact helper virus (Chapter 3). The nucleotide sequence of a 7.3 kilobase pair region of the AcMNPV genome, frequently involved in recombination in the deletion mutants, is presented in Chapter 4. Further analysis of the so-called defective interfering particles (DIPs) is described in Chapter 5. Extensive serial passaging of AcMNPV in insect cell culture resulted in the generation of DIPs, which retained only 5 percent of the original viral genome. This implied that *cis*-acting elements involved in DNA replication such as origins (*ori's*) must have been maintained in these DIPs. A replication assay was developed to locate these *cis*-acting elements, involved in DNA replication, in the conserved sequences. After the initial characterization of two *ori's* (Chapter 5), a more extensive search led up to the identification of five other *ori's* (Chapter 6), one of which was analyzed in some detail (Chapter 7). *Trans*-acting factors, involved in DNA replication, were identified using a transient complementation assay and one of the identified origins of DNA replication (Chapter 8 and 9). Finally, based on comparisons of proteins involved in DNA replication, the possible evolutionary relation between *Baculoviridae* and *Herpesviridae* is discussed (Chapter 10).

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CHAPTER 2

THE STRUCTURAL AND FUNCTIONAL ORGANIZATION OF THE *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS GENOME: AN OVERVIEW

SUMMARY

Baculoviruses are used as biological control agents of insect pests in agriculture and forestry. The multiple-nucleocapsid nuclear polyhedrosis virus of *Autographa californica* (AcMNPV) is the prototype baculovirus (Francki *et al.*, 1991). Recently, this virus has been widely used as vector for the high-level expression of foreign genes in insect cells. An updated physical map of restriction sites as well as the location of open reading frames (ORFs) and transcripts are presented. Most characteristic is the dispersal of "early", "late" and "very late" genes over the genome and the presence of nested sets of 3' and 5' coterminal transcripts.

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INTRODUCTION

The *Baculoviridae* are a family of occluded DNA viruses representatives of which are mainly encountered in insects (Granados and Federici, 1986). The interest in baculoviruses has been fostered for many years by their ability to control insect pests in agriculture and forestry. Recently, baculoviruses have been developed into vectors for the high level expression of heterologous proteins in insect cells. This so-called baculovirus expression system is capable of producing large amounts of recombinant proteins, which are similar if not identical to the authentic proteins and which are now extensively used in human and animal medicine, as diagnostic, therapeutic or prophylactic agents (vaccines). This development, in turn, has fostered the engineering of baculoviruses with improved insecticidal properties for insect control.

The development of the baculovirus expression vector system is the result of a detailed understanding of baculovirus gene regulation and has led, in turn, to an increased interest in the molecular genetics of baculoviruses in general, the nuclear polyhedrosis virus (NPV) of *Autographa californica* in particular. In this paper we give an updated account on the location of genes and transcripts of this prototype baculovirus, the multiple-nucleocapsid NPV of *A. californica* (AcMNPV).

STATE OF THE ART

More than four hundred baculoviruses have been reported in the literature, but less than twenty have been studied in some detail at the molecular level. AcMNPV is the most intensively studied baculovirus and most insight in gene structure and regulation has been derived from this virus. Limited information is available from other baculoviruses, such as the MNPVs of *Orgyia pseudotsugata*, *Lymantria dispar*, and *Spodoptera exigua*.

Many reviews have been written on the general biology (Granados and Federici, 1986), molecular biology (Doerfler and Bohm, 1986; Blissard and Rohrmann, 1990) and structural proteins (Rohrmann, 1986; Rohrmann, 1992) of baculoviruses. In addition, excellent accounts on the baculovirus expression vector system *in vitro* (Luckow and Summers, 1988; Miller, 1988) as well as *in vivo* (Maeda, 1989) have been prepared. Manuals are available

to outline the practical details of the system (Summers and Smith, 1987; O'Reilly *et al.*, 1992; King and Possee, 1992).

Many open-reading frames (ORFs) have been found dispersed over the AcMNPV genome and many transcripts have been mapped. Several proteins expressed by AcMNPV have been functionally characterized. However, a comprehensive physical map of restriction sites, ORFs, genes and transcripts, as well as of insertions, deletions and alterations in AcMNPV variants is lacking. Such a map would assist in the understanding of baculovirus gene regulation and would be very helpful in the design of new engineering strategies of this virus as expression vector as well as insect control agent with improved insecticidal characteristics.

PHYSICAL MAP

AcMNPV contains a double-stranded, circular DNA genome of approximately 131 kbp in size (Vlak and Odink, 1979). Sequences from about 70% of the genome could be retrieved from the literature and data banks, and the entire sequence is being assembled (R.D. Possee, personal communication). AcMNPV strain E2 is the baculovirus type strain (Francki *et al.*, 1991). Strains L1 and C6 differ from E2 by the presence of an additional *Hin*dIII site in the *Hin*dIII fragment A, whereas strains C6 and HR3 differ from L1 and E2 by a small insertion in fragment *Hin*dIII-C. There is no evidence that these strains are biologically different. Hence, the physical map as well as other data on the genetic organization are taken from the literature involving the above strains (Fig. 2.1).

The AcMNPV genome is presented, sectioned into seven segments of approximately 20 kilobase pairs (kbp). The map includes data, which are published, submitted or in press in scientific journals or which are deposited in data banks (EMBL, GENBANK). Sequences are not included in this paper, but the regions of the AcMNPV genome which are sequenced are indicated. Since the whole sequence of the AcMNPV genome is not yet available, the map of the restriction sites should be considered approximate. The genome orientation and fragment designations were kept in concord with the consensus reached previously on the AcMNPV and baculovirus genome organization (Vlak and Smith, 1982). However, the zero point of the map is now more precisely positioned at the left-most *Eco*RI site of the *hr*1 region between the *Eco*RI-I and *Eco*RI-B fragments. In this way the presented nomenclature

for the *hr* regions, *hr*1 to *hr*5, was maintained (Cochran *et al.*, 1983). These *hr* regions are known to act as enhancers for some early promoters in transient expression assays (Guarino and Summers, 1986; Guarino and Summers, 1987; Nissen and Friesen, 1989). There is also evidence now that the *hr* sequences play a role in viral DNA replication, because plasmids containing these sequences are able to replicate in an AcMNPV infection-dependent replication assay (Pearson *et al.*, 1992; Kool *et al.*, 1993a, b; Leisy and Rohrmann, 1993).

TRANSCRIPTION

Baculovirus gene expression is regulated in a cascaded manner, and divided into three phases (Blissard and Rohrmann, 1990). The early phase (*E*) is defined as the period preceding viral DNA replication. The early phase is subdivided into two functionally defined stages (immediate early and delayed early) involving two categories of genes: immediate early or α genes which can be transcribed by uninfected insect cells and require no viral gene products for their expression, and delayed early or ß genes which require other viral gene products for their transcription. However, evidence is accumulating that genes originally reported as α genes actually behave as ß genes because they are dependent on other viral gene products, and that some genes originally classified as ß genes should be considered as α-genes as they are transcribed at low levels by uninfected cells. Therefore, in accordance with the classification of Blissard and Rohrmann (1990) we designated all the α- and ß-genes as early genes (*E*) in this map. Late (*L*) and very late (*VL*) genes are dependent on early viral gene expression. Transcription of late, and probably also the very late genes occurs as or after viral DNA replication begins. Very late genes, however, are distinguished from other late genes by the fact that mRNAs from most late genes decline at very late times post infection, whereas mRNA levels of very late genes remain high throughout the infection cycle. In figure 2.1 only those transcripts are indicated of which the size, start and stop sites are reported. They are designated as early (*E*), late (*L*) or very late (*VL*).

GENES

Sequences of the AcMNPV genome revealed many open-reading frames (ORFs). In the map presented here (Fig. 2.1), however, only those ORFs are indicated, which are reported in the literature as being an ORF of certain size or as a gene of which it is known that it is transcribed and/or expressed.

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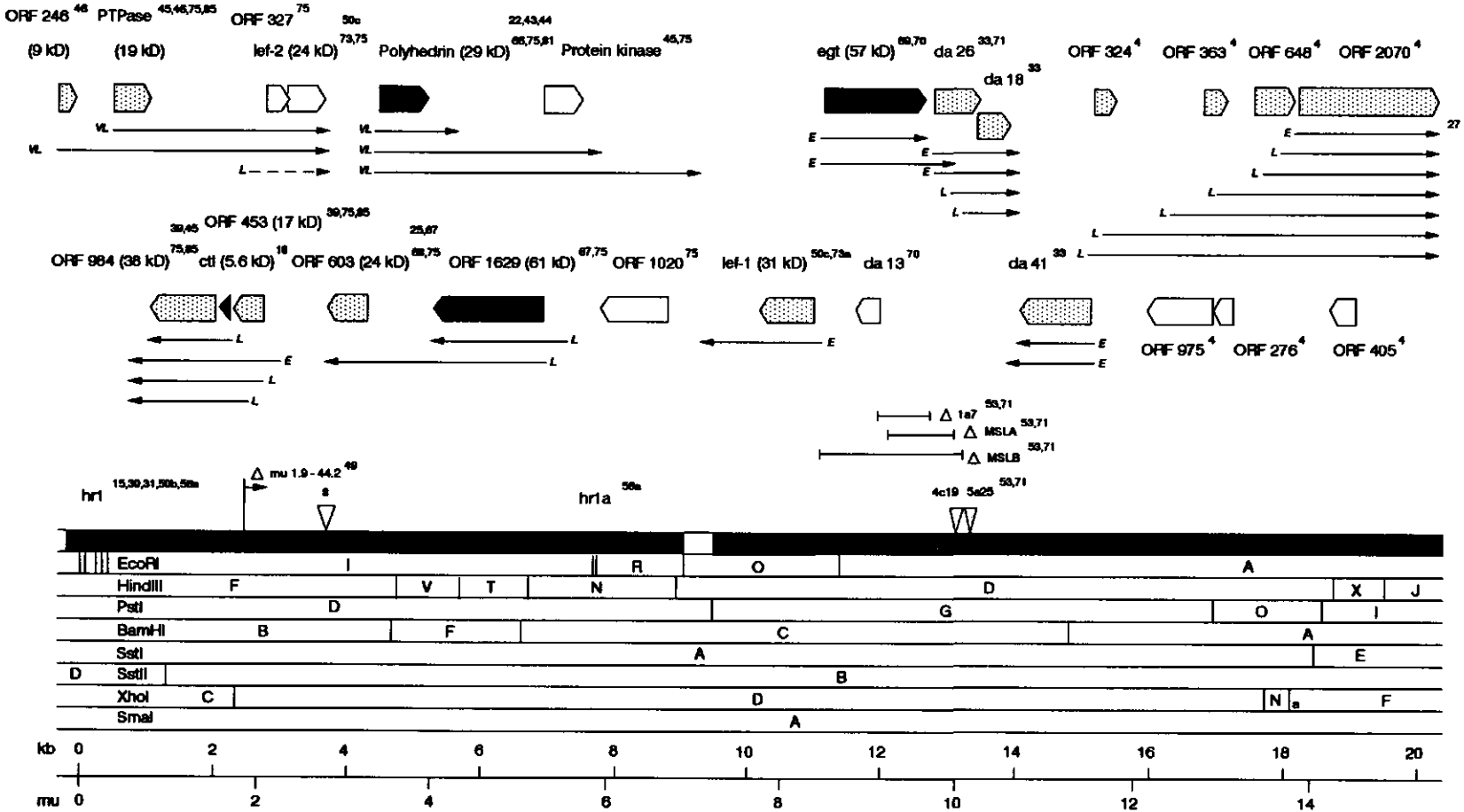
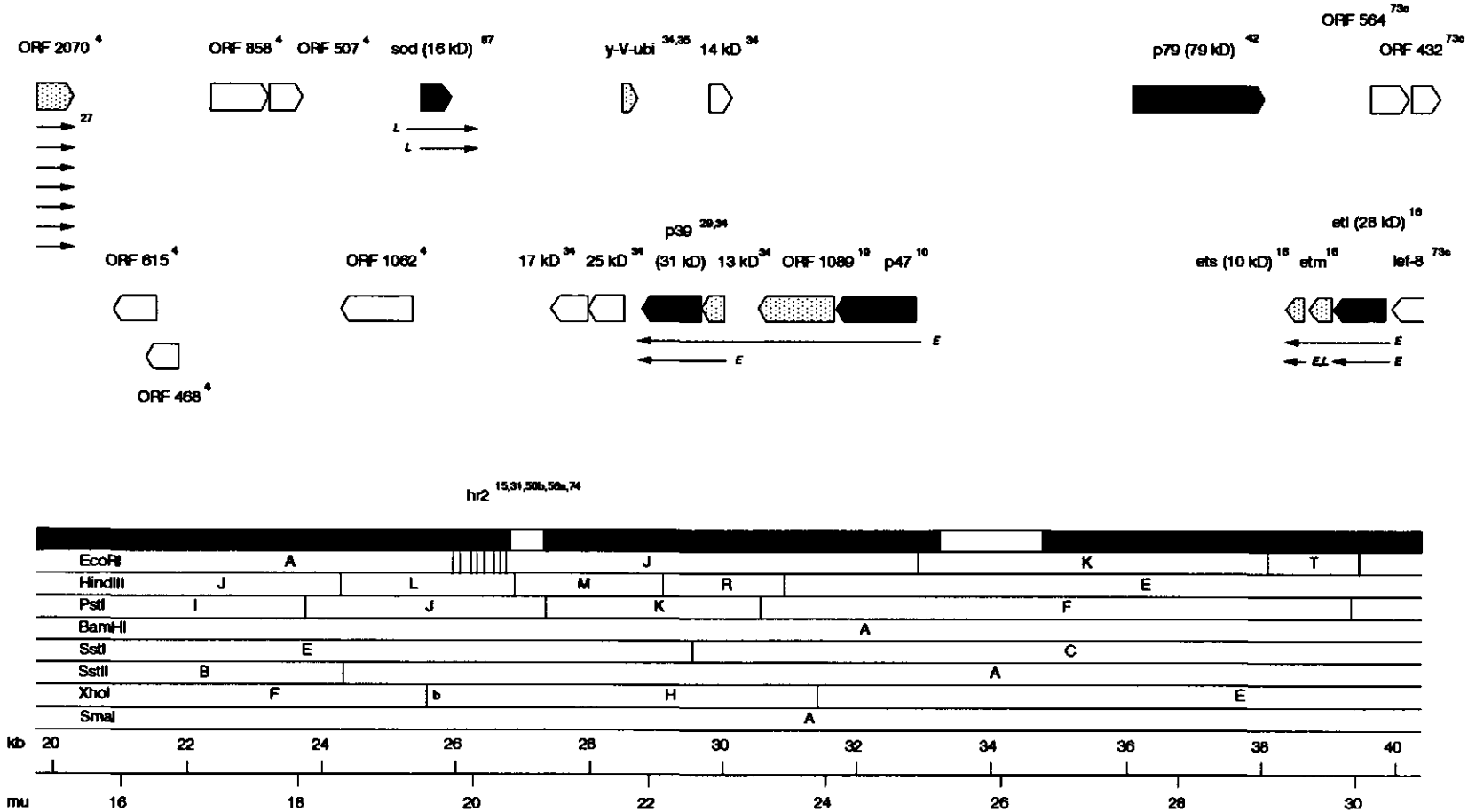
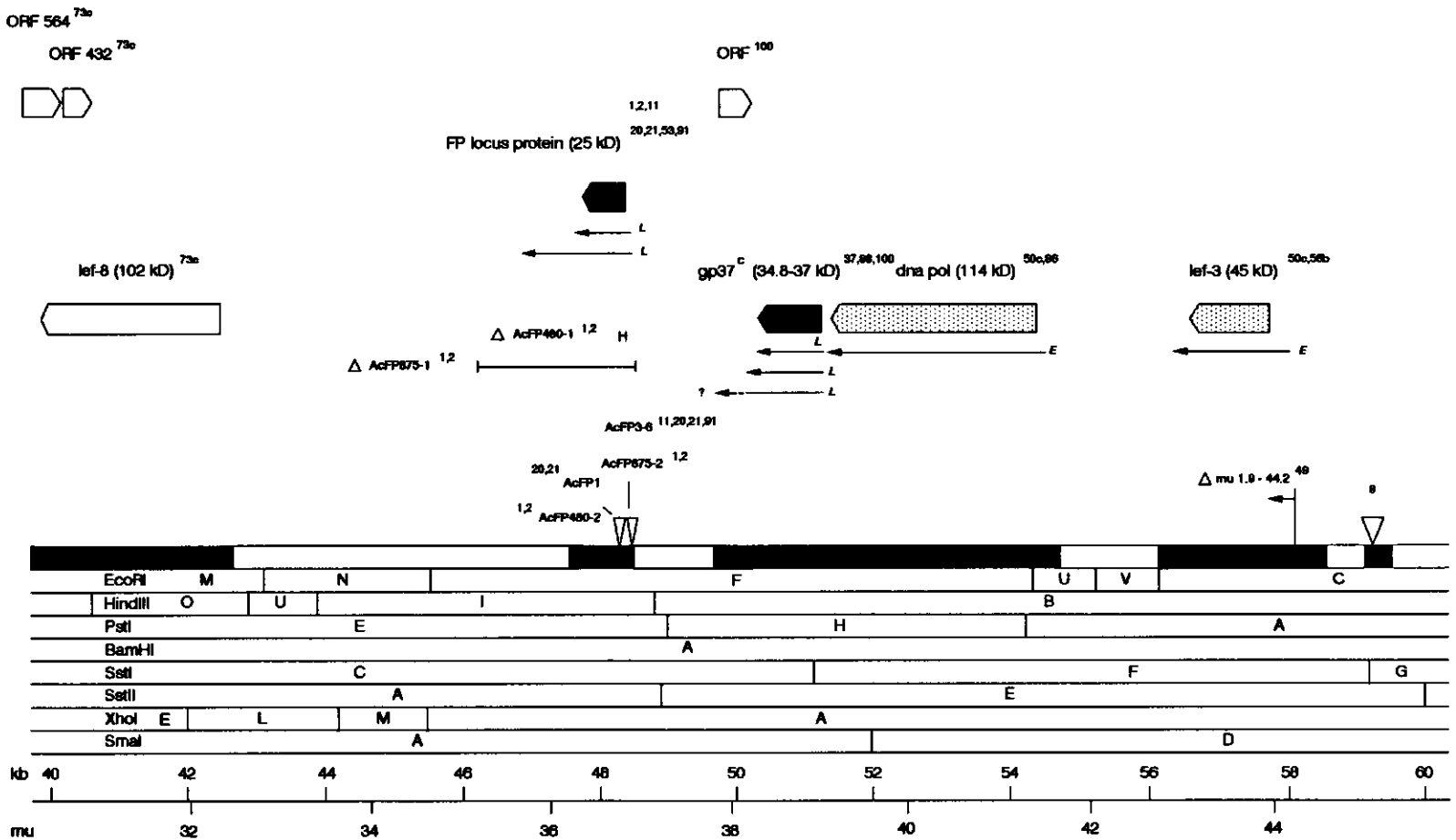
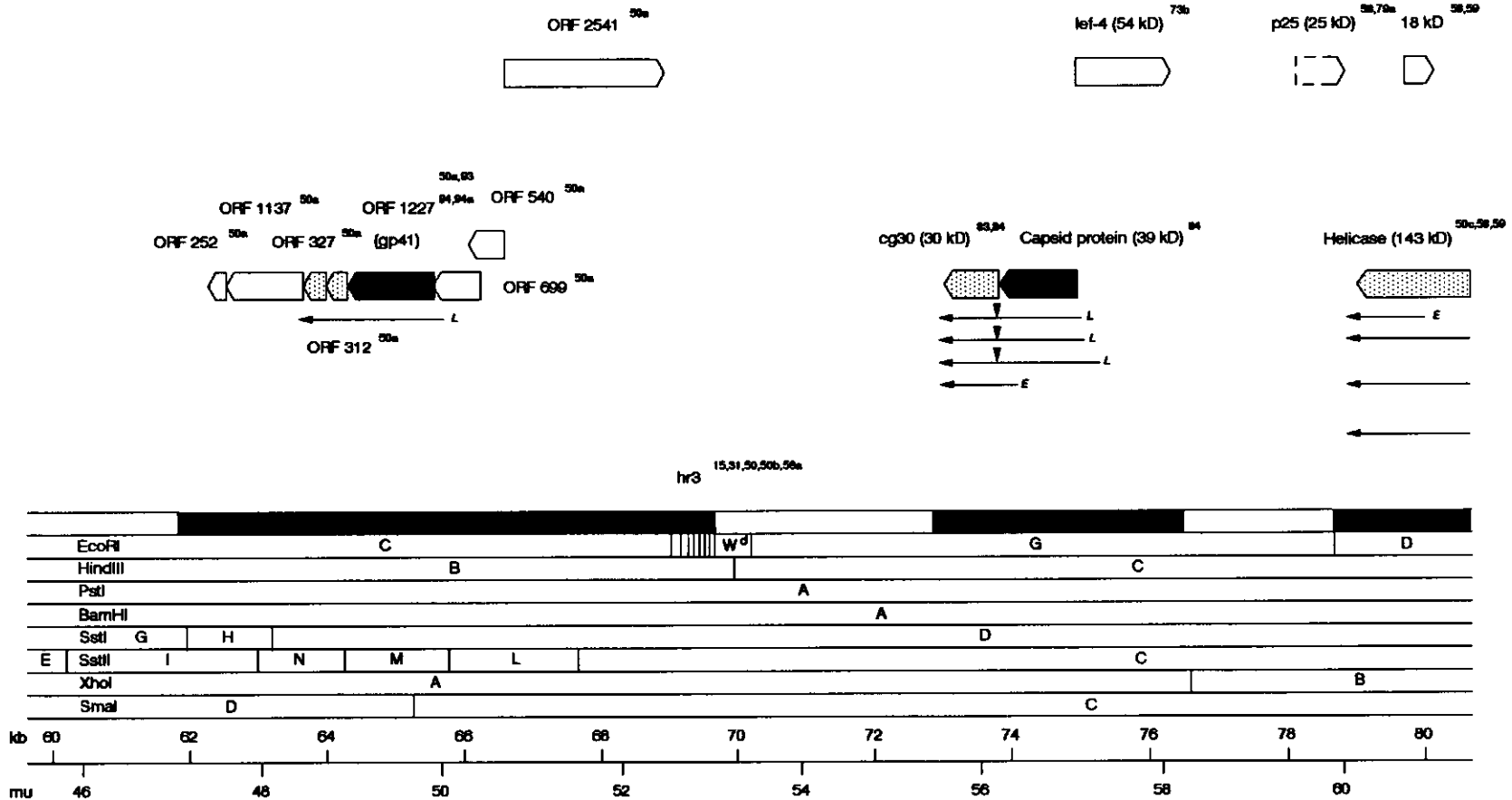
Figure 2.1. Structural and functional map of the AcMNPV genome. The map shows the positions of the cleavage sites of eight different restriction endonucleases (*Eco*RI, *Hin*dIII, *Pst*I, *Bam*HI, *Sst*I, *Sst*II, *Xho*I and *Sma*I) on a linear map of AcMNPV. As zero point the left most *Eco*RI-site of *hr*1 is taken (Guarino *et al.*, 1986). About the location of the fragments *Hin*dIII-A and *Hin*dIII-B there is some confusion in the literature (Cochran *et al.*, 1982; Vlak and Smith, 1982; O'Reilly *et al.*, 1992). In this map we labeled all the restriction fragments according to Cochran *et al.* (1982), which means that the *Hin*dIII-A fragment is located between *Hin*dIII-S and -K, and the *Hin*dIII-B fragment is located between *Hin*dIII-I and -C. A scale in kbp and one in map units (mu) is presented at the bottom. The entire genome is 131 kbp so that 1 mu = 1.31 kbp; 1 cm on the map corresponds to 1 kbp. The regions of the genome that have been sequenced are indicated with black bars. The locations and directions of characterized ORFs, genes, transcripts, deletions and insertions are presented at the top. Transcripts are designated as early (*E*), late (*L*) or very late (*VL*).

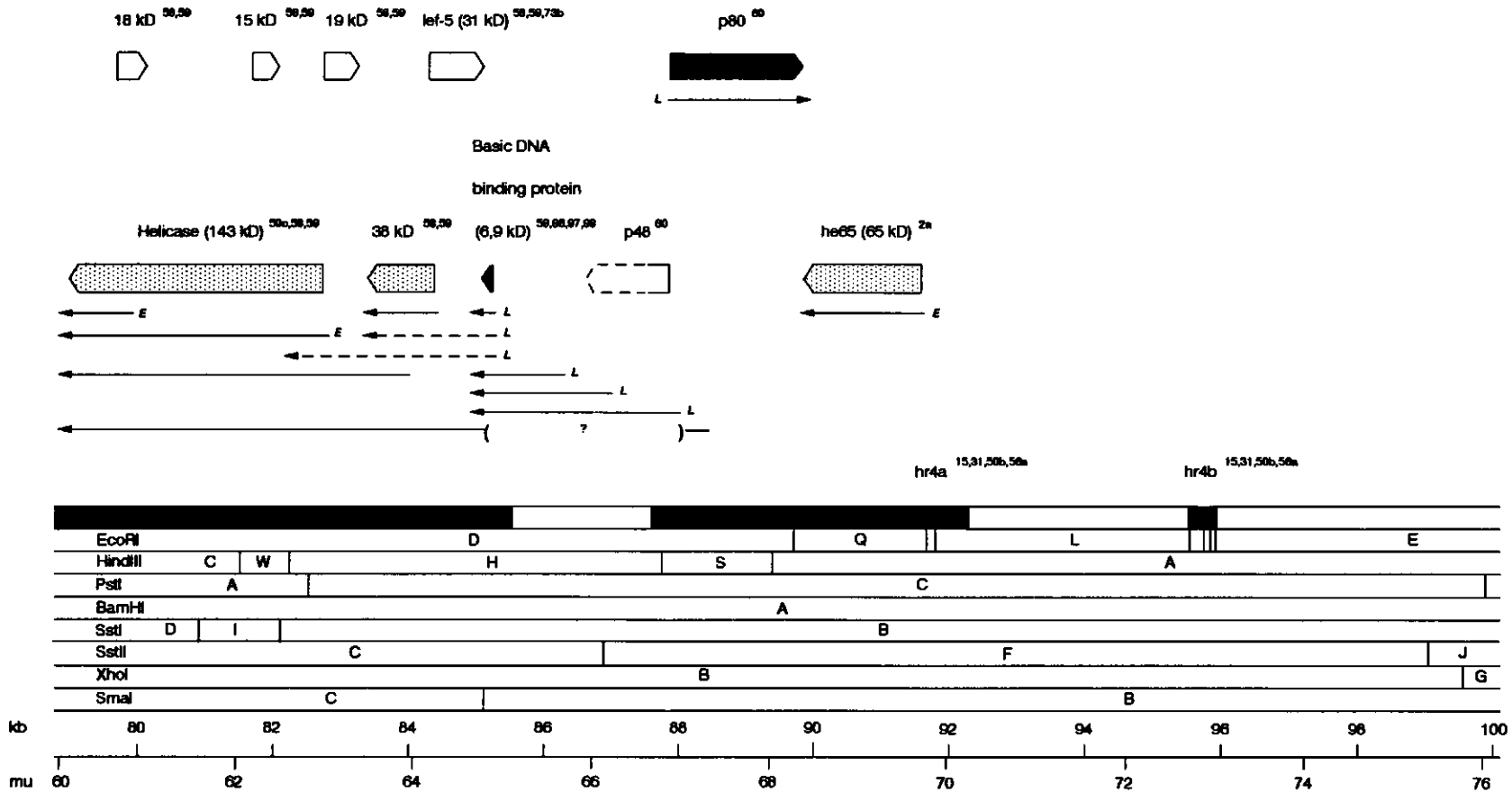
a. Sequencing of *Eco*RI-A by Braunagel and Summers (1992) revealed an extra *Xho*I-site at mu 13.8 creating the *Xho*I-N fragment of 376 bp. The existence of this fragment was already reported by Cochran *et al.* (1982), but the location was until now not clear.
b. The sequence of the *hr*2 region (Guarino *et al.*, 1986) revealed another extra *Xho*I site at mu 20, creating a new *Xho*I fragment of 769 bp. However, restriction analysis of AcMNPV-E2 with *Xho*I did not show this fragment, and it is therefore not marked on this map.
c. The *gp37* gene (mu 38-39) was formerly known as SLP (Wu and Miller, 1989; Vialard *et al.*, 1990).
d. Cochran *et al.* (1982) reported the location of the *Eco*RI-W fragment between *Eco*RI-G and *Eco*RI-D. However, it is now known that the *Eco*RI-W fragment is located at the other site of *Eco*RI-G, next to the *hr*3 region.
e. For *gp64* late transcripts were also found, but the exact mapping of these transcripts is not reported in the literature (J. Kuzio, personal communication).

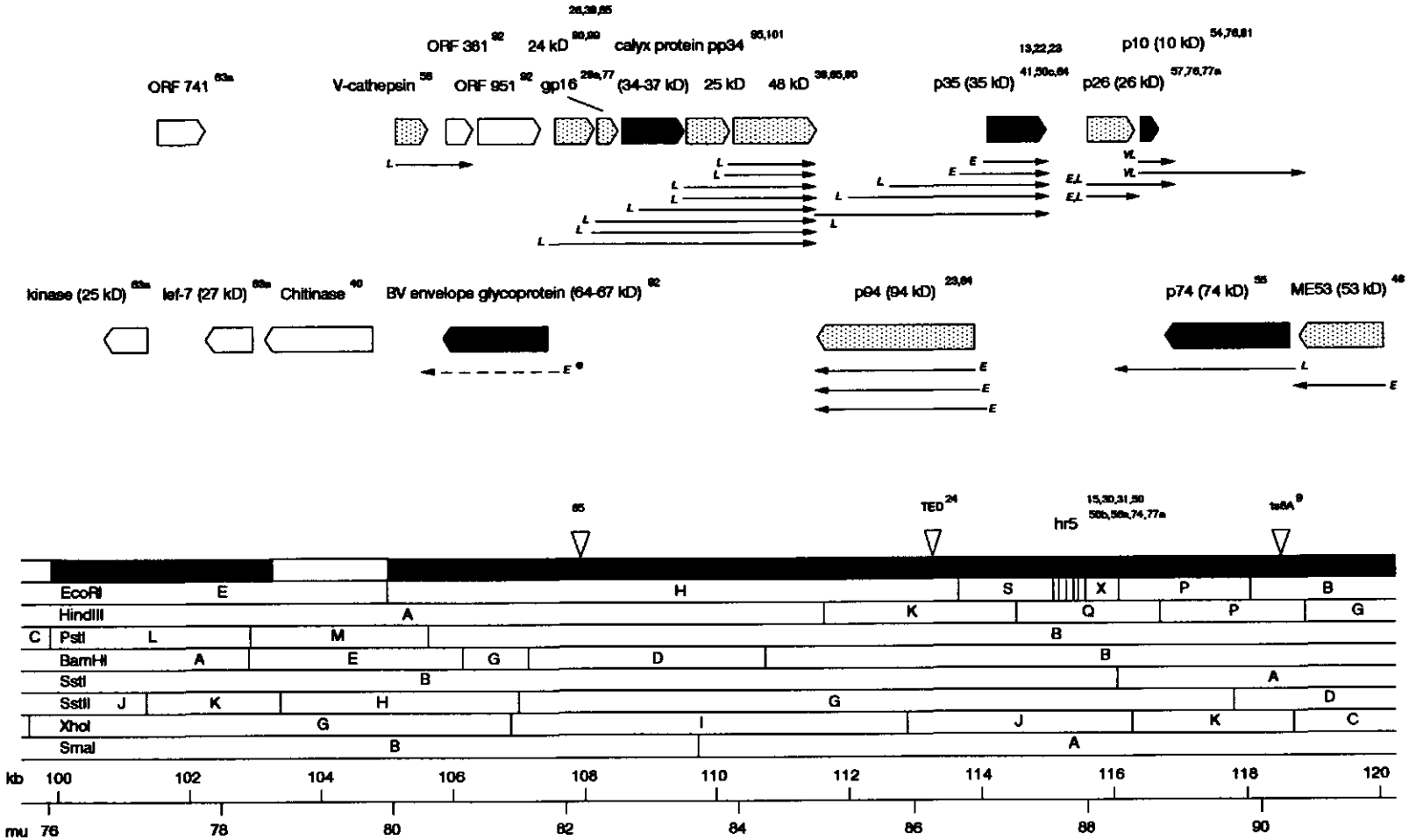

ORF 246 [46]
PTPase [45,46,75,85]
ORF 327 [75]
(9 kD)
(19 kD)
lef-2 (24 kD) [50c,73,75]
Polyhedrin (29 kD) [22,43,44,68,75,81]
Protein kinase [45,75]
egt (57 kD) [69,70]
da 26 [33,71]
da 18 [33]
ORF 324 [4]
ORF 363 [4]
ORF 648 [4]
ORF 2070 [4]
ORF 453 (17 kD) [39,75,85]
ORF 984 (38 kD) [39,45,75,85]
ctl (5.6 kD) [18]
ORF 603 (24 kD) [25,67,68,75]
ORF 1629 (61 kD) [67,75]
ORF 1020 [75]
lef-1 (31 kD) [50c,73a]
da 13 [70]
da 41 [33]
ORF 975 [4]
ORF 276 [4]
ORF 405 [4]
Δ 1a7 [53,71]
Δ MSLA [53,71]
Δ MSLB [53,71]
hr1 [15,30,31,50b,58a]
Δ mu 1.9 - 44.2 [49]
8
hr1a [58a]
4c19
5a25 [53,71]
EcoRI
HindIII
PstI
BamHI
SstI
SstII
XhoI
SmaI
kb
mu

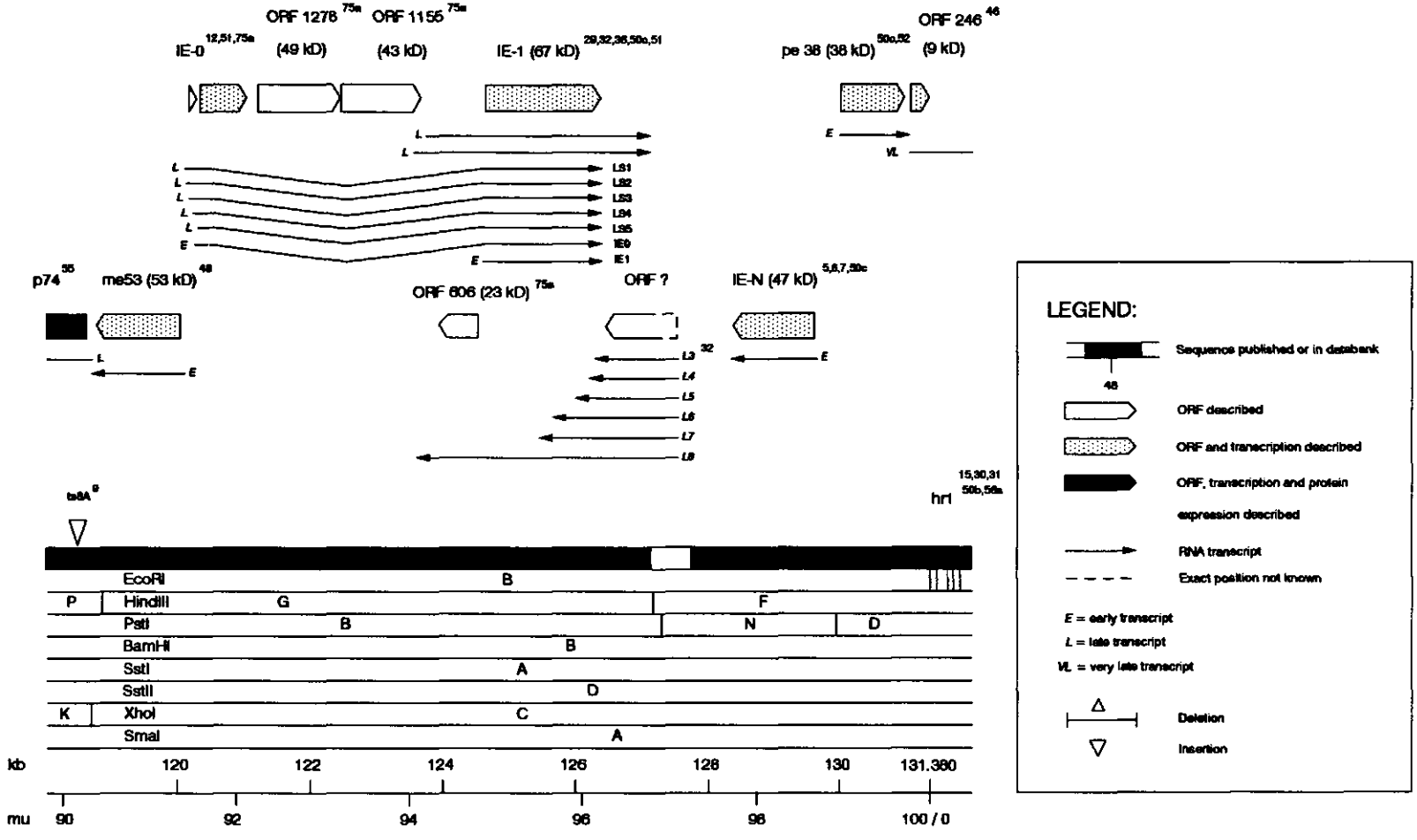

ORF 2070 4
ORF 858 4
ORF 507 4
sod (16 kD) 67
y-V-ubi 34,35
14 kD 34
p79 (79 kD) 42
ORF 564 73c
ORF 432 73c
27
L
L
ORF 615 4
ORF 1062 4
p39 29,34
17 kD 34
25 kD 34
(31 kD)
13 kD 34
ORF 1089 10
p47 10
etl (28 kD) 16
ets (10 kD) 16
etm 16
lef-8 73c
E
E
E
E L
E
ORF 468 4
hr2 15,31,50b,56a,74
EcoRI
HindIII
PstI
BamHI
SstI
SstII
XhoI
SmaI
A
J
K
T
J
L
M
R
E
I
J
K
F
A
E
C
B
A
F
b
H
E
A
kb
20
22
24
26
28
30
32
34
36
38
40
mu
16
18
20
22
24
26
28
30


ORF 564 73a
ORF 432 73a
ORF 100
1,2,11
20,21,53,91
FP locus protein (25 kD)
L
L
lef-8 (102 kD) 73a
gp37 C (34.8-37 kD) 37,88,100
dna pol (114 kD) 50c,86
lef-3 (45 kD) 50c,58b
Δ AcFP460-1 1,2
H
Δ AcFP675-1 1,2
L
E
L
?
L
E
AcFP3-6 11,20,21,91
20,21
AcFP675-2 1,2
AcFP1
1,2
AcFP460-2
Δ mu 1.9 - 44.2 49
8
EcoRI M N F U V C
HindIII O U I B
PstI E H A
BamHI A
SstI C F G
SstII A E
XhoI E L M A
SmaI A D
kb 40 42 44 46 48 50 52 54 56 58 60
mu 32 34 36 38 40 42 44


ORF 2541 [50a]
lef-4 (54 kD) [73b]
p25 (25 kD) [58,79a]
18 kD [58,59]
ORF 1137 [50a]
ORF 1227 [50a,83] [84,84a]
ORF 540 [50a]
ORF 252 [50a]
ORF 327 [50a]
(gp41)
ORF 699 [50a]
ORF 312 [50a]
cg30 (30 kD) [83,84]
Capsid protein (39 kD) [84]
Helicase (143 kD) [50c,58,59]
hr3 [15,31,50,50b,56a]
EcoRI
HindIII
PstI
BamHI
SstI
SstII
XhoI
SmaI
kb
mu


18 kD [58,59]
15 kD [58,59]
19 kD [58,59]
lef-5 (31 kD) [58,59,73b]
p80 [60]
L
Basic DNA
binding protein
Helicase (143 kD) [50a,58,59]
38 kD [58,59]
(6,9 kD) [58,68,97,98]
p48 [60]
he65 (65 kD) [2a]
E
L
?
hr4a [15,31,50b,58a]
hr4b [15,31,50b,58a]
EcoRI
HindIII
PstI
BamHI
SstI
SstII
XhoI
SmaI
kb
mu


ORF 741 63a
V-cathepsin 56
ORF 951 92
gp16 28a,77
(34-37 kD)
25 kD
48 kD 38,65,80
ORF 361 92
24 kD 80,89
calyx protein pp34 95,101
p35 (35 kD) 41,50c,84
p26 (26 kD) 57,76,77a
p10 (10 kD) 54,76,81
13,22,23
kinase (25 kD) 63a
lef-7 (27 kD) 63a
Chitinase 40
BV envelope glycoprotein (64-67 kD) 92
p94 (94 kD) 23,84
p74 (74 kD) 55
ME53 (53 kD) 48
65
TED 24
hr5
15,30,31,50
50b,56a,74,77a
ie0A 9
EcoRI
HindIII
PstI
BamHI
SstI
SstII
XhoI
SmaI
kb
mu


ORF 1278 75a
ORF 1155 75a
ORF 246 46
IE-0 12,51,75a
(49 kD)
(43 kD)
IE-1 (67 kD) 29,32,36,50a,51
pe 38 (38 kD) 50a,52
(9 kD)
L
L
E
VL
LS1
LS2
LS3
LS4
LS5
IE0
IE1
p74 35
me53 (53 kD) 48
ORF 606 (23 kD) 75a
ORF ?
IE-N (47 kD) 5,6,7,50c
L3 32
L4
L5
L6
L7
L8
ts8A 9
hr1 15,30,31 50b,56a
EcoRI
HindIII
PstI
BamHI
SstI
SstII
XhoI
SmaI
P
K
A
B
C
D
F
N
kb
120
122
124
126
128
130
131.380
mu
90
92
94
96
98
100 / 0
LEGEND:
Sequence published or in databank
ORF described
ORF and transcription described
ORF, transcription and protein expression described
RNA transcript
Exact position not known
E = early transcript
L = late transcript
VL = very late transcript
Deletion
Insertion

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CHAPTER 3

DETECTION AND ANALYSIS OF *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS MUTANTS WITH DEFECTIVE INTERFERING PROPERTIES

SUMMARY

Defective interfering particles (DIPs) were generated upon continuous production of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) in bioreactors. This configuration mimicked the serial undiluted passaging of virus, which is known to result in plaque-morphology mutants. Restriction enzyme analysis of DIP-containing preparations of extracellular virus showed the presence of many DNA fragments in less-than-equimolar amounts. These fragments were colinear on the physical map of AcMNPV and extended from map position 1.7 to 45. These DIPs thus lacked 43% of the genetic information of the standard virus, including the polyhedrin and DNA polymerase genes. The existence of DIPs was confirmed by electron microscopy, where virions were observed with reduced length. Among the less-than-equimolar fragments in DIP-containing preparations, fragments were observed linking sequences from map position 1.7 and 45 via a TGTT linker of unknown origin. The DIPs could not be plaque-purified and needed standard (helper) virus to replicate; DIP-containing preparations interfered with standard virus replication in an interference assay, which explained the reduction in productivity of an AcMNPV expression vector-insect cell system in continuous bioreactor operations. The origin of these DIPs and their possible generation mechanism are discussed.

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INTRODUCTION

Baculoviruses are attractive biological agents for the control of insect pests. They are highly specific for insects and cause a fatal disease (Granados and Federici, 1986). In addition, baculoviruses are successfully used as expression vectors for the production of heterologous proteins for various applications (see Luckow and Summers, 1988; Luckow, 1991, for review). In both cases large-scale systems for the production of recombinant baculovirus proteins are important. Production in insect larvae is difficult to scale up and to control. Insect-cell cultures offer an attractive alternative. In the case of pharmaceuticals and diagnostics insect-cell systems are preferred as these are better defined.

Baculoviruses can be produced on a large scale in insect-cell cultures using batch (Maiorella *et al.*, 1988), semicontinuous (Hink and Strauss, 1980) and continuous reactors (Kompier *et al.*, 1988). Continuous production of wild type (wt) *Autographa californica* multi-nucleocapsid nuclear polyhedrosis virus (AcMNPV) and recombinants was achieved in a system consisting of one bioreactor producing insect cells in series with a second bioreactor for virus infection and protein production (Kompier *et al.*, 1988; insert Fig. 3.1). After a few weeks of continuous operation, however, the productivity decreased to a low level. In case of wt AcMNPV, the number of polyhedra per cell decreased, the fraction of cells containing polyhedra, and the concentration of non-occluded virus all decreased (Kompier *et al.*, 1988). Continuous production of an AcMNPV recombinant where the polyhedrin gene was replaced by the lacZ gene of *Escherichia coli* essentially gave the same results (Van Lier *et al.*, 1992).

Since this continuous operation mimics the serial undiluted passaging of virus, it was assumed that the decrease in productivity was due to the multiple passaging (Tramper and Vlak, 1986). In the case of AcMNPV, serial undiluted passaging is often associated with the generation of plaque-morphology mutants, in particular the few polyhedra (FP) phenotype (Potter *et al.*, 1976; Fraser and Hink, 1982). Sequences derived from the insect (cell) hosts were found to be inserted into the genome of these mutants (Miller and Miller, 1982; Burand and Summers, 1982; Kumar and Miller, 1987), and in one case intervening a gene involved in polyhedron morphogenesis (Fraser *et al.*, 1983).

To elucidate the mechanisms responsible for the decrease in baculovirus productivity in the bioreactors, we analyzed the virus produced during the continuous operation. A deletion

mutant of AcMNPV is described, which became predominant during continuous production of a recombinant AcMNPV in *Spodoptera frugiperda* cells cultured in a bioreactor and which showed defective interfering (DI) properties.

MATERIALS AND METHODS

Cells and virus

Spodoptera frugiperda (*Sf*-AE-21) cells (Vaughn *et al.*, 1977) were cultured in TNM-FH medium (Hink, 1970), supplemented with 10% fetal calf serum. For culturing in bioreactors 0.1% (w/v) methylcellulose was added to reduce the effect of hydrodynamic forces on the cells. The E2-strain of *Autographa californica* multi-nucleocapsid nuclear polyhedrosis virus (AcMNPV) (Smith and Summers, 1978) was used as wild-type (wt) virus. The recombinant Acßgal (Summers and Smith, 1987) containing the lacZ gene downstream from the polyhedrin promoter was used as standard virus for the bioreactor experiments. Routine cell culture maintenance and virus infection procedures have been described (Summers and Smith, 1987). Virus stocks were titrated using the end-point dilution assay; the concentration of infectious virus was expressed as the median tissue culture infective dose ($TCID_{50}$).

Interference assay

To test if the DI-virus preparations interfered with the replication of the standard virus, an interference assay was developed. *S. frugiperda* cells (2.5×10^6) were inoculated with different dilutions of a virus sample taken from the bioreactor at 30 days post infection (p.i.), when the productivity had dropped considerably (Fig. 3.1). Standard virus was added to the inocula, so that the multiplicity of infection (MOI) based on the amount of infectious (standard) virus was the same (either 1, 5 or 10) for all dilutions. Only the amount of DI-particles varied. After incubation for 2 h at 27°C, unadsorbed viruses were removed by washing the cells two times with fresh medium. The cells were further incubated at 27°C for 72 h in 4 ml of medium. Production of infectious standard virus was measured with the end-point-dilution method.

Continuous production

Continuous production of virus and proteins was executed in a system consisting of two 1 dm^3 (working volume 0.8 dm^3) round-bottomed fermentors in series (insert Fig. 3.1). Reactor 0 is for culturing insect cells and reactor 1 is for virus and protein production (Kompier *et al.*, 1988). The reactors were equipped with marine impellers and air was passed through 0.2 μm filters into the headspace of the reactors at about 10 l/h. The temperature in the reactors was kept at 28°C. The mean residence time in the insect-cell reactor was set at 60 h by adding medium at 13.3 ml/h. The cell concentration in each reactor was 8-10 x 10^5/ml. Infection in the infection reactor was started by adding virus with a multiplicity of infection (MOI) of 0.01 $TCID_{50}$ units per cell. The production process was monitored daily by taking samples of about 5 ml from the infection reactor, in which cell number and cell viability were determined. Virus concentration was measured by the end point dilution method. Production of ß-galactosidase was determined using *o*-nitrophenyl-ß-D-galacto-pyranoside (ONPG) as a substrate (Miller, 1972).

DNA analysis

Extracellular virus was purified from infected-cell culture media by centrifugation and viral DNA as well as total DNA from infected cells was isolated as described by Summers and Smith (1987).

Viral DNA was digested with various restriction enzymes and the fragments were separated in a 0.8% agarose gel. DNA-fragments were isolated from the gel using the freeze-squeeze technique (Tautz and Renz, 1983). Fragments of AcMNPV were cloned into the plasmid pJDH118 (Hoheisel, 1989) and transfected into DH5α *E. coli* cells using standard techniques (Sambrook *et al.*, 1989). DNA-isolation, digestion with restriction enzymes (Bethesda Research Laboratories, Inc.), agarose gel electrophoresis, and transfer to nitrocellulose filters by the method of Southern (1975) were done using standard procedures (Sambrook *et al.*, 1989). Hybridizations were carried out with probes that were radioactively labeled by nick translation (Rigby *et al.*, 1977).

Double-stranded DNA (2-4 μg) was sequenced according to Sanger *et al.* (1977) using the procedures described in the protocol of the Sequenase Kit (United States Biochemicals, Inc.). The sequencing products were separated in 7 M urea- 6% polyacrylamide gels.

Electron microscopy

Extracellular virus was centrifuged from the culture medium (Summers and Smith, 1987) and the sediment was resuspended in TNM-FH medium without serum. The virus particles were negatively stained with 2% (w/v) phosphotungstic acid, pH 6.8 for 1 min and examined in a Philips CM12 electron microscope.

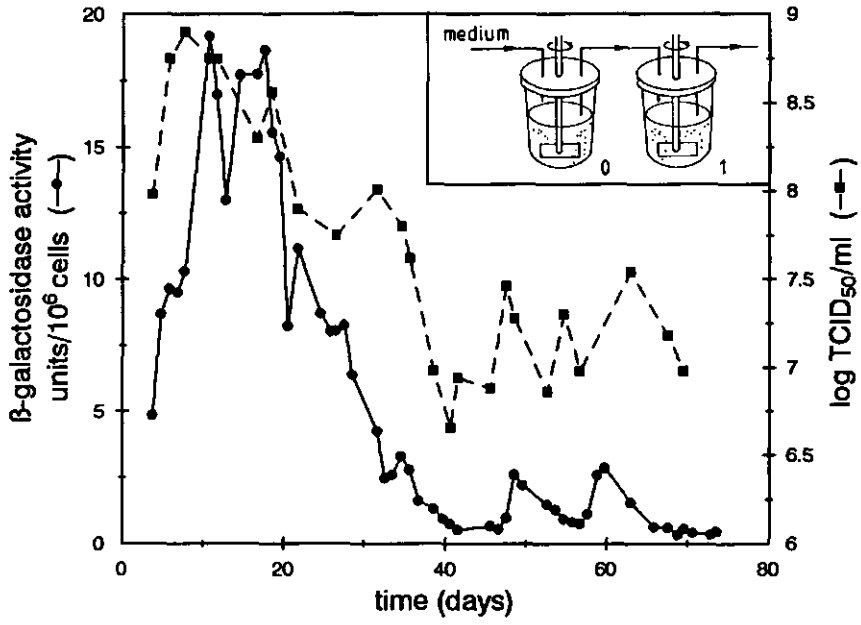


Figure 3.1. Activity (Units/10^6 cells) of ß-galactosidase and concentration of infectious virus ($TCID_{50}$/ml) present over time in a continuously operated *two-stage bioreactor system (insert). Reactor 0 is* the insect cell growth reactor; reactor 1 is the infection reactor.

RESULTS

Production of recombinant Acßgal in a continuous bioreactor

A recombinant baculovirus, containing the lacZ gene of *E. coli* downstream from the polyhedrin promoter was used to follow the productivity of the baculovirus/insect-cell system in continuous production. The use of ß-galactosidase proved a convenient assay to measure the productivity of the system. After reaching a maximum activity of 20 units per 10^6 cells

after 20 days of continuous running, the production of ß-galactosidase decreased rapidly to almost zero (Fig. 3.1). A similar reduction was noted following the production of polyhedra (Kompier *et al.*, 1988). Also, the infectious virus concentration, as measured by the end point dilution method, decreased over time from 10^9 to 10^7 $TCID_{50}$/ml after 40 days. This, however, also indicated that the virus was not completely eliminated in the effluent of the continuous infection reactor.

Samples of extracellular virus taken from the bioreactor at day 40 were subjected to a plaque assay to detect mutants which did not express (active) ß-galactosidase. Only blue plaques were found suggesting that such mutants were not present in high numbers in the bioreactor and that their presence could not have caused the reduction in productivity.

Analysis of virus from the bioreactor

DNA, isolated from cells infected with extracellular virus from the bioreactor, was analyzed with restriction enzymes *Eco*RI (Fig. 3.2A) and *Hin*dIII (Fig. 3.2B). The DNA-pattern of these digests (lanes 3) appeared to be distinct of DNA from plaque-purified standard virus (AcßgaI) (lanes 2). Several DNA-fragments, indicated by arrows, were present in less-than-equimolar amounts suggesting the presence of minor genotypic variants, possibly deletion mutants. Using other restriction enzymes (data not shown), it was possible to locate the fragments present in less-than-equimolar amounts on the physical map of AcMNPV (Fig. 3.3) (Vlak and Smith, 1982). The variants lacked segments spanning about 43% of the viral genome, provisionally located between 1.7 (*Eco*RI-I) and 45 (*Eco*RI-C) map units (m.u.) on the physical map (Fig. 3.3). The deletion included the lacZ gene. The physical existence of such variants was suggested by the presence of a minor, but new fragment of about 17 kbp in the *Hin*dIII digest.

Digestion with *Sst*II identified two additional DNA-fragments (3.3 kbp and 5.4 kbp) in the bioreactor-derived preparation which were absent in the plaque-purified DNA of standard virus (Fig. 3.4A, lanes 2 and 3, open arrows) and may therefore be specific for the mutant. Fragments A, B and E were present in less-than-equimolar amounts. Hybridization of this digest with either radioactively labeled *Eco*RI-C (Fig. 3.4B) or *Eco*RI-I (Fig. 3.4C) indicated that the unique 3.3 kbp DNA-fragment, named *Sst*II-BE, presumably contained sequences joining the left end of *Sst*II-B and the right-hand end of *Sst*II-E (Fig. 3.3). Hybridization was also found with the 5.4 kbp fragment, suggesting the presence of a mutant with a smaller

deletion. Radioactively labeled *Eco*RI-C and *Eco*RI-I also hybridized to the new 17 kbp fragment in the *Hin*dIII digest and with a minor fragment comigrating with *Eco*RI-C (data not shown).

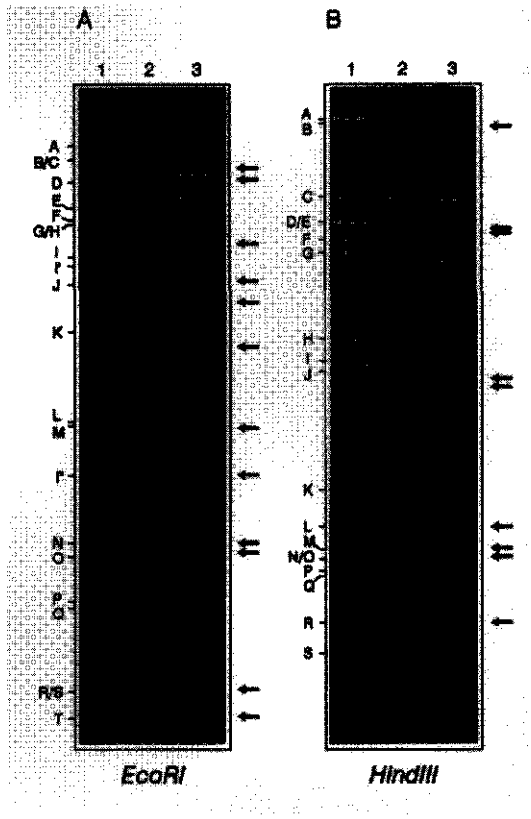


Figure 3.2. Restriction endonuclease analysis of DNA of wt AcMNPV (lane 1), plaque-purified standard AcβgaI (lane 2) and DNA isolated from *S. frugiperda* cells infected with virus preparations taken from the bioreactor, 40 days p.i. (lane 3), using *Eco*RI (panel A) and *Hin*dIII (panel B). Restriction fragment band designations are at left of each panel. The arrows on the right of each panel indicate DNA fragments which are present in less-than-equimolar amounts.

Sequence analysis

The *Sst*II-BE fragment of 3.3 kbp was cloned and various restriction sites were mapped (Fig. 3.5A). With the aid of restriction enzymes *Sst*I and *Xho*I subclones were made and the

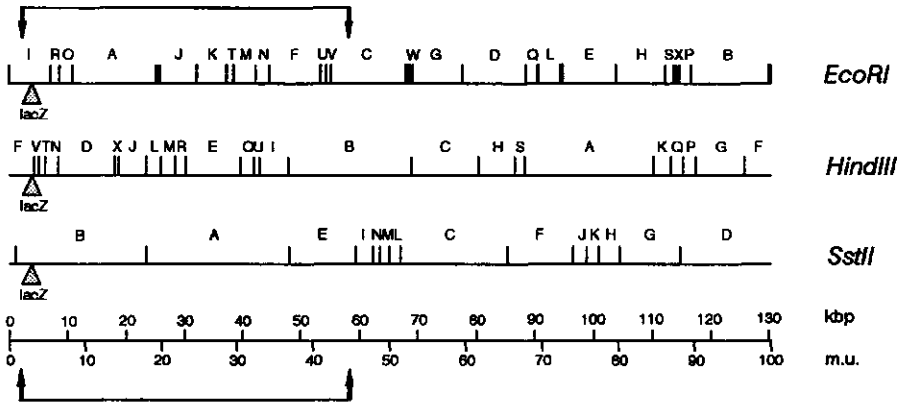


Figure 3.3. Physical map of AcMNPV for the restriction endonucleases *Eco*RI, *Hin*dIII and *Sst*II (Cochran *et al.*, 1982). The circular DNA is presented in a linear form with the polyhedrin gene located near the left end. (Vlak and Smith, 1982). The segment that is present in less-than-equimolar amounts is indicated (arrows). The site where the lacZ gene is inserted in the recombinant Acβgal is indicated by the dotted arrow.

sequence at the junction was determined (Fig. 3.5B). DNA of wt AcMNPV DNA encompassing the 45 m.u. region was cloned as a *Sst*I-*Eco*RI fragment of 2.4 kbp and partially sequenced to compare with the sequences found in *Sst*II-BE. The sequence around m.u. 1.7 has been published by Gearing and Possee (1990) and was used to confirm our sequence data. The sequence near the *SstI*-site partly overlaps with the sequence published by Carstens (1987). From the *Xho*I-site in fragment *Sst*II-BE towards the junction 131 bp were identical to the wt AcMNPV DNA sequence near m.u. 1.7. The junction further consisted of four nucleotides (TGTT) of unknown origin and was followed by the sequence identical to the 45 m.u. region of wt AcMNPV DNA (Fig. 3.4B). These nucleotide sequence analyses confirmed the restriction mapping and hybridization data, indicating that the *Sst*II-BE fragment of the mutant DNA originated from the joining of DNA sequences from m.u. 1.7 and 45.

Electron microscopy of extracellular virus

Extracellular virus from samples taken at day 40 from the bioreactor was sedimented by centrifugation and investigated using electron microscopy (Fig. 3.6). In addition to particles

of normal length (330 nm) many virus particles of about 190 nm were observed. This difference in length may reflect the difference in size of the viral DNA between standard virus and the deletion mutant.

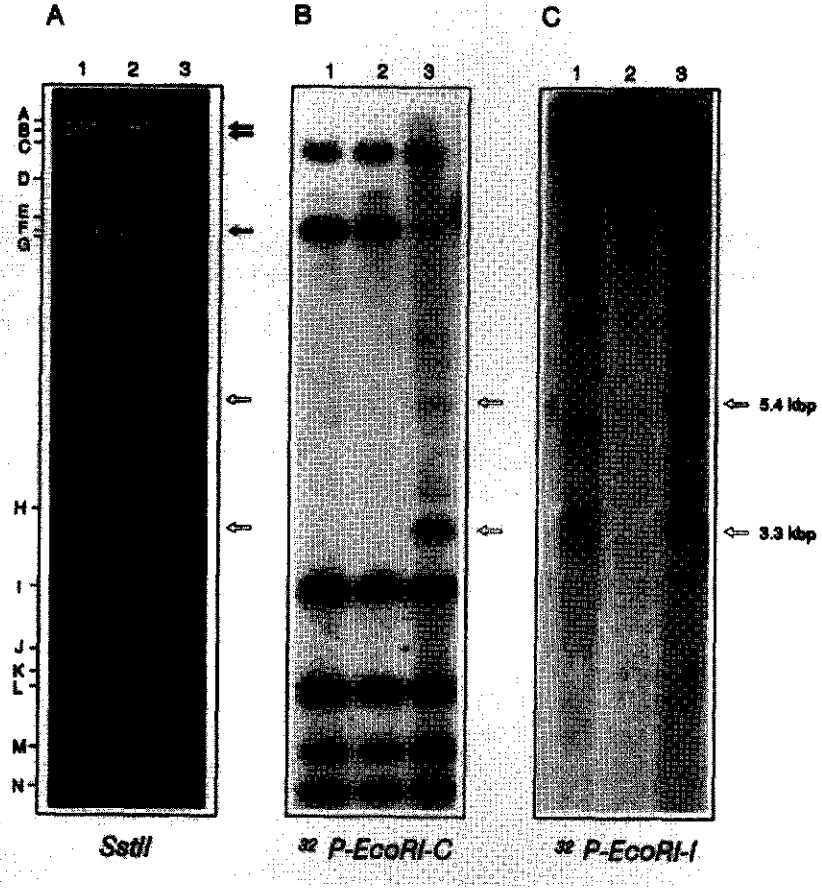
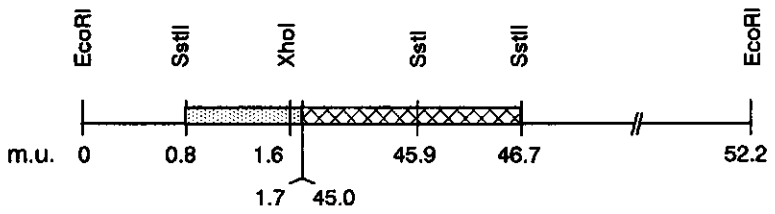


Figure 3.4. Electropherogram (A) and autoradiograms (B and C) of *Sst*II digests of wt AcMNPV (lane 1), plaque-purified standard virus (AcßgaI) (lane 2) and DNA isolated from *S. frugiperda* cells infected with virus, taken from the infection bioreactor, 40 days p.i. (lane 3). The electrophoretically separated fragments were stained with ethidium bromide (panel A), transferred to nitrocellulose, and hybridized with radioactively labeled *Eco*RI-C (panel B) and *Eco*RI-I (panel C). Restriction fragment band designations are at left of panel A. The solid arrows indicate DNA fragments which are present in less-than-equimolar amounts. The open arrows indicate the extra DNA-fragments, 3.3 and 5.4 kbp in size, found in bioreactor-derived viral DNA (lane 3) and which are not present in digests of wt AcMNPV (lane 1) or standard virus (lane 2).

A.



B. *Xho*I

```
CTCGAGCAGT TCGTTGACGC CTTCCTCCGT GTGGCCGAAC ACGTCGAGCG GGTGGTCGAT
        10         20         30         40         50         60

GACCAGCGGC GTGCCGCCGC GACGCACAAG TATCTGTACA CCGAATGATC GTCGGGCGAA
        70         80         90        100        110        120

GGCACGTCGG CTGTTGAGTA AAAATGTTGC AGCAAAAATT AAATAAACTC AAAGATGGTT
       130        140        150        160        170        180

TGAACACGTT CAGCAGCAAG TCGGTGGTTT GCGCTCGCTC AAAATTATTT GACAAACGCC
       190        200        210        220        230        240

CAACGCGCAG    -- ± 1000 bp  ---   TATTTTACAA ATGAAAGTTG TAAAGAGCTC
       250                              1250       1260       1270
                                                          SstI
```

Figure 3.5. A. Physical map of the 3.3 kbp junction fragment *Sst*II-BE. The location on the wt AcMNPV DNA-map is indicated in m.u. The light dotted area indicate sequences from *Eco*RI-I; the hatched area indicate sequences from *Eco*RI-C. **B.** Partial nucleotide sequence of the 3.3 kbp *Sst*II fragment spanning the junction between *Eco*RI-I and C. The *Xho*I and *Sst*I restriction sites are indicated in italics; the underlined sequence is not present in wt AcMNPV DNA at this location.

Interference analysis

To determine if the decrease in production of infectious virus from 10^9 to 10^7 in the bioreactor is due to interfering properties of the deletion mutant, an interference assay was carried out (Fig. 3.7). A sample was taken from the bioreactor, 30 days p.i., when there was already a decline in virus production (Fig. 3.1). With electron microscopy and restriction

analysis of isolated viral DNA it was found that mutant particles were present at this time. A series of dilutions from this bioreactor sample was made and standard virus was added to achieve the same final concentration (MOI 1, 5 or 10) of infectious (standard) virus for each dilution. With increasing amounts of DI-virus, the titer of newly produced infectious virus gradually decreased about 100-fold for all three MOIs (Fig. 3.7). At MOI 1 there is most likely some cell growth after infection, because not all the cells will be infected initially. This higher cell concentration probably explains the higher virus titer compared to MOI 5 and 10.

Figure 3.6. Electron micrograph of normal (N) and defective (D) NOV particles. The bar represents 300 nm.

DISCUSSION

The reduction in productivity of continuously propagated baculoviruses in *S. frugiperda* cells in bioreactors (Kompier *et al.*, 1988) was predicted by Tramper and Vlak (1986) and mimicked the effect of serial passage of a baculovirus in cell culture. In cell culture, the FP phenotype becomes predominant and polyhedra production is severely reduced (Potter *et al.*, 1976; Fraser and Hink, 1982; Burand and Summers, 1982). These FP mutants contain small insertions or deletions at specific sites in the genome, resulting in this altered phenotype (Fraser *et al.*, 1983; Kumar and Miller, 1987). However, from the bioreactor preparations we obtained evidence for the presence of another class of mutants characterized by defective

interfering properties. These mutants require a helper to replicate and hence escaped detection by plaque-purification (Carstens, 1987; Cusack and McCarthy, 1989).

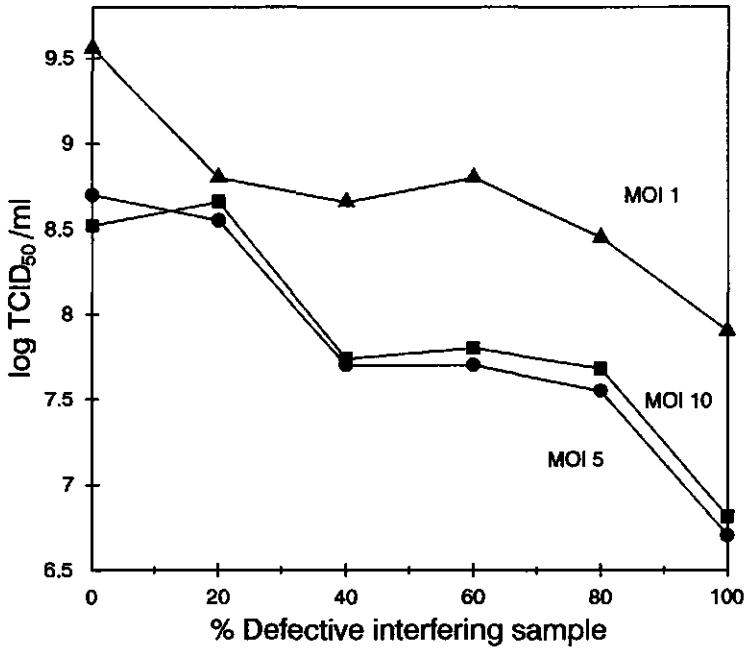


Figure 3.7. Effect of DIPs on the production of infectious standard NOVs. The MOI of infectious NOV was maintained at either 1 (▲), 5 (●) or 10 (■) $TCID_{50}$ units/cell at each dilution of the virus preparation containing DIPs.

Physical analysis of extracellular viruses from bioreactor preparations revealed the presence of viruses smaller in length (Fig. 3.6). These viruses most likely contain a genome which is 43% shorter in length than standard AcMNPV (Figs. 3.2-3.4); the deletion included the ß-galactosidase gene. Samples containing these mutant viruses interfered with the replication of standard virus (Fig. 3.7). This suggests that defective interference (DI) is an alternative mechanism to explain the passage effect during baculovirus replication. DI-viruses have been reported for both RNA- and DNA-viruses, and are also referred to as DI-particles. The name DI-particles was proposed by Huang and Baltimore (1970) to describe virus particles that lack an essential part of genomic RNA or DNA, that become packaged within the usual virus structural proteins and antigens, that require homologous infectious helper

virus in order to replicate, and that interfere specifically with homologous helper virus by replicating at their expense in doubly-infected cells (Holland, 1990). The information on the AcMNPV mutant described here meets the definition of a DI particle.

By definition a DIP is not able to establish an infection independently as it needs a helper virus to replicate. This may explain why the concentration of infectious NOV in the bioreactor is maintained at about 10^7 $TCID_{50}$ per ml. When it drops below this level the amount of helper virus is insufficient to assist in the replication of DIPs. When, as a consequence, the concentration of DIPs decreases, the concentration of helper viruses can increase due to the lack of interference. This so-called Von Magnus phenomenon (Von Magnus, 1954) is also observed in the bioreactor (Fig. 3.1).

For baculoviruses little is known about the incidence and role of DIPs. Burand *et al.* (1983) described the detection of defective particles in *Trichoplusia ni* cells, persistently infected with the *Hz*-1 isolate of the *Heliothis zea* baculovirus. These preparations contained viral DNA heterogeneous in size, with deletions ranging from 10-20% of the 228 kbp genome (Chao *et al.*, 1990), and showed reduced levels of protein synthesis in infected cells (Burand and Wood, 1986). In their study on the effect of serial passage on the genetic homogeneity of *Lymantria dispar* NPV in cell culture, Cusack and McCarthy (1989) observed subpopulations of minor variants which could not be plaque-purified and which may have interfering properties. The DNA of these DIP-containing preparations appear to contain small deletions dispersed throughout the viral genome. It is known that infections with DIPs of herpesviruses can induce persistent infections (O'Callaghan, 1981). Therefore, it is of interest to isolate the AcMNPV DIPs to test whether they can cause a persistent infection in insect cells.

Carstens (1982, 1987) described a mutant of AcMNPV, called M5, which contained, in addition to wt genotype, a smaller DNA genome which could not be plaque-purified. This molecule had a specific deletion of 42% of the genome, which extended from map position 2.6 and 45, and carried short DNA insertions. The deletion in our mutant is similar in that the break points in the DNA genome occurred in both the *Eco*RI-I fragment and in the *Eco*RI-C fragment, but about 1300 bp to the left in both fragments. In contrast to the M5 mutant, no DNA insertions other than the 4 nucleotides TGTT were found. The origin of these 4 nucleotides is unknown, but it is possible that they are remnants of a DNA insertion or rearrangement at this site present in an intermediate mutant. The evidence that such

intermediate mutants may exist is supported by the detection of other unique fragments in the *Sst*II digests (Fig. 3.4A and B).

The data presented in this paper suggest two possible mechanisms for the emergence of DIPs in serially propagated baculovirus preparations. Firstly, deletion mutants with progressive deletions are generated over time. Indeed, mutant viruses with smaller deletions are observed earlier after infection (P.A. Koetsier, personal communication). Secondly, a heterogeneous population of defective viruses is produced during the initial rounds of replication, during plaque assaying. With continued passaging in bioreactors, a selection occurs for viruses with a genome of smaller size at the end of the serial passaging. This also leads to a more or less homogeneous population of DIPs. This hypothesis is supported by our finding that the DIP lacking 43% of the genome is predominant 30 days p.i. in the bioreactor. Most interestingly, this DIP appeared to be present in the plaque-purified standard isolate (P.A. Koetsier, personal communication). A parallel case is the generation of SV40 DIPs, which became genetically more homogeneous with continued passaging (Norkin and Tirrell, 1982). It is also possible that origins of replication have been amplified in the DIPs, as has been the case for SV40 (Lee and Nathans, 1979). However, from the restriction endonuclease analysis no reiteration of large DNA segments was observed (Fig. 3.2), and the sequences flanking the junctions (Fig. 3.5B) were not different from those present in DNA of standard virus.

The initial events in DIP generation are unclear. It is possible that, as is the case for SV40, non-specific cellular nucleases induce breaks in the DNA which are improperly repaired (Johnson *et al.*, 1982) or that homologous and heterologous recombination occurs (O'Neill *et al.*, 1987). In the case of herpes simplex virus (Mocarski *et al.*, 1985) and equine herpesvirus (Yalamanchili *et al.*, 1990) DIPs are thought to be generated through recombination mediated by limited homology. Since baculoviruses are highly recombinogenic (Smith and Summers, 1979), the latter property may contribute to the generation of DIPs. Since many of the FP mutants have acquired insertions of transposon-like host sequences (Fraser, 1987), it is possible that these FP mutants are intermediates in the generation of DIPs. Many sites of these insertions are located within the deleted sequences of the DIP.

The mechanism of interference of baculovirus DIPs is yet to be disclosed. DIPs most often interfere at the level of virus replication, by competing for enzymes essential for DNA replication (Holland, 1990). This may also be the case for baculoviruses as the DIPs lack the

DNA polymerase gene, which is located in the deleted area (Tomalski *et al.*, 1989). Alternatively, interference may also take place at the level of assembly, i.e. competition for encapsidation (packaging) or maturation.

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CHAPTER 4

NUCLEOTIDE SEQUENCE AND GENETIC ORGANIZATION OF A 7.3 KB REGION (MAP UNIT 47 TO 52.5) OF *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS FRAGMENT *ECO*RI-C

SUMMARY

The nucleotide sequence and genetic organization of a 7297 bp region within the *Eco*RI-C fragment of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) are presented. Eight putative open reading frames were found and their respective amino acid sequences compared with a number of data libraries. ORF 1227 corresponded with *gp41* and its predicted protein sequence was found to be 55 amino acids longer at its C-terminus than reported previously (Whitford and Faulkner, 1992b). Moreover, the main part of the ORF 1227 product, including the additional 55 amino acids, showed a high degree of homology with protein P40 of *Helicoverpa zea* single nucleocapsid nuclear polyhedrosis virus (HzSNPV). Three other ORFs in the analysed AcMNPV region showed homology with ORFs in the HzSNPV sequence, indicating that the general organization of this region is similar in both viruses. However, one ORF found in the AcMNPV sequence, was absent from the corresponding HzSNPV sequence.

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INTRODUCTION

The genus nuclear polyhedrosis virus (NPV) of the family *Baculoviridae* includes the single (S) and multiple (M) nucleocapsid NPVs (Francki *et al.*, 1991). The MNPV of *Autographa californica* (AcMNPV) represents the prototype baculovirus and its DNA is approximately 131 kilobase pairs (kbp) in size. Baculoviruses contain circular, double-stranded DNA genomes and their gene organization and sequence are being investigated (see Blissard and Rohrmann, 1990; Kool and Vlak, 1993 for review). About 65% of the AcMNPV genome has been sequenced. The replication of baculoviruses can be studied in much detail in insect cell culture systems.

Serial, undiluted passage of AcMNPV in *Spodoptera frugiperda* insect cell culture results in the generation of defective viruses (Kool *et al.*, 1991; Wickham *et al.*, 1991; Lee and Krell, 1992). These defective viruses are characterized by the presence of major genomic deletions and their dependence of helper virus for replication in insect cells. In addition, these defective viruses have interfering properties (Kool *et al.*, 1991). DNA analysis showed that in these defective viruses up to 43% of the standard viral genome, a segment extending from map unit (m.u.) 1.7 to 45.0 on the physical map of AcMNPV, has been deleted. Further analysis of the defective genomes in serially passaged virus preparations revealed the presence of many different classes of deletion mutants all with their deletions ending in the *Eco*RI-C fragment (M. Kool, unpublished data).

From the observation that some regions of the AcMNPV genome are maintained in defective viral DNA molecules it is logical to assume that they contain *cis*-acting elements necessary for DNA replication and/or encapsidation. Regions with highly repetitive sequences in the retained fragments could serve as an origin of DNA replication (Pearson *et al.*, 1992; Kool *et al.*, 1993a, 1993b). These regions are also implicated as enhancers of transcription (Guarino *et al.*, 1986). Encapsidation signals have not yet been described, but it was postulated that they are present near the junctions in the defective genomes (Kool *et al.*, 1991). One junction is within the *Eco*RI-C fragment.

To investigate the genetic functions of the *Eco*RI-C fragment in the defective genomes and their possible role in the generation of these genomes, the sequence of this fragment needs to be determined. Whitford and Faulkner (1992b) sequenced the AcMNPV *Sst*II-M fragment, which is located entirely within the *Eco*RI-C fragment and which contained an

open reading frame (ORF) coding for a structural glycoprotein (GP41) of polyhedra-derived virions (Whitford and Faulkner, 1992a). Here, we present the nucleotide sequence of 7297 base pairs (bp) of the right part of the *Eco*RI-C fragment, extending from the left *Sst*I site of *Sst*I-H to the first *Eco*RI site of the *hr*3 region of AcMNPV. Eight putative ORFs were found. Evidence is presented that the overall organization of this region is similar in both MNPVs and SNPVs.

MATERIALS AND METHODS

Virus

The E2-strain of *Autographa californica* multi-nucleocapsid nuclear polyhedrosis virus (AcMNPV) (Smith and Summers, 1978) was used.

Plasmid constructions

AcMNPV fragment *Eco*RI-C was cloned into plasmid pBR322. Overlapping subfragments, generated by a number of restriction enzymes, were cloned into pUC19 or pJDH119 (Hoheisel, 1989), and transformed into *Escherichia coli* JM101 using standard techniques (Sambrook *et al.*, 1989). DNA-isolation, purification in CsCl gradients, digestion with restriction enzymes, and agarose gel electrophoresis were carried out using standard procedures (Sambrook *et al.*, 1989).

DNA sequencing and computer analysis

Overlapping DNA fragments were sequenced on both strands using the dideoxy chain-termination method (Sanger *et al.*, 1977) following the procedures described in the protocol of the Circumvent Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs). For fragments which could not be subcloned further, oligonucleotides were synthesized for primers. Sequence analyses were performed using the UWGCG computer programs (Devereux *et al.*, 1984) (version 7). The DNA sequence and the deduced amino acid sequences of the open reading frames were compared with sequences in the GenBank (release 75.0), EMBL (release 33.0), SWISSPROT (release 24.0) and NBRF databases, using the FASTA and TFASTA programs.

RESULTS

AcMNPV fragment *Sst*I-H (1.3 kbp) and fragment *Sst*I-*Eco*RI (6 kbp), which form a contiguous segment on the right-hand end of fragment *Eco*RI-C located next to homologous region *hr*3, were sequenced (Fig. 4.1). The approximate map units (m.u.) for this region are 47.0 to 52.5 (Vlak and Smith, 1982). The DNA sequence encompassed 7297 nucleotides, i.e. about 5% of the AcMNPV genome (Fig. 4.1). The locations of the most commonly used restriction sites are indicated in the nucleotide sequence (Fig. 4.2) and these sites were used to construct a restriction map for these enzymes (Fig. 4.1).

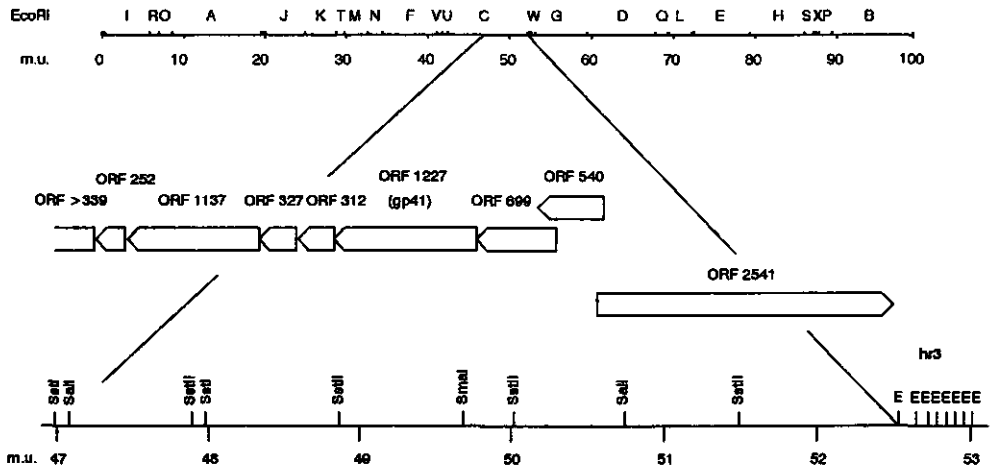
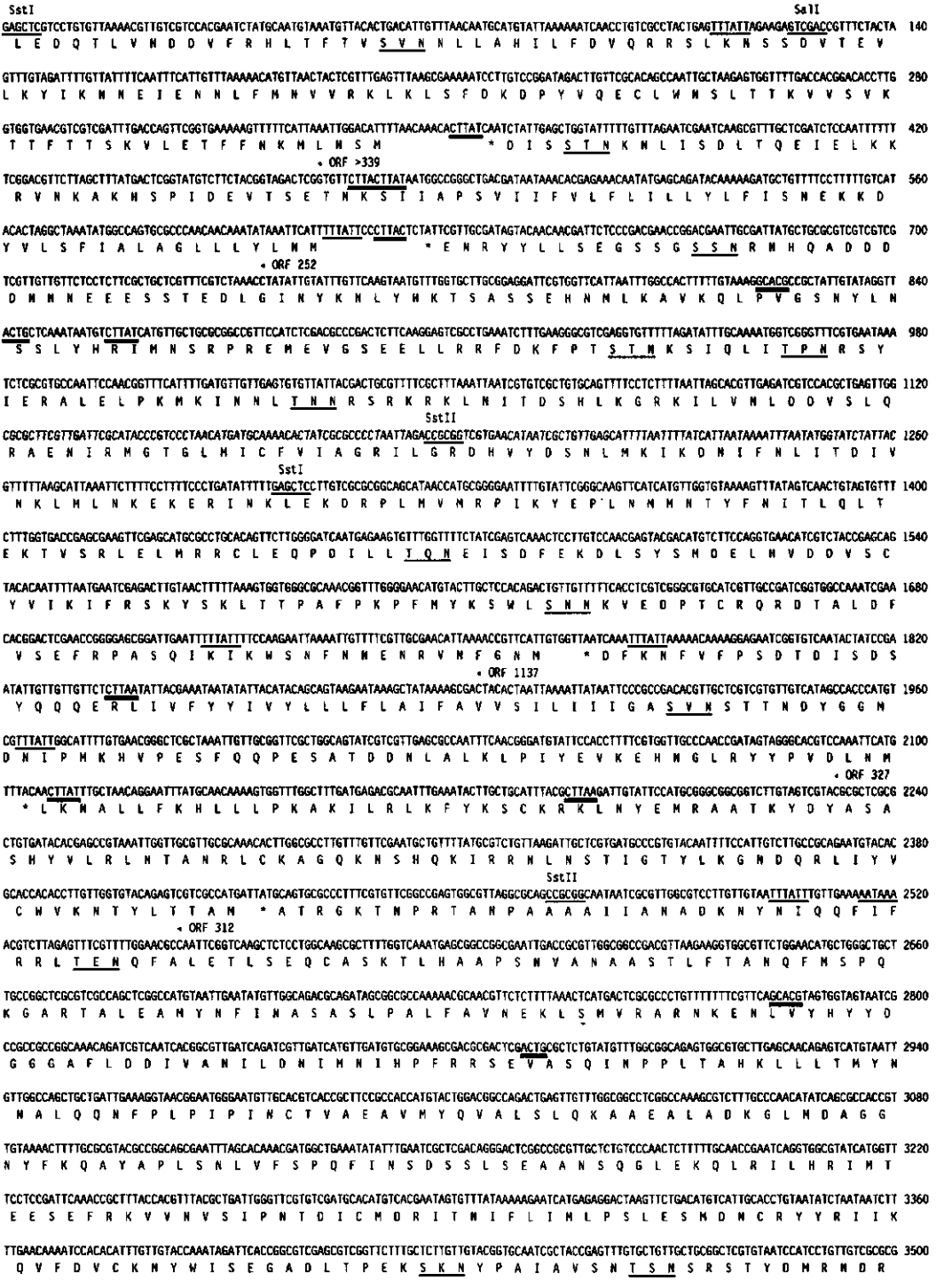
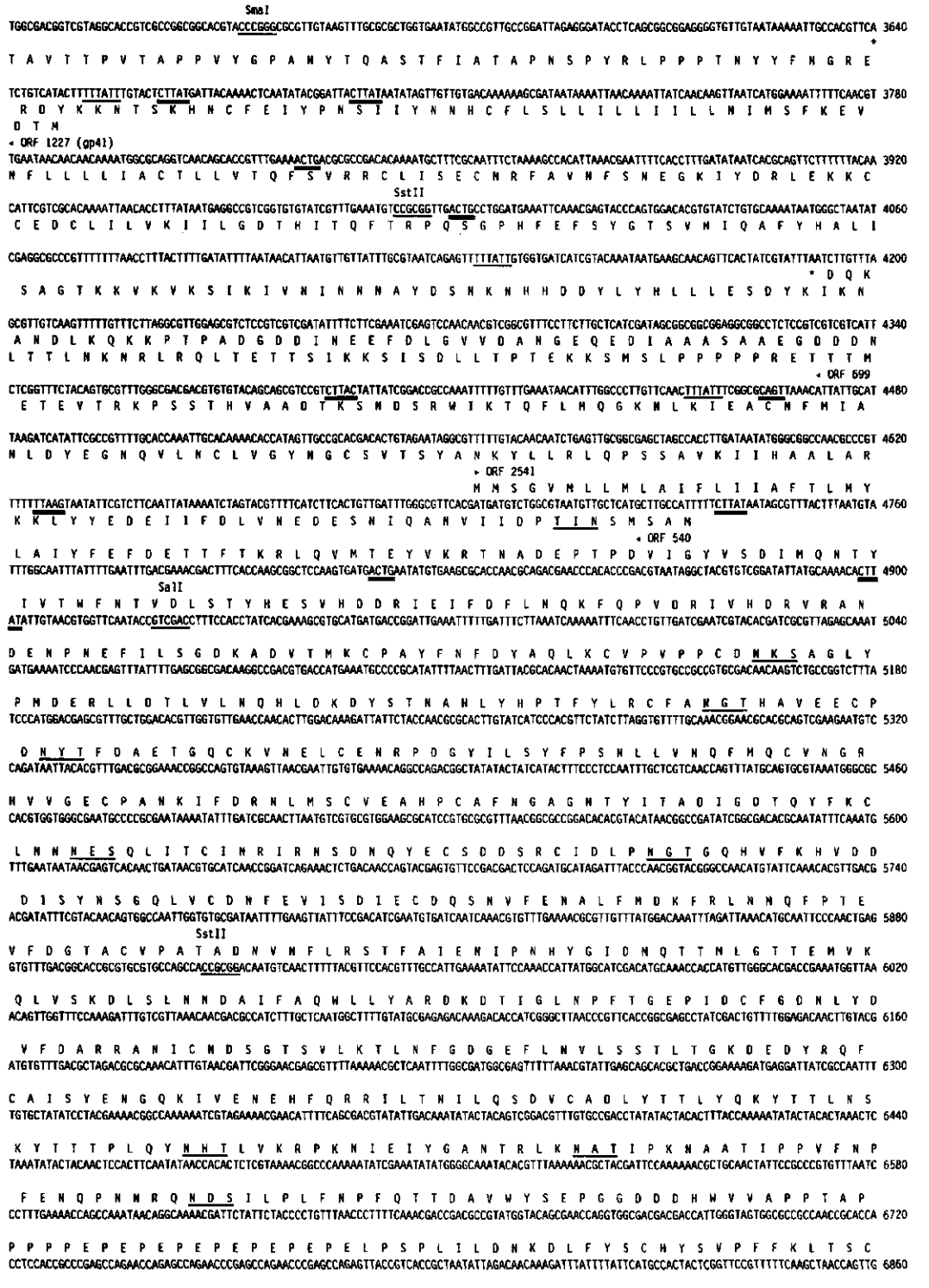


Figure 4.1. *Eco*RI restriction map of the AcMNPV genome and a partial restriction map of the region that has been sequenced. The position of the ORFs and the direction of transcription are indicated. m.u. = map unit, E in *hr*3, *Eco*RI.

Eight ORFs were identified within the sequenced region and these are schematically shown in Fig. 4.1. The ORF annotations refer to the putative number of nucleotides. The predicted protein sequences for these eight ORFs are presented in Fig. 4.2. A partial ORF of 113 amino acids extends in the region upstream of m.u. 47. One of the 8 ORFs (ORF 2541) has the same genomic DNA orientation as the polyhedrin gene (Vlak and Smith, 1982) and hence runs from left to right.


SstI
SalI
ORF >339
ORF 252
SstII
SstI
ORF 1137
SstII
ORF 312


SmaI
SstII
SalI
◂ ORF 1227 (gp41)
▸ ORF 2541
◂ ORF 599
◂ ORF 540

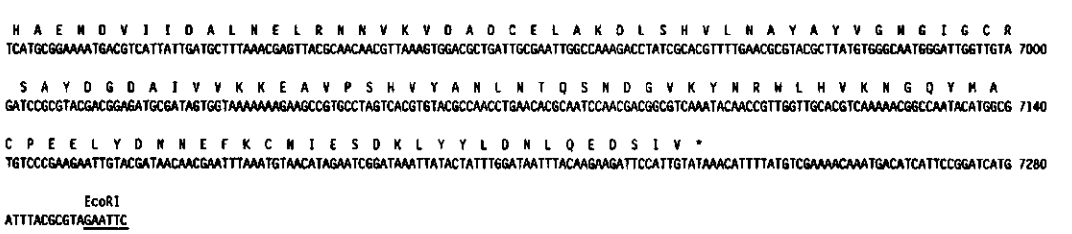


Figure 4.2. Nucleotide and predicted protein sequences of 7297 bp of *Eco*RI-C. For ORFs running from left to right the amino acid sequence is placed above the DNA sequence. The amino acid sequences derived from the opposite strand are placed below the DNA sequence. Several restriction enzyme sites are underlined and named. Polyadenylation signals and putative *N*-glycosylation sites are underlined. Potential consensus early and late transcription initiation sites are doubly underlined.

All but two ORFs (ORF 1227 and ORF 2541) are putative baculovirus genes since they contained putative transcriptional start and stop signals at appropriate locations up- and downstream from the ORF (Fig. 4.2, Table 4.1). Most early genes contain a promoter with the consensus sequence CAGT, whereas late AcMNPV gene promoters contain a motif (A/T/G)TAAG in which transcription initiates (see Blissard and Rohrmann, 1990, for review). Another promoter motif, CGTGC, found at the early start sites of the *helicase* (*p143*) gene (Lu and Carstens, 1992), the DNA polymerase gene (Tomalski *et al.*, 1988), and at the early start site of *p47* (Carstens *et al.*, 1993) is also found upstream of several ORFs in the sequence presented here. All these motifs are indicated in Fig. 4.2, and their positions are also summarized in Table 4.1, together with the positions and sizes of all the putative ORFs. No consensus polyadenylation signals were found immediately downstream from ORF 1227 or in the sequence immediately downstream from ORF 2541 or the sequence of *hr*3 (Guarino *et al.*, 1986) which is located further downstream of ORF 2541.

Computer-assisted analysis of the sequence data indicated that there was no nucleotide or amino acid sequence homology with AcMNPV sequences deposited in data libraries, except for ORF 1227. This ORF, located on fragment *Sst*II-M (m.u. 48.9 - 50), exhibited high sequence homology with the *gp41* gene reported by Whitford and Faulkner (1992b), although this gene was much shorter (1062 bp) than ORF 1227, and had an opposite orientation. Recently, Whitford and Faulkner (1993) have corrected the orientation and, as a

consequence, the *gp41* ORF now runs from right to left according to the conventional orientation of the AcMNPV genome (Fig. 4.1).

The data presented for nucleotides 2466-3988 agree with those published by Whitford and Faulkner (1992b), except for an extra G residue on position 2618, and an A on position 3071 instead of a G. Furthermore, in the upstream sequence of ORF 1227, a T residue after positions 3916, 3947 and 3960 is missing as compared to the Whitford and Faulkner (1992b) sequence.

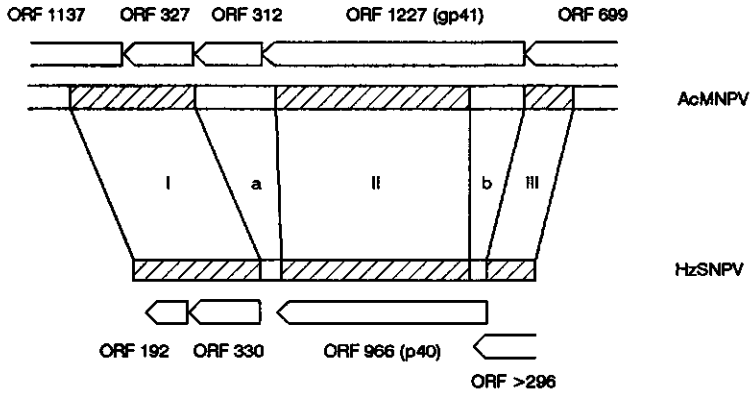


Figure 4.3. Schematic presentation of regions of the AcMNPV and HzSNPV genomes with nucleotide sequence homology (hatched areas). For percentage nucleotide and amino acid sequence identities, see text.

Further computer-assisted sequence comparisons indicated a considerable homology of AcMNPV ORFs 1137, 327, 1227 (*gp41*) and 699 (Fig. 4.2) with a 1890 bp nucleotide sequence of *Helicoverpa zea* single-nucleocapsid nuclear polyhedrosis virus (HzSNPV) (Ma *et al.*, 1993). The HzSNPV sequence contains an ORF coding for a virion structural protein P40, which may represent the homologue of AcMNPV GP41 (Fig. 4.3). Three separate regions (I, II and III) of 580, 896 and 227 bp, respectively, in the AcMNPV sequence showed homology with three separate similar-sized regions in the HzSNPV genome. The nucleotide sequence identity for each of these three regions is 64.1%, 65.4% and 56.8%, respectively. In both viruses these conserved regions are interspaced by non conserved sequences (a and b) of different sizes (385 and 260 bp, respectively for AcMNPV, and 79 and 80 bp, respectively for HzSNPV) (Fig. 4.3).

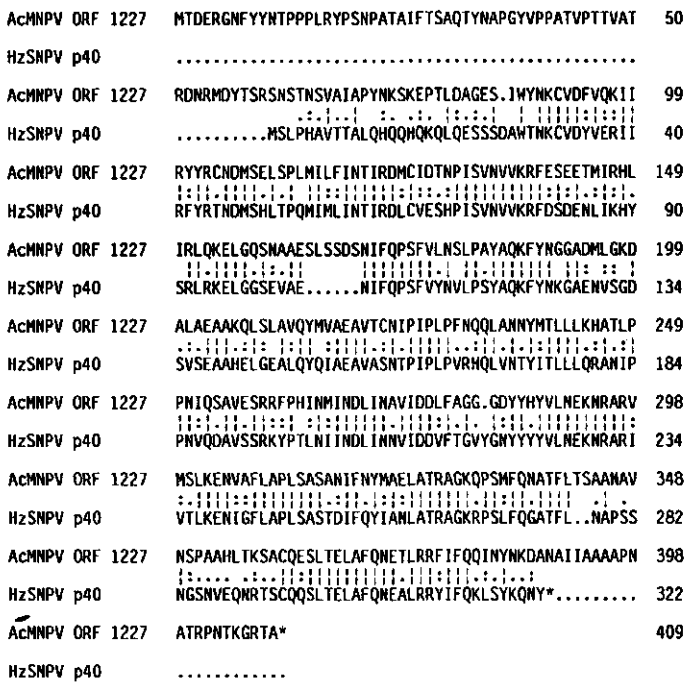


Figure 4.4. Comparison of amino acid sequences of AcMNPV ORF 1227 (GP41) and HzSNPV ORF 966 (P40).

The largest conserved region (II) corresponds with the C-terminal parts of the AcMNPV ORF 1227 product and HzSNPV P40. When the amino acid sequences of these proteins are compared a similarity of 79.1% and an identity of 62.4% was found over a stretch of approximately 300 amino acids (Fig. 4.4). In the C-terminal part of AcMNPV ORF 1227 a stretch of six amino acids, four of which are serines, is present (amino acids 164 to 169), which is absent from the HzSNPV P40 sequence. The major difference between AcMNPV ORF 1227 and HzSNPV P40 is found in the N-terminal sequence (Fig. 4.3, region b; Fig. 4.4). AcMNPV GP41 has an additional 86 amino acids at the N-terminus, whereas P40 of HzSNPV has only 27 additional amino acids with almost no sequence homology with AcMNPV. This implies that the two ORFs code for polypeptides of different size, 409 amino acids for AcMNPV ORF 1227 and 322 amino acids for HzSNPV P40 (Fig. 4.4). The promoter regions of both ORFs (region III, Fig. 4.3) from position -227 (nucleotide residue

3875, Fig. 4.2) on to the translational startcodon (ATG) do show considerable sequence homology. A late ATAAG transcription signal is found at position -52 for AcMNPV and at position -49 for HzSNPV. In both viruses region III overlaps with the 3' end of AcMNPV ORF 699 and with an as yet unidentified ORF in HzSNPV showing 73.1% similarity and 46.2% identity over a stretch of 78 amino acids.

Table 4.1. Summary of locations of ORFs, putative transcription initiation and termination signals, sizes of the ORFs in nucleotides and amino acids, and the predicted sizes of the proteins.

ORF	Name	Position	Coding direction	Size (nt)	Transcription signals	Kozak consensus ANNATGPu		Poly A signal	Size (aa)	Mw (kDa)
1	ORF >339	1-339	-	>339	ATAAG (-16) GTAAG (-140) ATAAG (-144)	A	T	?	>113	13.2
2	ORF 252	357-609	-	252	GTAAG (-14) CGTGC (-215) CAGT (-235) ATAAG (-252)	A	A	+241	84	9.5
3	ORF 1137	627-1764	-	1137	TTAAG (-77)	A	A	+12	379	44.4
4	ORF 327	1772-2099	-	327	ATAAG (-13) TTAAG (-94)	A	A	-12 +55	109	12.5
5	ORF 312	2104-2416	-	312	CGTGC (-380) CAGT (-470)	A	G	+136	104	12.2
6	ORF 1227 (gp41)	2421-3648	-	1227	ATAAG (-21) ATAAG (-52) CAGT (-182) CAGT (-347)	A	A	-	409	45.4
7	ORF 699	3640-4339	-	699	GTAAG (-57)	A	A	-18	233	26.9
8	ORF 540	4190-4730	-	540	ATAAG (-9) CAGT (-32) ATAAG (-172)	A	G	+50	180	20.0
9	ORF 2541	4694-7234	+	2541	TTAAG (-71) TTAAG (-214) CAGT (-232) CAGT (-342)	A	A	-	847	93.2

Region I of AcMNPV contains the complete ORF 327 and the 5' end of ORF 1137. In this region of the HzSNPV sequence an ORF of almost the same size (ORF 330) was found (Fig. 4.3). The predicted amino acid sequences of AcMNPV ORF 327 and HzSNPV ORF 330 showed a similarity of 64.4% and an identity of 44.6%. The homology between regions I ends near the ATG of both AcMNPV ORF 327 and HzSNPV ORF 330. The promoter regions for these ORFs show no homology (Fig. 4.3, region a), but share a late transcriptional motif (TTAAG), at position -94 for AcMNPV and at position -93 for HzSNPV. In this region AcMNPV has one unique ORF (Fig. 4.3, ORF 312). The 5' end

of AcMNPV ORF 1137 and HzSNPV ORF 192 have a similarity of 73.3% and a identity of 61.7% at the amino acid level. The promoter regions of these ORFs show significant identity (64%), since they are part of ORF 327 and ORF 330, respectively. Again for both these ORFs a late TTAAG signal was found, at position -77 for AcMNPV and at position -60 for HzSNPV.

Computer-assisted analysis further indicated the absence of leucine zipper or zinc-finger motifs in the putative amino acid sequences of the ORFs presented here. Hydrophobicity analysis of these ORFs indicated that ORF 2541 contained a highly hydrophobic amino terminus (25 aa), which may represent a signal peptide. In most ORFs the N-terminal methionine is located in accordance with the Kozak translation initiation sequence ANNATGPu (Table 4.1) (Kozak, 1983).

DISCUSSION

A contiguous sequence of 7297 nucleotides located on the *Eco*RI-C fragment of the AcMNPV genome has been determined (Fig. 4.1) and revealed eight different ORFs. The presence of transcriptional start and stop signals up- and downstream of most of these ORFs suggest that they might be transcriptionally active, although this has yet to be proven experimentally. For five of the eight identified ORFs an early CAGT motif was found in the upstream sequence. To be transcriptionally active, this CAGT sequence is generally preceded by an upstream TATA box (Blissard and Rohrmann, 1991). However, no TATA boxes were found upstream of any of the CAGT sequences. Several transcripts, both early and late, have been mapped to the AcMNPV *Eco*RI-C fragment (Lübbert and Doerfler, 1984; Mainprize *et al.*, 1986). However, in view of the many ORFs on this fragment, a further allocation requires more detailed transcriptional analysis using ORF sequences as probes.

Only ORF 1227 is known to be transcriptionally active and this ORF has now been found to be homologous to the *gp41* gene reported by Whitford and Faulkner (1992b). The nucleotide sequence of fragment *Sst*II-M revealed an additional G residue on position 2618 resulting in an ORF for *gp41* which is 165 nucleotides longer than previously reported (Whitford and Faulkner, 1992b). Owing to this extra nucleotide, the last C-terminal 10 amino acids in the sequence of Whitford and Faulkner (1992b) are replaced by 65 additional amino

acids. As a consequence the ORF codes for a polypeptide of 409 amino acids with a predicted size of 45.4 kDa instead of 39.6 kDa. The enlarged sequence of GP41 also reveals no hydrophobic C-terminus, which supports the suggestion that GP41 is not an integral membrane protein (Whitford and Faulkner, 1992b). The GP41 is the major glycoprotein of polyhedra-derived virions with an M_r of 42 kDa by PAGE (Stiles and Wood, 1983) and contains *O*-linked carbohydrates (Whitford and Faulkner, 1992a). According to our sequence data GP41 is thus in fact larger (>45 kDa). Antisera raised against the C-terminal part (Fig. 4.3, region b) of GP41 using an oligopeptide as antigen might be used to confirm the existence of the ORF 1227 product in polyhedra-derived virions.

An alternative explanation for the difference in GP41 sequence could be that another strain of AcMNPV (HR3) was used in the study of Whitford and Faulkner (1992b). The extra C-terminal 65 amino acids of ORF 1227 show homology to the C-terminus of HzSNPV P40, an observation which supports the sequence of ORF 1227. However, the last 22 amino acids of ORF 1227 are not present in P40. A difference in size is also found at the N-termini of both ORFs. AcMNPV ORF 1227 has 86 amino acids at the N-terminus which show almost no homology with the only 27 amino acids at the N-terminus of HzSNPV (Fig. 4.3, region b; Fig. 4.4). The high degree of homology in this region between AcMNPV and HzSNPV suggests a conserved sequence organization in MNPVs and SNPVs.

A 2.1 kbp transcript encoding the GP41 protein was detected by Whitford and Faulkner (1992b). The size of this transcript corresponds with the observation that no polyadenylation signal was found immediately downstream of ORF 1227 (*gp41*), as this would have given a transcript of about 1.5 kbp. When the sequence downstream of the *gp41* ORF was searched for transcription termination signals in agreement with the size of the actual transcript, two were found at residues 1717 and 1784. This suggests that in AcMNPV this transcript most likely proceeds through two other ORFs, ORF 312 and ORF 327, and terminates at or near one of the polyadenylation signals in or behind the sequence of ORF 327. This may suggest that the transcript is tricistronic. Several other polycistronic transcripts have been mapped to the AcMNPV genome (see Kool and Vlak, 1993, for review). The major *p39* capsid gene and the downstream *cg30* polypeptide gene are present on a bicistronic mRNA (Thiem and Miller, 1989). It is not clear whether both genes are translated from the same message, since *cg30* is expressed early and *p39* late (see Kool and Vlak, 1993, for review). Using baculoviruses as an expression vector, Haseman and Capra (1990) showed that downstream

ORFs on a bicistronic messenger RNA are not translated. The short intergenic regions between ORF 1227, ORF 312 and ORF 327, and the compatible size of these three ORFs with the transcript of 2.1 kbp (Whitford and Faulkner, 1992b) supports the hypothesis of a tricistronic message. In HzSNPV ORF 312 is missing (Ma *et al.*, 1993).

Regions of the *Eco*RI-C fragment that were maintained in defective genomes (Kool *et al.*, 1993a), map predominantly within ORF 2541. Further analysis of this region is now facilitated by the availability of the nucleotide sequence. This analysis may reveal *cis*-acting elements involved in the generation and/or encapsidation of the defective viruses.

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CHAPTER 5

LOCATION OF TWO PUTATIVE ORIGINS OF DNA REPLICATION OF *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS

SUMMARY

Previously, we described a defective *Autographa californica* nuclear polyhedrosis virus (AcMNPV) which must contain *cis*-acting elements required for DNA synthesis, such as origin(s) of replication (*ori*) (Kool *et al.*, 1991). Defective genomes of AcMNPV generated after serial undiluted passage were analysed further. Three small separated regions were retained in DNA of defective AcMNPV and accumulated in extracellular defective interfering viruses as well as in intracellular DNA after 40 passages. Two of these regions were now identified as containing putative *ori*'s. They were located on the *Hin*dIII-B fragment between map units (m.u.) 50.1 and 53.2, and on the *Hin*dIII-Q fragment between m.u. 87.2 and 88.9 of the physical map of AcMNPV DNA, respectively. Transfection of *Spodoptera frugiperda* cells with plasmids containing these sequences followed by superinfection with intact helper AcMNPV resulted in amplification of these plasmids, as demonstrated by the *Dpn*I sensitivity assay. The replicating activity of *Hin*dIII-Q was putatively located within the 1,000 bp region containing highly repetitive DNA (*hr*5), which is also ascribed to enhance delayed-early gene expression (Guarino and Summers, 1986).

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INTRODUCTION

Autographa californica multi-nucleocapsid nuclear polyhedrosis virus (AcMNPV) is an invertebrate virus and the type member of the family *Baculoviridae* (Francki *et al.*, 1991). AcMNPV, which is representative of the MNPV subtype in which virions may contain many nucleocapsids within a single viral envelope, has a circular, double stranded DNA genome of about 131 kbp in size (Vlak and Odink, 1979; Vlak and Smith, 1982) and replicates, like all baculoviruses, in the nucleus of infected insect cells. Progeny virus particles are occluded in large polyhedron-shaped protein crystals providing protection of the virus from environmental decay. The molecular genetics of AcMNPV are studied in much detail (Blissard and Rohrmann, 1990) due to the great interest in this virus as biological insecticide (Payne, 1988) and as an expression vector of foreign genes (Luckow and Summers, 1988; Miller, 1988).

In contrast to other large animal DNA viruses such as vaccinia-, adeno-, and herpesviruses (see Fields and Knipe, 1990, for review), the process of viral DNA replication of AcMNPV is poorly understood. A few genes have been found which are thought to be involved in AcMNPV DNA replication, such as a putative DNA polymerase (Tomalski *et al.*, 1988), a proliferating cell nuclear antigen-like protein (O'Reilly *et al.*, 1989) and a helicase (Lu and Carstens, 1991). Despite some efforts (Blinov *et al.*, 1984; Hooft van Iddekinge *et al.*, 1986), the sequences associated with the origin of AcMNPV DNA replication have not yet been detected.

Recently, defective interfering virus particles (DIP) have been described which were generated in continuous production of AcMNPV in bioreactors (Kool *et al.*, 1991) and upon serial, undiluted passage of virus in cell culture (Wickham *et al.*, 1991). The viral genome lacked considerable portions, up to 43%, of the standard viral genome extending from map unit (m.u.) 1.7 to 45.0 on the physical map of AcMNPV, but apparently retained the *cis*-acting sequences which are essential for DNA replication and encapsidation (Kool *et al.*, 1991). In this report we describe the detection and functional analysis of supermolar sequences present in defective viral DNA, that may serve as origins of replication (*ori*) of AcMNPV DNA in insect cells.

MATERIALS AND METHODS

Cells and virus

Spodoptera frugiperda (*Sf*-AE-21) cells (Vaughn *et al.*, 1977) were cultured in TNM-FH medium (Hink, 1970), supplemented with 10% fetal calf serum (FCS). The E2-strain of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) (Smith and Summers, 1978) was used as wild-type (wt) virus. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Summers and Smith, 1987).

Serial passaging of AcMNPV was done in tissue culture flasks with undiluted virus inocula. *S. frugiperda* cells (2.5×10^6) were inoculated with 0.5 ml of the undiluted virus inoculum of the previous passage. After incubation for 2 h at 27°C, unadsorbed viruses were removed by washing the cells two times with fresh medium. The cells were further incubated at 27°C for 72 h in 4 ml of medium. Virus stocks were titrated (Vlak, 1979) and the titer was expressed as the median tissue culture infective dose ($TCID_{50}$) in units per ml. Virus inocula of passage 5 (AcMNPV-P5) and 40 (AcMNPV-P40) were prepared.

DNA analysis

Extracellular virus (ECV) was purified from culture medium of infected cells by centrifugation. DNA from ECV as well as from infected *S. frugiperda* cells, denoted as intracellular DNA, was isolated 48 h post infection (p.i.) as described by Summers and Smith (1987).

ECV and intracellular DNA were digested with various restriction enzymes (*Eco*RI, *Hin*dIII and *Sst*II) (Bethesda Research Laboratories, Inc.) and the fragments were separated in a 0.8% agarose gel. DNA-fragments were isolated from the gel using the freeze squeeze technique (Sambrook *et al.*, 1989). Fragments of AcMNPV DNA were cloned into the plasmids pUC19 or pJDH119 (Hoheisel, 1989) and transfected into *Escherichia coli* JM101 using standard techniques (Sambrook *et al.*, 1989). DNA-isolation, purification in CsCl gradients, digestion with restriction enzymes, agarose gel electrophoresis, and transfer to membrane filters (Hybond-N) by the method of Southern (1975) were done using standard procedures (Sambrook *et al.*, 1989). DNA probes for hybridization were radioactively labeled by nick translation (Rigby *et al.*, 1977).

Double-stranded DNA (2-4 μg) was sequenced using the dideoxy chain-termination

method (Sanger *et al.*, 1977) following the procedures described in the protocol of the Sequenase Kit (United States Biochemicals, Inc.). The sequencing products were separated in 7 M urea- 6% acrylamide gels.

Replication assays

The assay used to test for origin function was based on the fact that, in the presence of helper AcMNPV which provides *trans*-acting viral functions, plasmids carrying an AcMNPV origin of replication will be amplified in insect cells (Stow and McMonagle, 1983). Test plasmids were amplified in *E. coli* JM101, which is Dam^+. The adenine residue within the GATC recognition sequence for *Dpn*I is thus methylated and hence sensitive to *Dpn*I digestion as *Dpn*I cleaves only methylated GATC sequences. DNA which is replicated in insect cells is not methylated at GATC sequences and therefore resistant to *Dpn*I digestion. The plasmids were purified in CsCl gradients and 1 μg of plasmid DNA was employed to transfect 2 x 10^6 *S. frugiperda* cells using lipofectin (Bethesda Research Laboratories, Inc.) (Groebe *et al.*, 1990).

The cells (in 35 mm ϕ Petri dishes) were infected with AcMNPV either 3 h before transfection or 8 h after transfection. When cells were infected before the transfection, AcMNPV was added with a multiplicity of infection (MOI) of 25 $TCID_{50}$ units per cell. After incubation of 1 h at 27°C, unadsorbed virus was removed by washing the cells with Hink's medium (Hink, 1970) without BSA and FCS. The cells were further incubated at 27°C in this medium for 2 h before transfection was carried out. When cells were infected after transfection the complete Hink's medium was replaced by Hink's medium without BSA and FCS 2 h before transfection. For transfection 1 μg plasmid DNA was mixed with 35 μl H_2O and 15 μl of lipofectin in 1 ml of medium and added to the cells. After incubation for 8 h at 27°C, the lipofectin-containing supernatant was removed by washing the cells two times with complete Hink's and the cells were superinfected with AcMNPV using a MOI of 25 $TCID_{50}$ units per cell. After 1 h of incubation 2 ml of fresh medium was added and the cells were further incubated at 27°C.

The cells were harvested 48 h after the infection with virus and total DNA from the infected cells was isolated as described by Summers and Smith (1987). The DNA was digested with the restriction enzyme *Hin*dIII. To distinguish input plasmid DNA from plasmid DNA that has replicated in insect cells, DNA was digested with *Dpn*I.

RESULTS

Serial passage of AcMNPV

Continuous production of AcMNPV in bioreactors results upon time in a 100-fold reduction of infectious ECVs and a considerable decrease of polyhedra (Kompier *et al.*, 1988) or recombinant protein production (Van Lier *et al.*, 1992). This decrease is the result of the occurrence of defective interfering virus particles, which lack a considerable part of the genome including the polyhedrin gene (Kool *et al.*, 1991). The bioreactor system mimics the serial, undiluted passage of AcMNPV in insect cells, which is known to result in aberrant polyhedra formation, such as 'few polyhedra' (FP) (Potter *et al.*, 1976; Fraser and Hink, 1982) and deletion mutants (Kumar and Miller, 1987). Since these mutants were isolated after a relatively small number of passages and plaque-purified on the basis of the occurrence of polyhedra before analysis, we analyzed the ECV and intracellular DNA after 40 serial, undiluted passages (AcMNPV-P40) in *S. frugiperda* cells.

After 40 passages of AcMNPV 95% of the cells did not produce polyhedra anymore, although all cells showed classical signs of infection, i.e. enlargement of the nuclei and formation of a ring zone at 48 h p.i. (Volkman and Keddie, 1990). The concentration of infectious ECV, expressed as $TCID_{50}$ per ml decreased from 5×10^8 at low passage numbers (< 8) to 5×10^6 at passages > 40 mimicking the results with the bioreactor producing AcMNPV continuously (Kool *et al.*, 1991; Van Lier *et al.*, 1992).

Restriction enzyme analysis of intracellular DNA

*Eco*RI digestion of DNA isolated from AcMNPV-P40 infected cells (IC) and analysis in 0.8% agarose gels showed the presence of seven supermolar fragments (AE-1 to AE-7) (Fig. 5.1, lane 3). Four of these fragments had the same electrophoretic mobility as *Eco*RI-L or -M (AE-1), *Eco*RI-P (AE-2), *Eco*RI-R or -S (AE-5) and *Eco*RI-W or -X (AE-7). The other three fragments had sizes of 1.85 kbp (AE-3), 1.65 kbp (AE-4) and 1.2 kbp (AE-6) and did not comigrate with any of the authentic AcMNPV *Eco*RI fragments (Fig. 5.1, lane 1). Fragments AE-2, -3, -5, -6, and -7 were present in comparable amounts, whereas fragments AE-1 and AE-4 were present in much lower amounts. This contrasted strongly with the restriction pattern of AcMNPV-P5 ECV DNA which displayed the normal distribution of fragments (Fig. 5.1, lane 1). In AcMNPV-P40 DNA the fragments AE-1 to -7 were

superimposed on a background of *Eco*RI fragments of AcMNPV DNA (lane 1) which in turn were superimposed on host cell DNA (lane 3).

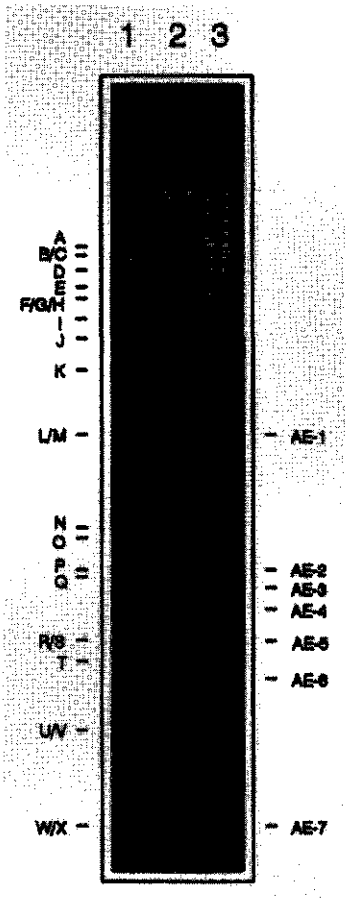

Figure 5.1. Analysis of intracellular supermolar *Eco*RI fragments in AcMNPV-infected *S. frugiperda* cells. Restriction enzyme analysis of DNA from extracellular AcMNPV P5 (lane 1) and P40 (lane 2), and of intracellular DNA from AcMNPV-P40 infected *S. frugiperda* cells (lane 3). DNA was digested with *Eco*RI, separated in a 0.8% agarose gel, stained with ethidium bromide, and visualized with UV light. At left are the normal restriction fragment band designations of *Eco*RI, and at right are indicated the *Eco*RI fragments of P40.

When DNA was isolated from AcMNPV-P40 ECV and digested with *Eco*RI, these seven *Eco*RI fragments, as in intracellular DNA, were also present in the ECV DNA, but in much lower concentration relative to other viral fragments (Fig. 5.1, lane 2). In this ECV preparation all the original *Eco*RI fragments of AcMNPV represented the complete genome of AcMNPV. This authentic virus in the medium of AcMNPV-P40 infected cells served as a helper for defective AcMNPV.

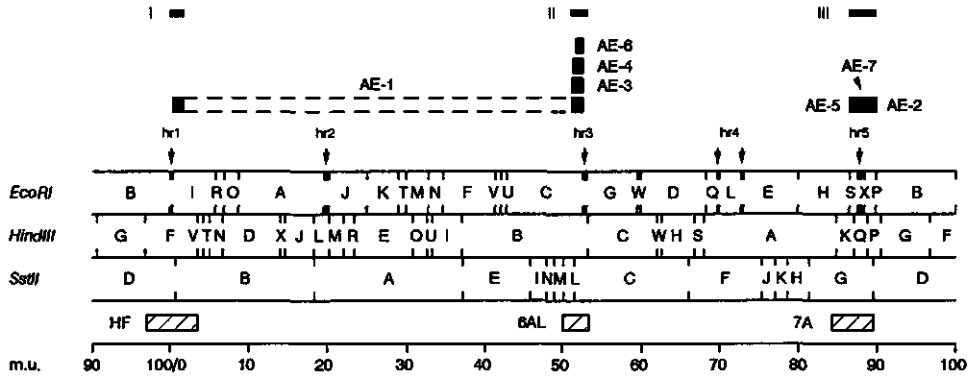

Figure 5.2. Physical map of the AcMNPV genome (strain E2, Smith and Summers, 1978; Cochran *et al.*, 1982) for restriction enzymes *Eco*RI, *Hin*dIII and *Sst*II, and location of the supermolar *Eco*RI (AE-1 to AE-7) fragments. Dashed lines represent joint fragments. The *hr* regions 1-5 are indicated. The regions I - III are indicated and the clones used in the replication assay are shown by hatched bars.

Hybridization of supermolar AcMNPV fragments

To investigate the nature of the *Eco*RI fragments AE-1 to AE-7 from AcMNPV-P40 infected cells, these fragments were isolated from the agarose gel, radio-actively labeled with ^{32}P-dATP and used separately as probes on Southern blots with *Eco*RI, *Hin*dIII and *Sst*II digests of AcMNPV-P5 DNA. All AE fragments hybridized with AcMNPV-P5 ECV DNA and did not with host DNA indicating the viral nature of these fragments. Although some *Eco*RI fragments and also some *Hin*dIII fragments of AcMNPV-P5 DNA comigrated in an agarose gel, the hybridization patterns gave an unequivocal answer as to the nature, identity and location of AE-1 to AE-7 (Fig. 5.2).

Fragment AE-1 hybridized both to *Eco*RI-I and *Eco*RI-C, and to *Hin*dIII-B and -F suggesting that this 3.7 kbp fragment is a fragment joining the left-hand end of *Eco*RI-I and the right-hand end of *Eco*RI-C (Fig. 5.2). This structure was confirmed by sequencing the ends of AE-1. The presence of this junction fragment and the fact that no hybridization was detected with any of the other *Eco*RI-fragments with sequences between m.u. 3.6 (the right-hand limit of *Hin*dIII-F) and m.u. 50.1 (the left-hand end of *Sst*II-L), suggested that the chimeric nature of AE-1 is a result of a major deletion.

Fragments AE-3, AE-4 and AE-6 hybridized to *Eco*RI-C, *Hin*dIII-B and *Sst*II-C, whereas AE-3 and AE-4 also shared homology with *Sst*II-L. This located fragments AE-3, AE-4 and AE-6 at the *Sst*II-L and -C junction at m.u. 52.5. Cloning, mapping and sequencing of the ends of these three fragments revealed that they have extensive overlap, with coterminal ends at m.u. 53, but variable left-hand ends (Fig. 5.2).

Fragments AE-2, AE-5 and AE-7, of similar size as *Eco*RI-P, -R or -S and -W or -X respectively (Fig. 5.1), hybridized with *Eco*RI fragments of AcMNPV-P5 ECV DNA of similar size. Sequencing of AE-2, AE-5 and AE-7 confirmed their identity as *Eco*RI-P, -S and -X.

Analysis of these seven supermolar *Eco*RI fragments indicated that during serial, undiluted passage of AcMNPV in *S. frugiperda* cells, three separated, but distinct regions of the AcMNPV genome, designated regions I, II and III, were retained. The same three regions were also found to be retained in defective genomes of a recombinant of AcMNPV after 40 serial, undiluted passages. The three regions are located in close proximity to the homologous regions *hr*1, *hr*3 and *hr*5 (Cochran and Faulkner, 1983), respectively (Fig. 5.2). Since AE-2, AE-5 and AE-7 form a contiguous segment except for *hr*5 and since *Eco*RI fragmented *hr*5 due to the numerous recognition sites (Cochran and Faulkner, 1983), it is assumed that region III includes *hr*5. The presence of *hr*1 in region I and *hr*3 in region II is unclear, since no fragments hybridizing 'across' *hr*1 or *hr*3 could be observed. We cannot rule out the possibility that they are part of these regions in the defective viral DNA's.

In analogy with a similar phenomenon after serial passage of other DNA viruses (Roizman and Sears, 1990), certain regions of AcMNPV are likely to be retained in defective viral DNA, because they contain *cis*-acting elements of DNA replication, such as an origin or terminus. In order to investigate whether this is the case, AcMNPV fragments encompassing the entire regions I, II and III were cloned into individual plasmids and tested for their replication capabilities in a replication assay (Fig. 5.2 and 5.3).

Replication assays

The following constructs were employed in the replication assay using *S. frugiperda* cells. To make sure that these constructs contained the entire region spanning the supermolar fragments, some additional flanking sequences were included. *Hin*dIII-F containing region I, which includes *hr*1 (m.u. 96.8 - 100/0 - 3.6; Fig. 5.2) was cloned into pBR322 to give

plasmid pAcHF. To obtain region II, a 2260 bp *Sst*II-*Hin*dIII fragment (m.u. 50.1 - 53.2) of AcMNPV including *hr*3 was transferred into plasmid pJDH119 resulting in plasmid pAc6A. By inserting the 1905 bp *Sst*II-L fragment in the *Sst*II-site in the authentic orientation with respect to the AcMNPV physical map (Vlak and Smith, 1982) a plasmid pAc6AL was obtained with an insert spanning the entire region II and sufficient flanking sequences. The 7084 bp *Bam*HI-*Sst*II fragment (m.u. 84.3 - 89.7) spanning region III was isolated from AcMNPV and cloned in pJDH119 to create pAc7A which contained *hr*5 (Fig. 5.2).

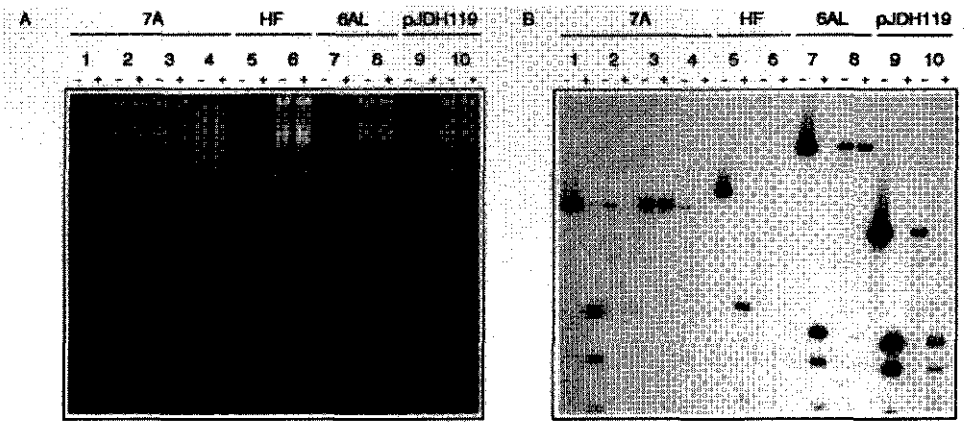

Figure 5.3. Replication activity of AcMNPV DNA regions I - III. Restriction enzyme (A) and Southern blot (B) analysis of AcMNPV plasmids pAc7A, pAcHF and pAc6AL (lanes 1, 5 and 7), control plasmid pJDH119 (lanes 9), and cellular DNA of uninfected (lanes 4) and AcMNPV-infected (lanes 2, 3, 6, 8 and 10) *S. frugiperda* cells isolated 48 h p.i. and digested with *Hin*dIII (-) or with *Hin*dIII plus *Dpn*I (+). *S. frugiperda* cells were infected with AcMNPV 3 h prior to transfection (lanes 2), or 8 h after transfection (lanes 3, 6, 8 and 10) or not infected at all after transfection (lanes 4). Transfections were carried out with plasmid pAc7A (region III) (lanes 2-4), plasmid pAcHF (region I) (lanes 6), plasmid pAcAL6 (region II) (lanes 8) and plasmid pJdH119 (control) (lanes 10). Southern hybridization was carried out using ^{32}P-labeled pUC19 as a probe.

S. frugiperda cells were transfected with 1 μg of each plasmid using the lipofectin method (Groebe *et al.*, 1990) and, 8 h after transfection, superinfected with AcMNPV (Fig. 5.3, lanes 3, 6, 8 and 10). Total cell DNA was extracted, digested with *Hin*dIII alone (-) or with

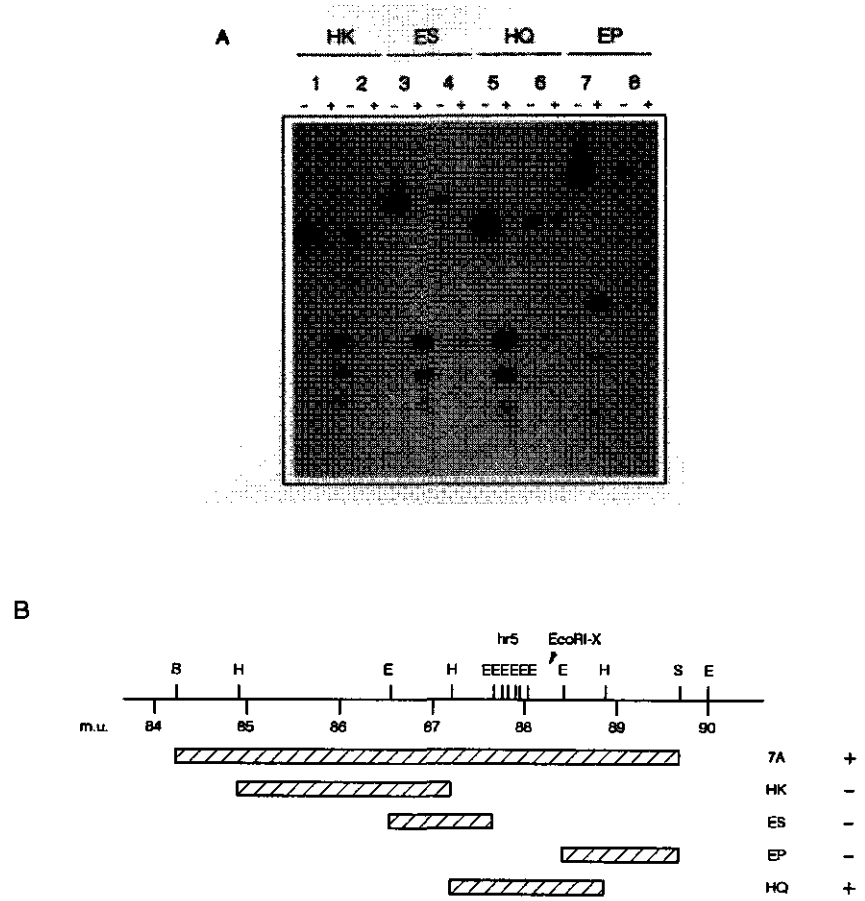

Figure 5.4. Southern blot analysis (A) and mapping (B) of the origin of replication in region III. Southern blot analysis of AcMNPV plasmids pAcHK, pAcES, pAcHQ and pAcEP (lanes 1, 3, 5 and 7) and AcMNPV-infected cellular DNA (lanes 2, 4, 6 and 8) treated with *Hin*dIII (-) or with *Hin*dIII plus *Dpn*I (+). *S. frugiperda* cells were infected with AcMNPV 8 h after transfection with pAcHK (*Hin*dIII-K) (lanes 2), pAcES (*Eco*RI-S) (lanes 4), pAcHQ (*Hin*dIII-Q) (lanes 6) and pAcEP (*Eco*RI-P) (lanes 8). Detailed physical map (B) of region III of the AcMNPV genome with the various fragments found positive (+) or negative (-) in the replication assay. B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII, S = *Sst*II.

*Hin*dIII and *Dpn*I (+), and analyzed by agarose electrophoresis (Fig. 5.3A) and Southern blot hybridization (Fig. 5.3B) using ^{32}P-labeled pUC19 as a probe. Methylated plasmid DNA digested with *Hin*dIII (-) or with *Hin*dIII + *Dpn*I (+) was included in the analysis (Fig. 5.3,

lanes 1, 5, 7 and 9) to locate the position of *Dpn*I insensitive and sensitive plasmid sequences.

Plasmids pAc7A (region III) and pAc6AL (region II) were amplified in infected *S. frugiperda* cells (Fig. 5.3, lanes 3 and 8), as indicated by the *Dpn*I-resistant *Hin*dIII fragments following digestion of total cell DNA. This amplified plasmid was even directly visible in ethidium bromide-stained gels (Fig. 5.3A, lanes 3 and 8). The plasmids alone did not show replicating activity in insect cells, as is shown only for pAc7A (Fig. 5.3, lanes 4). It remained sensitive to *Dpn*I, indicating that functions provided in *trans* by a helper virus are essential for replication. Hybridization with ^{32}P-labeled pUC19 (Fig. 5.3B) was carried out to detect only the replicated and input plasmid DNA without the background of AcMNPV helper DNA.

Plasmid pAcHF did not replicate in the presence of helper AcMNPV in *S. frugiperda* cells (Fig. 5.3, lanes 6). In this case the plasmid detected in the cells by hybridization represented only input material and remained *Dpn*I sensitive. A similar result was obtained when the parental plasmid pJDH119 lacking AcMNPV sequences was used (lanes 10). The latter experiment also confirmed that *Dpn*I resistance was not the result of cellular inhibition of *Dpn*I activity.

When *S. frugiperda* cells were infected with AcMNPV (MOI = 25) 3 h *before* transfection with test plasmids, no amplification of any of the plasmids with DNA sequences of AcMNPV could be detected. The region III containing plasmid pAc7A, for example, remained sensitive to *Dpn*I (Fig. 5.3, lanes 2), whereas it was amplified when transfected well before infection (lanes 3). The former procedure is more routinely used to demonstrate replication activity of transfer plasmids (Handermann *et al.*, 1992). The lack of amplification when administered after infection was not due to breakdown since the plasmids were taken up by the insect cells and remained intact for at least 48 h (Fig. 5.3, lanes 2).

Region III was further investigated to locate the replicating activity in more detail. Several subfragments of the *Bam*HI-*Sst*II insert of plasmid pAc7A (*Hin*dIII-K, *Hin*dIII-Q and *Eco*RI-S) were cloned into pUC19 (giving pAcHK, pAcES and pAcHQ, respectively) and tested for replication in *S. frugiperda* cells (Fig. 5.4). An *Eco*RI-*Sst*II fragment, which is a subfragment of *Eco*RI-P was isolated from pAc7A and cloned into pJDH119 resulting in plasmid pAcEP. Only pAcHQ (*Hin*dIII-Q) was amplified, as indicated by the presence of a *Dpn*I-resistant *Hin*dIII fragment (Fig. 5.4, lanes 6); the other plasmids, pAcHK (lanes 2),

pAcES (lanes 4) and pAcEP (lanes 6) were not. These results indicated that the putative *ori* is located in *Hin*dIII-Q and most likely in the *hr*5 region or in *Eco*RI-X (Fig. 5.4B). Plasmid pAc6A, lacking the *Sst*II-L fragment in pAc6AL, was also amplified in infected cells as evidenced by *Dpn*I resistance (data not shown). These data suggest that a putative *ori* in this region is located in the 2260 bp *Sst*II-*Hin*dIII fragment, which includes *hr*3.

DISCUSSION

We demonstrated that two segments of the AcMNPV genome induced amplification of test plasmids in *S. frugiperda* cells in the presence of helper AcMNPV. One of these segments is located between m.u. 87.2 and 88.9 on the physical map of AcMNPV DNA (Fig. 5.2), and includes the 500 bp region of *hr*5 and *Eco*RI-X. The *hr* region contains highly repetitive DNA and multiple *Eco*RI sites (Cochran and Faulkner, 1983). Since *Hin*dIII-Q was amplified in *S. frugiperda* cells in the presence of helper AcMNPV and *Eco*RI-P and -S were not (Fig. 5.4), *hr*5 and/or *Eco*RI-X is a most likely candidate segment to contain at least one *ori* of AcMNPV. The other segment with *ori* activity is located in a region extending from m.u. 50.1 - 53.2 and includes *hr*3 (Fig. 5.2). Sequences of both regions were maintained in defective interfering AcMNPV, which was obtained after serial propagation of AcMNPV in bioreactors (Kool *et al.*, 1991) and in the form of supermolar intracellular viral DNA (Fig. 5.1). However, from the analysis of supermolar fragments it is not clear whether *hr*3 is retained, since they did not hybridize with *Hin*dIII-G. Sequences of a third region that was retained in these viral forms did not show replicating activity in infected insect cells, but may have another, yet undefined function in AcMNPV DNA replication.

The *ori* activity in region III appears to be located in a 1,000 bp segment containing *hr*5 and *Eco*RI-X (Fig. 5.4B). *Hr*5 is known as enhancer of delayed early gene expression (Guarino and Summers, 1986) and it has been hypothesized that *hr*'s may serve as *ori* (Cochran and Faulkner, 1983). *Hr*5 contains multiple 30 bp near-perfect inverted repeats with only 2-bp mismatches. Computer-assisted analysis of *hr*5 showed various stem-loop structures in this A-T rich sequence. Such structures are characteristic for *ori*'s of other DNA viruses (Camp *et al.*, 1991). One copy of such an inverted repeat is sufficient for enhancer function

(Guarino *et al.*, 1986). If *hr*5 also has a function as *ori*, then this region is bifunctional. In simian virus 40 and polyoma virus enhancer sequences also function as *ori* (Bergsma *et al.*, 1983; Tyndall *et al.*, 1981).

Despite the high degree of homology between the five *hr* regions (Cochran and Faulkner, 1983; Guarino *et al.*, 1986), in this study *ori* activity was only detected from those AcMNPV segments containing *hr*5 and *hr*3. It is possible, however, that the other *hr* regions are also serving as *ori*, but that their loss in defective AcMNPV is compensated for by the amplification of other *hr* sequences, such as *hr*5. Direct testing of each of the other *hr* regions in the replication assay should answer this question.

*Eco*RI-X is also maintained in region III in most of the defective AcMNPV (Fig. 5.4). Most of this fragment contains an open reading frame coding for a 26 kDa protein of unknown function (Liu *et al.*, 1986). It is interesting to note that it appeared impossible to retrieve insertional mutants of the *p26* gene of AcMNPV (R.P. Possee, personal communication). This also corroborates with an *ori*-related function of *Eco*RI-X. Additional sequences flanking the 1,000 bp segment may have auxiliary functions. By using deletion mutagenesis of this 1,000 bp segment and *Hin*dIII-Q the sequences and functional domains essential for *ori* activity will be determined.

Our results indicate that at least two potential *cis*-acting sequences on the AcMNPV genome appear to serve as *ori*. These sequences differ from an *ori* sequence reported from a closely related variant of AcMNPV, *Galleria mellonella* NPV (Blinov *et al.*, 1984). Plasmids with this *ori* sequence were claimed to replicate autonomously in SCLd135 insect cells, i.e. without a helper virus, based on the persistence of plasmids containing this putative *ori* rather than on replication. The two putative AcMNPV *ori* sequences reported here also differed from those sequences (m.u. 25 - 29 and 34.5 - 37) showing autonomous replicating activity in yeast (Hooft van Iddekinge *et al.*, 1986), but which are deleted in the defective viruses (Kool *et al.*, 1991). The presence of more than one *ori* is not unusual in large DNA viruses, such as herpesviruses (Roizman and Sears, 1990).

In herpes simplex virus type 1 (Spaete and Frenkel, 1982; Stow and McMonagle, 1983) and Marek's Disease virus (Camp *et al.*, 1991) defective DNA molecules consist of concatemers of repeated sequences. There is evidence that the seven supermolar viral *Eco*RI AcMNPV fragments are organized in high molecular weight forms, most likely arranged in concatemers (Lee and Krell, 1992). Some of the supermolar fragments (AE-1 and AE-4; Fig.

5.1) were not present in equimolar amounts suggesting that there is some variation in regions non-essential for replication. Further analysis of the defective intracellular forms of AcMNPV may shed light on this question.

Plasmids containing *hr*5 and/or *Eco*RI-X and the p10 promoter, such as pAcAs3 (Vlak *et al.*, 1990), would now become useful in transient assays for foreign gene expression in insect cells due to the amplification of the plasmid, and thus an increase of gene dose, in the presence of helper AcMNPV.

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CHAPTER 6

IDENTIFICATION OF SEVEN PUTATIVE ORIGINS OF *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS DNA REPLICATION

SUMMARY

Seven putative origins of DNA replication (*ori's*) were identified and located on the genome of *Autographa californica* nuclear polyhedrosis virus (AcMNPV), when an improved infection-dependent replication assay was used. A threefold higher yield of amplified plasmid was achieved when a MOI of 1 was used (instead of 25), and another twofold increase was obtained when the interval between transfection and infection was extended from 5 to 24 h. Six of the putative *ori's* were located in *hr* regions with homologous sequences. This suggests that all *hr's* in AcMNPV are bifunctional, i.e. have both *ori* activity and enhancer activity for transcription (Guarino *et al.*, 1986). In addition to the six *hr's*, the *Hin*dIII-K fragment of AcMNPV was also identified to carry a putative *ori*, although this fragment does not contain an *hr* region. However, the individual role of these seven *ori's* during viral DNA replication, and whether they are all active simultaneously *in vivo*, is still unclear. The replication of an *ori*-containing plasmid starts at the same time (6 h p.i.) and proceeds at the same rate as viral DNA replication. A circular topology of *ori*-containing plasmids was a prerequisite for replication. Linear DNA, with an *ori*, did not replicate. Therefore, we suggest a theta structure or a rolling-circle as a model for baculovirus DNA replication.

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INTRODUCTION

The genome of *Autographa californica* multiple-nucleocapsid nuclear polyhedrosis virus (AcMNPV) is a double-stranded, circular, covalently closed, supercoiled DNA molecule of approximately 131 kilobase pairs (kbp). The viral DNA consists primarily of unique sequences with the potential to encode more than 75 average-sized proteins (for reviews, see O'Reilly *et al.*, 1992; Kool and Vlak, 1993). In addition, six homologous regions, designated *hr*1 through *hr*5 (*hr*4 contains two distinct *hr's*, designated *hr*4a and *hr*4b), of approximately 500 to 800 bp in length are interspersed at specific sites along the length of the genome (Cochran and Faulkner, 1983; Guarino *et al.*, 1986).

These *hr* regions contain sequence repeats centred around *Eco*RI sites, separated by 72 to 215 bp. Initial studies demonstrated that the *hr* regions function as enhancers for transcription, when placed in *cis* to the promoter of the AcMNPV delayed early gene *39k* and *trans*-activated by the *ie-1* gene (Guarino *et al.*, 1986; Guarino and Summers, 1986). These results suggested that the *hr* enhancer function is dependent on *ie-1 trans*-activation but subsequent studies with the *p35* gene (Nissen and Friesen, 1989) and *ie-n* gene (Carson *et al.*, 1991) have shown that the *hr* region can also increase the expression of early genes in the absence of *ie-1*.

Homologous regions have been identified in four other baculoviruses to date, *Choristoneura fumiferana* MNPV (Arif and Doerfler, 1984; Kuzio and Faulkner, 1984), *Lymantria dispar* MNPV (McClintock and Dougherty, 1988), *Bombyx mori* MNPV (Maeda and Majima, 1990), and *Orgyia pseudotsugata* MNPV (Theilmann and Stewart, 1992). However, only for OpMNPV is it known that the identified *hr* regions can function as enhancer elements (Theilmann and Stewart, 1992).

Repeated sequences have also been described for a number of other eukaryotic DNA viruses. Unlike the baculovirus *hr's*, repeated sequences of vertebrate viral genomes usually consist of perfect or nearly perfect repeats of similar size. A variety of functions have been ascribed to such repeated sequences: the direct repeats of simian virus 40 and polyoma virus have a dual function, since both serve as enhancers of gene expression and as origins (*ori*) of DNA replication (Bergsma *et al.*, 1983; de Villiers *et al.*, 1984); the replication of adenovirus DNA is initiated within the inverted terminal repeats (Tamanoi and Stillman, 1983), whereas in herpesvirus genomes terminal repeats are required for circularization

during replication (Kitner and Sugden, 1979).

Recently, defective interfering virus particles (DIPs) have been described which are generated in continuous production of AcMNPV in bioreactors or upon serial, undiluted passage of virus in cell culture (Kool *et al.*, 1991; Wickham *et al.*, 1991; Lee and Krell, 1992). The defective viral genomes lack considerable portions of the standard viral genome, but apparently have retained the *cis*-acting sequences essential for DNA replication and encapsidation. At least two segments of the AcMNPV genome, containing the *hr*3 and *hr*5 regions, were maintained in defective genomes and could possibly serve as an origin of DNA replication (Kool *et al.*, 1993). *Hr*1 which was also present in the DIPs was found to be negative in the replication assay used. Pearson *et al.* (1992) showed that *hr*2 and *hr*5 could serve as origins of DNA replication and demonstrated in addition that a part of *hr*5, containing a single, complete palindrome, was sufficient to promote replication.

In this paper we investigated whether all known *hr* regions can function as *ori* in an infection-dependent replication assay, and whether the negative result for *hr*1 is due to the conditions used in this assay. Therefore, the AcMNPV replication assay was optimized and all six known *hr* regions and adjacent sequences were tested for replication activity. The results indicated that all six *hr* regions as well as one additional non-*hr* sequence can, in principle, serve as origins of DNA replication of AcMNPV. However, it is not known whether the DNA replication of the AcMNPV genome involves the use of all these origins.

MATERIALS AND METHODS

Cells and virus.

Spodoptera frugiperda (*Sf*-AE-21) cells (Vaughn *et al.*, 1977) were cultured in TNM-FH medium (Hink, 1970), supplemented with 10% fetal calf serum (FCS). The E2-strain of *Autographa californica* multi-nucleocapsid nuclear polyhedrosis virus (AcMNPV) (Smith and Summers, 1978) was used as wild-type (wt) virus. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Summers and Smith, 1987).

Plasmid constructions.

AcMNPV fragments containing *hr* regions and surrounding sequences were cloned into pBR322, pUC19 or pJDH119 (Hoheisel, 1989), in *Escherichia coli* JM101 using standard techniques (Sambrook *et al.*, 1989). DNA-isolation, purification in CsCl gradients, digestion with restriction enzymes, and agarose gel electrophoresis were carried out using standard procedures (Sambrook *et al.*, 1989).

Replication assays.

The assay used to test for *ori* function was based on the observation that, in the presence of helper AcMNPV which provides *trans*-acting viral functions, plasmids carrying an AcMNPV origin of replication will be amplified in insect cells (Kool *et al.*, 1993; Stow and McMonagle, 1983). Test plasmids were amplified in *E. coli* JM101, which is Dam^+. The adenine residue within the GATC recognition sequence for *Dpn*I is thus methylated and hence sensitive to *Dpn*I digestion as *Dpn*I cleaves only methylated GATC sequences. DNA that is replicated in insect cells is not methylated at GATC sequences and therefore resistant to *Dpn*I digestion. The plasmids were purified in CsCl gradients and 1 μg of plasmid DNA was employed to transfect 2 x 10^6 *S. frugiperda* cells using lipofectin (Bethesda Research Laboratories, Inc.) (Groebe *et al.*, 1990).

The replication assay was performed basically as described previously (Kool *et al.*, 1993), but a few modifications were made. Before transfection the Hink's medium was removed and the cells were washed with Hink's medium without BSA and FCS. For transfection 1 μg plasmid DNA was mixed with 35 μl H_2O and 15 μl of lipofectin in 1 ml of this medium and added to the cells. After incubation for 6 h at 27°C, the lipofectin-containing supernatant was removed by washing the cells twice with complete Hink's medium. The cells were further incubated in 2 ml of complete Hink's medium at 27°C. The next day, or 24 h after transfection, the cells were superinfected with AcMNPV using a MOI of 1 $TCID_{50}$ unit per cell. After 1 h of incubation 2 ml of fresh medium was added and the cells were further incubated at 27°C.

DNA analysis.

The cells were harvested 48 h post infection with AcMNPV and total DNA was isolated from infected cells as described by Summers and Smith (1987). The DNA was digested with

the restriction enzyme *Hin*dIII. To distinguish input plasmid DNA from plasmid DNA that has replicated in insect cells, DNA was digested with *Dpn*I. After gel electrophoresis, the DNA was transferred to membrane filters (Hybond-N) by the method of Southern (1975). DNA probes for hybridization were radioactively labeled by nick-translation (Rigby *et al.*, 1977).

RESULTS

Optimization of the replication assay

As reported previously, two segments of the AcMNPV genome, containing *hr*3 and *hr*5, have been identified as harbouring putative *ori's* of DNA replication (Kool *et al.*, 1993). Since all *hr* regions have homologous sequences, it was logical to assume that they all could serve as *ori's* and in view of their transcriptional enhancing activity (Guarino *et al.*, 1986) would be bifunctional. However, when the *Hin*dIII-F fragment of AcMNPV, containing *hr*1, was tested for replication activity, no replication of the plasmid was found (Kool *et al.*, 1993). In addition, under the conditions of the replication assay described by Pearson *et al.* (1992) we were unable to detect replication of our test plasmids. This prompted us to re-evaluate and optimize the assay, before testing all *hr* regions.

Starting with the assay conditions, established and discussed in a previous paper (Kool *et al.*, 1993), different amounts of plasmid were used for transfection, the MOI of helper AcMNPV used for superinfection was varied, and different time intervals between infection and plasmid transfection were tested (Fig. 6.1). The plasmid used for these experiments was pAc7A (Kool *et al.*, 1993), which contains *hr*5. Increase of the amount of transfected plasmid to 2 or 4 μg gave no higher yield of replicated plasmid, but resulted in a decrease of replication efficiency (Fig. 6.1A). Amounts of less than 1 μg were not further tested as the hybridization signal of the input plasmid then became too weak. Hence, for all the following experiments 1 μg of plasmid was used as the standard amount for transfection. The Southern hybridizations with pUC19 as probe also gave an non-specific signal of about 2.4 kbp with viral DNA, but this signal could easily be distinguished from the signals caused by hybridization to the plasmid with a size of 3.7 kbp (Fig. 6.1C). When transfections were preceded by AcMNPV infection with a MOI of 25, no plasmid replication could be detected

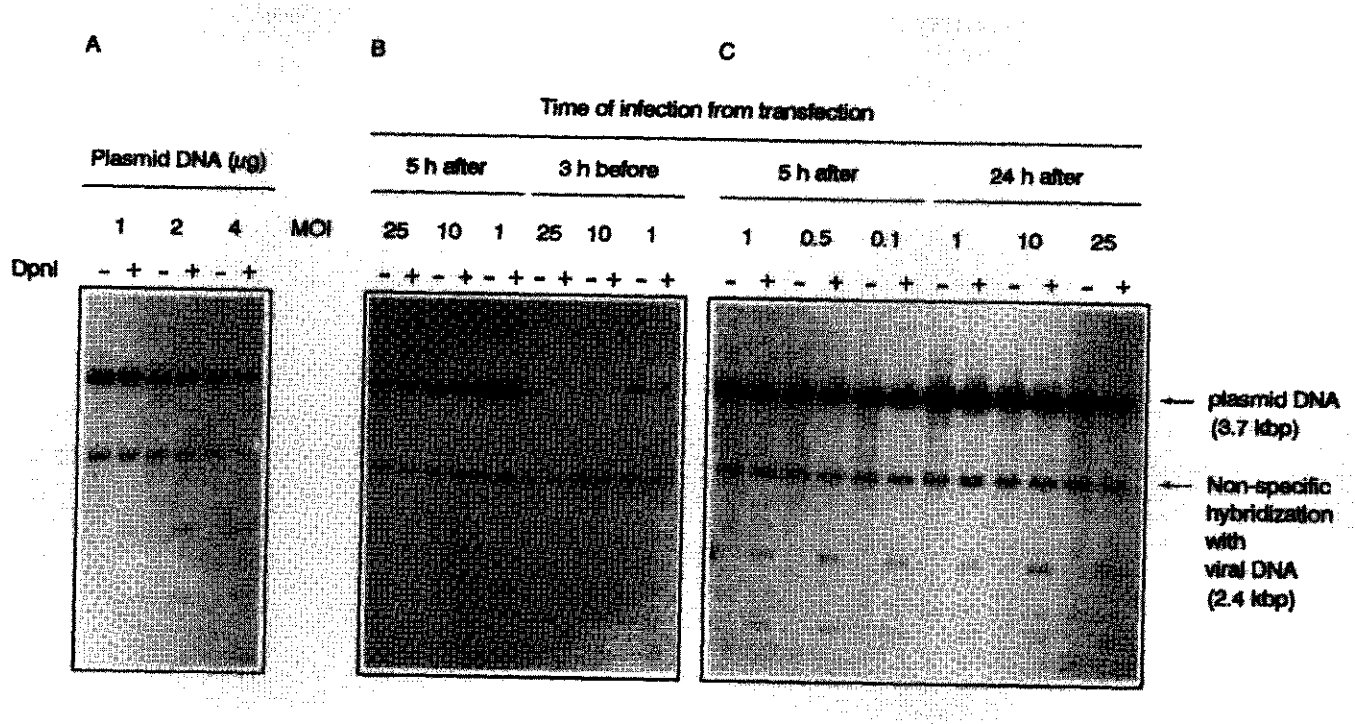


Figure 6.1. Optimization of the DNA replication assay of AcMNPV *ori*-containing plasmids in insect cells. A. *S. frugiperda* cells were transfected with 1, 2 or 4 μg plasmid DNA, and infected with AcMNPV (MOI = 10) 5 h post transfection. B. *S. frugiperda* cells were transfected with 1 μg plasmid DNA and infected with AcMNPV (MOI = 25, 10 or 1) 3 h prior to transfection or 5 h after transfection. C. *S. frugiperda* cells were transfected with 1 μg plasmid DNA and infected with AcMNPV (MOI = 1, 0.5 or 0.1) 5 h after transfection or 24 h after transfection (MOI = 1, 10 or 25). For all transfections cellular DNA was isolated 48 h p.i. and digested with *Hin*dIII with (+) or without (-) *Dpn*I. Southern blot hybridization was carried out using ^{32}P-labeled pUC19 as a probe.

(Kool *et al.*, 1993). This result was also obtained when a MOI of 10 was used (Fig. 6.1B). Surprisingly, at a MOI of 1, replication did occur, although at a low level. Much higher levels of replication were obtained when the infection was carried out after the transfection, with the highest level at a MOI of 1 (Fig. 6.1B). No further improvement of replication level was obtained when infections were carried out 5 h after transfection with a MOI of 1, 0.5 or 0.1 (Fig. 6.1C). On the other hand, when infection was performed 24 h after transfection, this resulted in higher levels of replication, especially when a MOI of 1 was used (Fig. 6.1C). Therefore, for all further experiments cells were transfected with 1 μg of plasmid and, 24 h later, infected with AcMNPV with a MOI of 1.

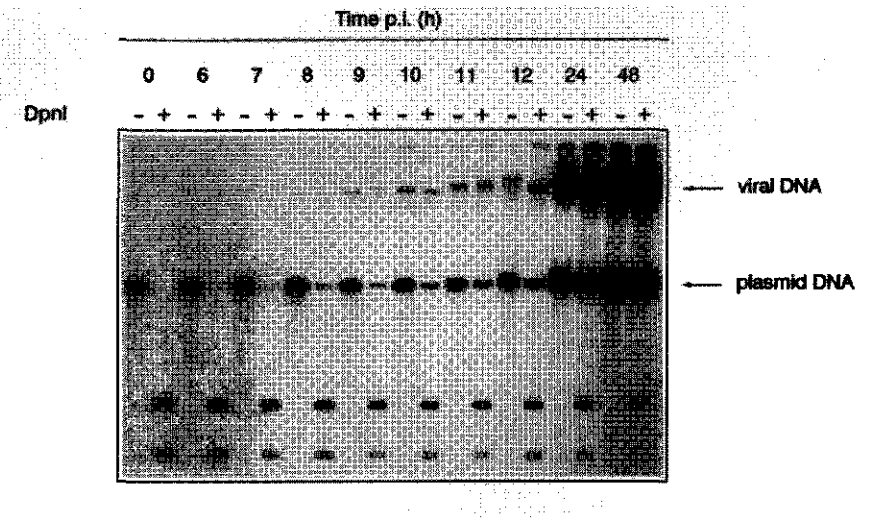
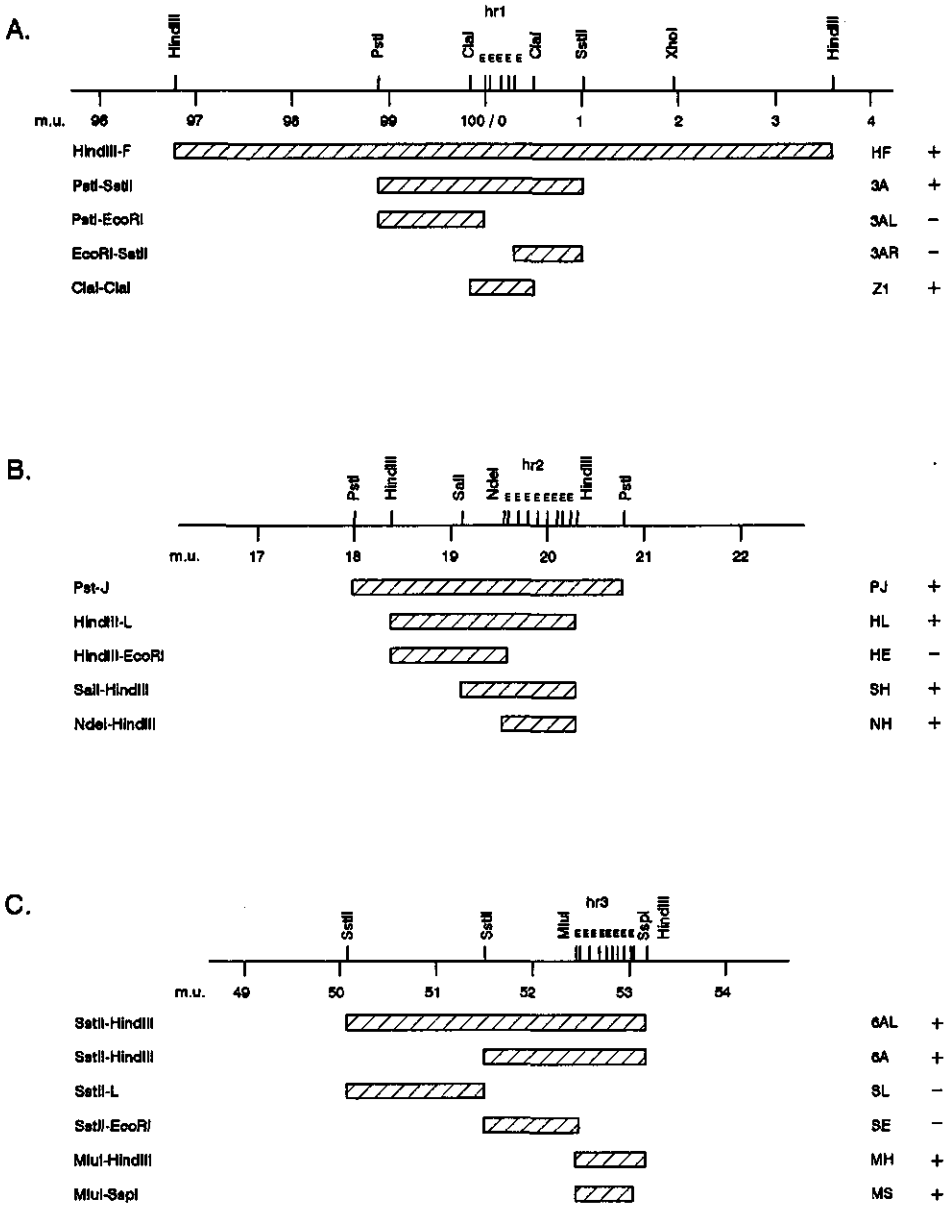


Figure 6.2. Temporal replication of plasmid pAc7A in insect cells as compared to viral DNA replication. *S. frugiperda* cells were transfected with 1 μg of plasmid pAc7A DNA and infected with AcMNPV (MOI = 1) 24 h after transfection. Cellular DNA was isolated at 0, 6, 7, 8, 9, 10, 11, 12, 24, and 48 h after infection and digested with *Hin*dIII with (+) or without (-) *Dpn*I. Southern blot hybridization was carried out using the ^{32}P-labeled *Sst*I-G fragment, cloned in pUC19, as a probe.

Time course of replication

To gain more insight in the replicative behavior of a plasmid containing an *ori*, the amplification of plasmid pAc7A was followed and compared with the replication of the wild-type viral genome. Ten 35 mm dishes with *S. frugiperda* cells were transfected with 1 μg of


A.
hr1
HindIII
PstI
ClaI
ClaI
SstII
XhoI
HindIII
m.u.
96
97
98
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100 / 0
1
2
3
4
HindIII-F
PstI-SstII
PstI-EcoRI
EcoRI-SstII
ClaI-ClaI
HF +
3A +
3AL −
3AR −
Z1 +
B.
hr2
PstI
HindIII
SalI
NdeI
HindIII
PstI
m.u.
17
18
19
20
21
22
Pst-J
HindIII-L
HindIII-EcoRI
SalI-HindIII
NdeI-HindIII
PJ +
HL +
HE −
SH +
NH +
C.
hr3
SstII
SstII
MluI
SapI
HindIII
m.u.
49
50
51
52
53
54
SstII-HindIII
SstII-HindIII
SstII-L
SstII-EcoRI
MluI-HindIII
MluI-SapI
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6A +
SL −
SE −
MH +
MS +

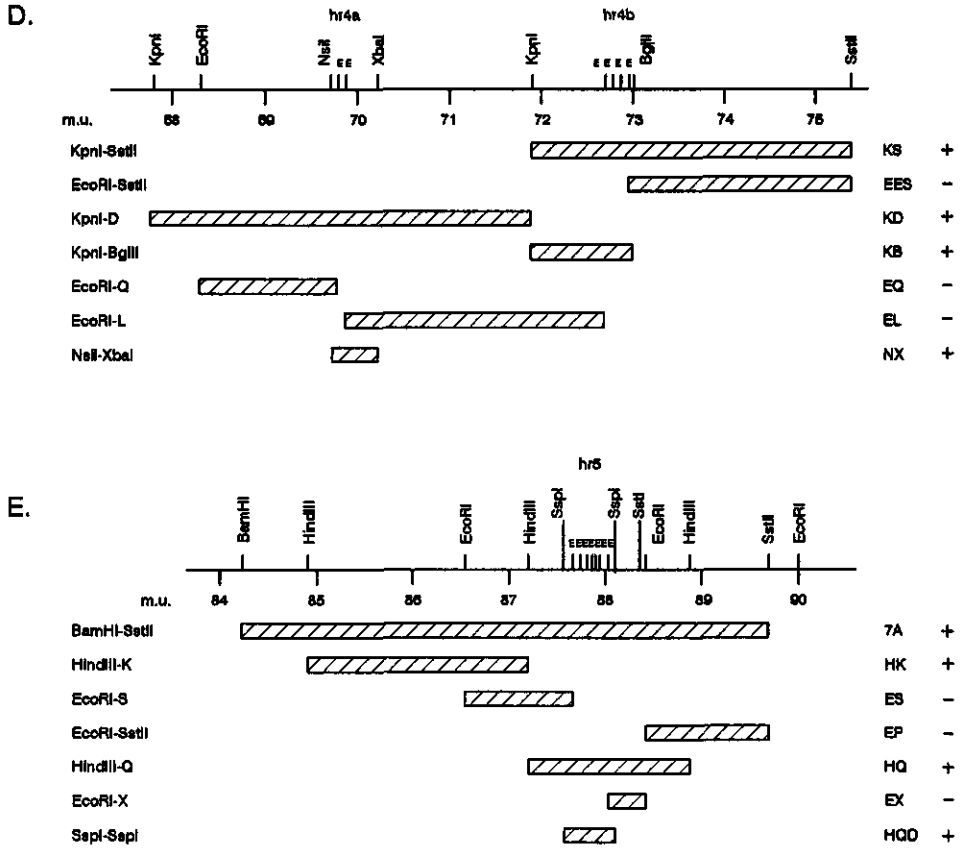

Figure 6.3. Physical maps of all then known *hr* regions of the AcMNPV genome with the various fragments (hatched bars) found positive (+) or negative (-) in the replication assay. E = *Eco*RI; m.u. = map unit (see Kool and Vlak, 1993).

pAc7A, and infected with AcMNPV (MOI = 1) 24 h later. The cells were subsequently incubated and harvested after 1, 6, 7, 8, 9, 10, 11, 12, 24 and 48 h after infection, respectively. Newly replicated plasmid and viral DNA was analyzed by digestion with *Hin*dIII with and without *Dpn*I, and monitored by hybridization using the *Sst*I-G fragment cloned in pUC19 as a probe (Fig. 6.2). The choice for *Sst*I-G was based on its length, which closely corresponds with that of pUC19, and therefore will give similar hybridization signals for both the viral DNA and plasmid DNA. Progeny viral DNA and newly amplified plasmid

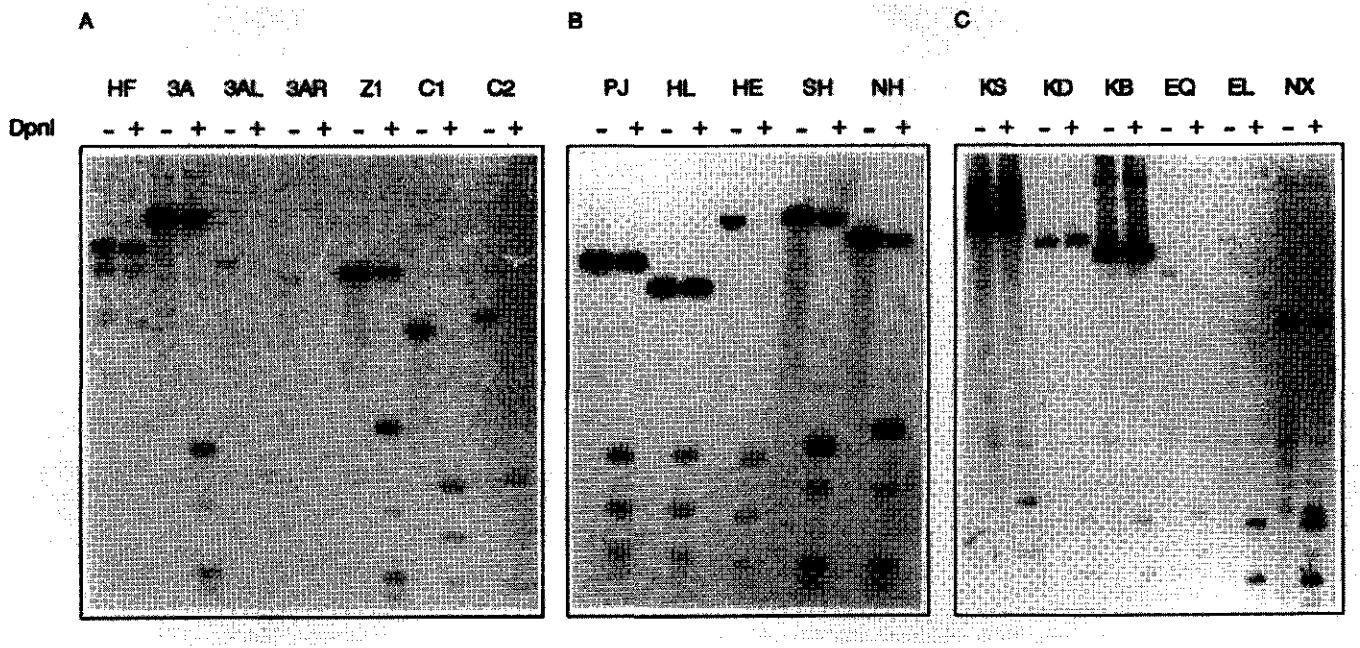

Figure 6.4. Replication activity of AcMNPV regions *hr*1 (A), *hr*2 (B), and *hr*4 (C). Transfections were carrried out with plasmids containing various AcMNPV fragments as indicated in Fig. 6.3A, 6.3B, and 6.3D respectively, except for plasmid EES (Fig. 3D), which is not shown in Fig. 6.4C. Also the plasmids pUC19 (C1) and pJDH119 (C2) were tested as negative controls (A). *S. frugiperda* cells were transfected with 1 μg plasmid DNA and infected with AcMNPV (MOI = 1) 24 h after transfection. Cellular DNA was isolated 48 h p.i. and digested with *Hin*dIII with (+) or without (-) *Dpn*I. Southern hybridization was carried out using ^{32}P-labeled pUC19 as a probe.

DNA, i.e. *Dpn*I-insensitive DNA, both became detectable 6 h after infection. Other experiments confirmed that *Dpn*I-insensitive plasmid DNA was not detectably produced prior to 6 h post infection (data not shown). This demonstrates that the replication of an *ori*-containing plasmid starts at the same time (6 h p.i.) as viral DNA replication begins (Tjia *et al.*, 1979), and also that the rate of DNA synthesis in both processes is roughly comparable (Fig. 6.2).

Construction and testing of plasmids containing different AcMNPV *hr* regions

After optimization of the replication assay using a plasmid with *hr*5 as *ori*, all other known *hr* regions of the AcMNPV genome were tested for their ability to initiate DNA replication. The AcMNPV fragments used for cloning the *hr* regions and surrounding sequences are schematically drawn in Fig. 6.3. The *hr*1 region was cloned as *Hin*dIII-F fragment in pBR322 (pAcHF). A *Pst*I-*Sst*II subclone of this plasmid (pAc3A) was used to make three other constructs containing the left-hand (pAc3AL) and the right-hand (pAc3AR) border sequence of *hr*1, and the sequence of *hr*1 alone (pAcZ1) (Fig. 6.3A). For testing the *hr*2 region the *Pst*I-J fragment (pAcPJ) and *Hin*dIII-L fragment (pAcHL) of AcMNPV were used to construct three subclones containing, respectively, the left-hand border of *hr*2 (pAcHE), and the sequence of *hr*2 alone in a longer (pAcSH) and shorter (pAcNH) version (Fig. 6.3B). For testing the *hr*3 region constructs pAc6AL and pAc6A were used (Kool *et al.*, 1993) to generate 4 different subclones, i.e. pAcSL, pAcSE, pAcMH and pAcMS (Fig. 6.3C). For analysis of the *hr*4 region, which includes both *hr*4a and *hr*4b, constructs pAcKD, pAcKS, pAcEQ, pAcEL, pAcNX, pAcKB and pAcEES were made (Fig. 6.3D). For testing region *hr*5, seven different constructs, pAc7A, pAcHK, pAcES, pAcHQ, pAcEP, pAcEX and pAcHQD, were used (Fig. 6.3E).

All 30 constructs obtained, containing either one of the six different *hr* regions and / or their surrounding sequences, were tested for their replication ability using the optimized assay conditions. As negative controls, the plasmids pUC19 and pJDH119 were used. Only for the regions *hr*1, *hr*2 and *hr*4 are the experimental data shown (Fig. 6.4A-C); the replication ability of all constructs is presented in Fig. 6.3. All plasmids that contained a *hr* region, including those with *hr*1 (Fig. 6.4A), were amplified in the presence of helper AcMNPV; all plasmids that did not contain a *hr* region were not amplified (Fig 6.4).

One exception, however, was plasmid pAcHK, which contains the *Hin*dIII-K fragment

of AcMNPV and which does not contain *hr* sequences (Fig. 6.3). This plasmid was also amplified, although this was not found in previous experiments (Kool *et al.*, 1993). Inspection of the sequence of the *Hin*dIII-K fragment (Friesen and Miller, 1987) revealed no sequence homology with any of the *hr* regions. This means that either a non-*hr* sequence can function as *ori* or that a secondary structure, not the sequence itself, is important and is recognized by the replication machinery. No further attempts were made at this point to locate this origin in the *Hin*dIII-K fragment more precisely (see Chapter 7).

Replication of *Hin*dIII-K and *Hin*dIII-Q without vector sequences.

Because *hr* regions of AcMNPV have been also reported to possess enhancer activity (Guarino *et al.*, 1986), the possibility that these regions would activate a bacterial *ori* or other cryptic, *ori*-like motifs in the bacterial vector sequences cannot be ruled out *a priori*. To exclude this possibility, fragment *Hin*dIII-Q, containing *hr*5, was tested in the absence of any vector sequences. Also, fragment *Hin*dIII-K with no *hr* region was tested without vector sequences to see whether it could independently replicate. To this end, plasmid pAc7A, which contains both *Hin*dIII-K and -Q (Fig. 6.3E), was digested with *Hin*dIII and the fragments *Hin*dIII-K and -Q were isolated from agarose gels and circularized using T4 DNA ligase. Transfections were performed for both fragments with 1 μg DNA of a) intact plasmid pAcHK, pAcHQ and pUC19 (Fig. 6.5, lanes 1) b) circularized fragments *Hin*dIII-K and -Q (Fig. 6.5, lanes 2), and c) linear fragments *Hin*dIII-K and -Q (Fig. 6.5, lanes 3). Plasmids pAcHK and pAcHQ served as positive controls, whereas plasmid pUC19 served as negative control. Transfections with linear fragments were carried out to see whether a circular form of the DNA was essential for replication. Cellular DNA was isolated 48 h after infection of the cells and replication of transfected DNA was investigated by incubating *Eco*RI digested DNA (*Hin*dIII-K and pUC19) or *Sst*I digested DNA (*Hin*dIII-Q) with *Dpn*I. Digestion with *Hin*dIII was not appropriate in this case, since it would have been impossible to distinguish between newly amplified *Hin*dIII-K or -Q fragment and the corresponding fragments of replicated viral DNA. DNA blots were probed with ^{32}P-labeled pAcES (Fig. 6.3E), which contained the *Eco*RI-S fragment and thus is able to detect both the *Hin*dIII-K and -Q fragments as well as pUC19 sequences.

The results obtained unequivocally demonstrated that both fragments *Hin*dIII-K and -Q were amplified in the absence of any vector sequences, but only when transfected in

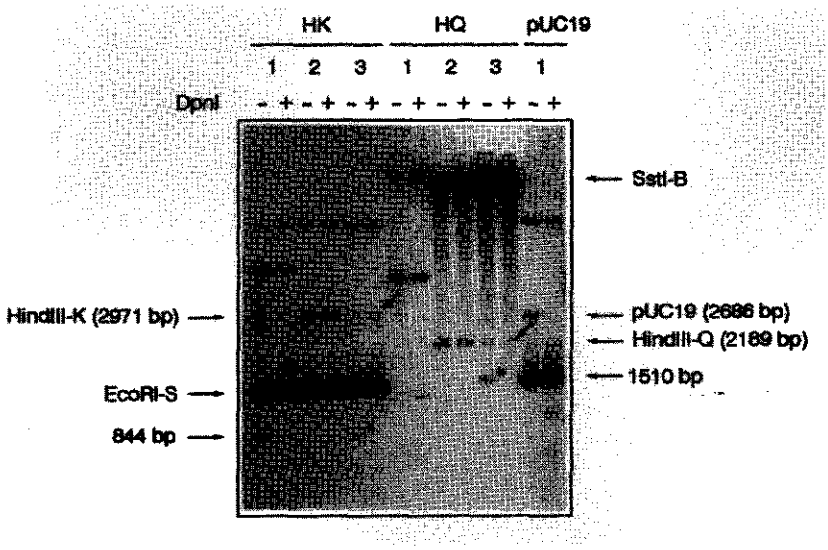

Figure 6.5. Southern analysis of transfections with the *Hin*dIII-K (HK) and -Q (HQ) fragments, and pUC19. *S. frugiperda* cells were transfected with 1 μg DNA of the plasmids pAcHK, pAcHQ or pUC19 (lanes 1), with the fragments *Hin*dIII-K or -Q alone in circularized form (lanes 2), or with the fragments *Hin*dIII-K or -Q alone in linear form (lanes 3). The cells were infected with AcMNPV (MOI = 1) 24 h after transfection. Cellular DNA was isolated 48 h p.i. and digested for the HK and pUC19 transfections with *Eco*RI with (+) or without (-) *Dpn*I, and for the transfections with HQ with *Sst*I with (+) or without (-) *Dpn*I. As probe for the Southern hybridization the ^{32}P-labeled *Eco*RI-S fragment, cloned in pUC19, was used.

circularized form (Fig. 6.5, HK and HQ, lanes 1 and 2), whereas vector sequences alone, i.e. pUC19, did not replicate (Fig. 6.5, pUC19, lanes 1). The linear *Hin*dIII fragments also gave a weak signal at the same position as found for the circularized fragments (Fig. 6.5, HK and HQ, lanes 3, arrow). This did not mean that the linear fragments were amplified, because digestion of the linear fragments *Hin*dIII-K or -Q with *Eco*RI or *Sst*I, respectively, results in two smaller fragments, only one of which will hybridize with *Eco*RI-S (Fig. 6.3E). These smaller fragments, respectively 844 and 1510 bp in size, can be seen in lanes 3 without *Dpn*I digestion (asterisk), and are *Dpn*I sensitive, because they are not present in lanes 3 with *Dpn*I digestion. Therefore the weak signals in lanes 3, which are *Dpn*I insensitive, are probably the result of amplification of spontaneously recircularized linear fragments. Digestion of the circularized forms of *Hin*dIII-K or -Q with either *Eco*RI or *Sst*I

indeed resulted in only a single fragment with the size of *Hin*dIII-K or -Q itself (2971 bp and 2189 bp, respectively) (lanes 2). From the results obtained it is concluded that both *Hin*dIII-Q, containing the *hr*5 region, and *Hin*dIII-K, without an *hr* region, can function as *ori's* in the absence of any vector sequences but only when they are present in circularized form.

DISCUSSION

Using an optimized replication assay at least seven distinct regions of the AcMNPV genome displayed replicating activity. These regions include the six *hr* regions, originally described by Cochran and Faulkner (1983), and one non-*hr* region. The results confirm previous observations for *hr*2, *hr*3 and *hr*5 (Pearson *et al.*, 1992; Kool *et al.*, 1993) and indicate that all these regions contain sequences that may serve as *ori's* of AcMNPV DNA replication. The six *hr* regions contain homologous repetitive sequences, which are also involved in enhancement of transcription (Guarino *et al.*, 1986). This result implies that all *hr's* in AcMNPV are bifunctional, i.e. have both enhancer and *ori* activity, as has been reported also for certain sequences in simian virus 40 and polyoma virus (Bergsma *et al.*, 1983; Tyndall *et al.*, 1981). In addition to the six *hr's*, an additional genomic segment (*Hin*dIII-K) was found to contain a putative *ori*, although this segment did not contain a conventional *hr* region.

A possible reason that *hr*1 and the *Hin*dIII-K fragment could now also be identified as *ori's*, while these elements were negative in previous experiments (Kool *et al.*, 1993), could be that the assay conditions to promote replication were improved, resulting in a tenfold higher rate of amplification of *ori* containing plasmids. Using a MOI of 1 for infection (instead of 25) another threefold higher yield of amplified plasmid was achieved (Fig. 6.1B), and another twofold increase was obtained by extending the interval between transfection and infection from 5 to 24 h (Fig. 6.1C).

Seven putative origins of DNA replication, six of which are located in the *hr* regions, have now been identified in the AcMNPV genome. The individual role of these *ori's* during viral DNA replication, and whether they are all active simultaneously *in vivo*, is still unclear. From experiments with DI particles generated by serial passaging, it can be deduced that not all the *ori's* are necessary for replication of the genome. After 40 serial, undiluted passages

three small segments of the genome were predominantly found to be retained, harboring only the *hr*1, *hr*3 and *hr*5 regions (Kool *et al.*, 1993). This observation may suggest that *hr*2 and *hr*4 are less important or not essential. Analysis of viral DNA of multiply passaged virus showed that the defective genomes mainly contain a small 2.8 kbp fragment which maps to the *Hin*dIII-K fragment (Lee and Krell, 1992). This would mean that *hr*1, *hr*3 and *hr*5 are also not essential and that the *ori* in the *Hin*dIII-K fragment alone is sufficient to support (DI) replication. The construction of viable recombinant virus, in which *hr*5 was deleted, also means that not all *hr* regions need to be present in AcMNPV for replication (Rodems and Friesen, 1993).

The occurrence of multiple *ori's* is not unique for baculoviruses, but has also been reported for herpes simplex virus 1 (HSV-1) and Chilo iridescent virus (CIV). The genome of HSV-1 contains three *ori's*, ori_L and two copies of ori_S (see Fields and Knipe, 1990, for review) which can support replication in a assay similar to that used in this paper (Stow and McMonagle, 1983). For HSV-1 it has been reported that the presence of any single *ori* is sufficient for replication (Longnecker and Roizman, 1986; Polvino-Bodnar *et al.*, 1987; Igarashi *et al.*, 1993). In CIV at least six putative *ori's* have been identified (Handermann *et al.*, 1992). It remains to be seen whether in the case of baculoviruses each of the seven putative *ori's* is necessary for viral replication. When the *ori's* are indeed functionally redundant, then the presence of multiple origins in the viral genome may increase the frequency of initiation and thus increase the speed of DNA replication. Subsequent deletion of each of the possible *ori's* may shed light on their relative importance.

The experiments in this paper also demonstrated that a circular topology is a prerequisite for replication of *ori*-containing plasmids. Linear DNA, even if it contained an *ori*, was not replicated (Fig. 6.5). These results are in line with the circular nature of baculovirus DNA and suggest a theta structure or a rolling-circle model for baculovirus replication. The latter model is supported by the finding of defective genomes with many reiterations (concatemers) of a 2.8 kbp segment, mainly mapping in the *Hin*dIII-K fragment (Lee and Krell, 1992). However, in a rolling-circle model the dependence on the origins is minimal and occurs at most only once at the initiation of DNA synthesis (Kornberg and Baker, 1992). It is possible that *in vivo* the *ori's* are not equivalent or that they are not all functional. The relative abundance of *Hin*dIII-K in defectives thus requires further investigation.

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CHAPTER 7

CHARACTERIZATION OF A NON-*HR* ORIGIN OF DNA REPLICATION IN THE *HIN*DIII-K FRAGMENT OF *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS

SUMMARY

In addition to the seven now known homologous regions (*hr's*), the *Hin*dIII-K fragment of *Autographa californica* MNPV (AcMNPV) was also found to carry a putative *ori*, although this fragment does not contain an *hr* region. Deletion analysis showed that this *ori* contains several regions, some of which are essential for its activity, whereas others contain auxiliary sequences that enhance the *ori* activity. Sequence analysis of these regions identified several structures often found in other viral replication *ori's*, such as palindromes and other repeated motifs. The importance of the *ori* in the *Hin*dIII-K fragment is supported by sequence data of the corresponding region in *Bombyx mori* MNPV (Kamita *et al.*, 1993). Although most of the auxiliary sequences of this *ori* were found to be deleted in the BmMNPV genome, the essential part of this *ori*, containing the palindromes and the A/T-rich region, was retained, suggesting that this could not be deleted. These sequence data and the observation that after prolonged serial passage of AcMNPV (80 passages) large replicating DNA molecules are found in which repeated sequences derived from the *Hin*dIII-K fragment accumulate (Lee and Krell, 1992) together point to the importance of this region as a genuine *ori in vivo*.

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INTRODUCTION

The *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) is the type member of the insect virus family *Baculoviridae* (Francki *et al.*, 1991). It has a circular, double-stranded DNA genome of approximately 131 kilobase pairs and replicates in the nucleus of infected insect cells. The knowledge of the molecular genetics of AcMNPV has greatly increased (for reviews, see Blissard and Rohrmann, 1990; Kool and Vlak, 1993) due to the potentials of this virus as biological insecticide (Payne, 1988) and as expression vector of foreign genes (Luckow and Summers, 1988; Miller, 1988).

Despite their widespread use, little is known about the mechanism by which baculoviruses replicate their DNA. Recently, eight *cis*-acting elements that may play a role in DNA replication have been identified (Pearson *et al.*, 1992; Leisy and Rohrmann, 1993; Kool *et al.*, 1993a, b). The nature and location of some these *cis*-acting elements has firstly been inferred from the structure of defective viral genomes, which are generated in continuous production of AcMNPV in bioreactors or upon serial, undiluted passage of virus in cell culture (Kool *et al.*, 1991; Wickham *et al.*, 1991; Lee and Krell, 1992). These defective viral genomes lacked considerable portions of the standard viral genome, but apparently had retained *cis*-acting elements that are essential for DNA replication and encapsidation. Plasmids containing these *cis*-acting elements replicated transiently in insect cells infected with AcMNPV and, hence, contained putative origins (*ori's*) of DNA replication.

Seven of the eight putative *ori's* have been located in the homologous regions of AcMNPV (Kool *et al.*, 1993b; Leisy and Rohrmann, 1993), which are interspersed along the genome (Cochran and Faulkner, 1983) (Fig. 7.1). Sequence analysis showed that *hr's* contain two to eight 30 bp imperfect palindromes, interspaced by other repeated sequences, and that each palindrome contains a naturally occurring *Eco*RI site at its core (Guarino *et al.*, 1986).

In addition to the seven *hr's*, a putative *ori* was also found in the *Hin*dIII-K fragment of AcMNPV, although this fragment does not contain an *hr* region (Kool *et al.*, 1993b). Lee and Krell (1992) reported that defective AcMNPV genomes, generated after extensive serial passage of this virus in insect cell culture, were comprised largely of reiterations of this *Hin*dIII-K region. This also suggested that this region contained important *cis*-acting sequences sufficient to initiate viral DNA replication.

MATERIALS AND METHODS

Cells and virus

Spodoptera frugiperda (*Sf*-AE-21) cells (Vaughn *et al.*, 1977) were cultured in TNM-FH medium (Hink, 1970), supplemented with 10% fetal calf serum (FCS). The E2-strain of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) (Smith and Summers, 1978) was used as wild-type (wt) virus. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Summers and Smith, 1987).

Plasmid constructions

AcMNPV fragments were cloned into pUC19 and transformed into *Escherichia coli* JM101 using standard techniques (Sambrook *et al.*, 1989). DNA-isolation, purification in CsCl gradients, digestion with restriction enzymes, and agarose gel electrophoresis were carried out using standard procedures (Sambrook *et al.*, 1989).

Replication assays

The assay used to test for *ori* function was based on the observation that, in the presence of helper AcMNPV which provides *trans*-acting viral functions, plasmids carrying a putative AcMNPV origin of replication will be amplified in insect cells (Kool *et al.*, 1993a; Stow and McMonagle, 1983). Test plasmids were amplified in *E. coli* JM101, which is Dam^+. The adenine residue within the GATC recognition sequence for *Dpn*I is thus methylated and, hence, sensitive to *Dpn*I digestion as *Dpn*I cleaves only methylated GATC sequences. DNA that is replicated in insect cells is not methylated at GATC sequences and therefore resistant to *Dpn*I digestion. The plasmids were purified in CsCl gradients and transfected into *S. frugiperda* cells using lipofectin (Bethesda Research Laboratories, Inc.) (Groebe *et al.*, 1990).

The replication assay was performed basically as described previously (Kool *et al.*, 1993a, b). Briefly, *S. frugiperda* cells were plated onto 35-mm-diameter Petri dishes at a density of 2×10^6 cells per dish 24 h before transfection. Approximately 2 h prior to transfection the medium was removed and the cells were washed with Hink's medium without BSA and FCS. For the transfections 1 μg plasmid DNA was used. The plasmid DNA

was mixed with 35 μl H_2O and 15 μl of lipofectin (GIBCO BRL) in 1 ml of Hink's medium without BSA and FCS and added onto the cells. After incubation for 6 h at 27°C, the lipofectin-containing supernatant was removed by washing the cells twice with complete Hink's medium. The next day, or 24 h after transfection, the cells were superinfected with AcMNPV at a multiplicity of infection (MOI) of 1 $TCID_{50}$ per cell. After 1 h of incubation 2 ml of fresh medium was added and the cells were further incubated at 27°C.

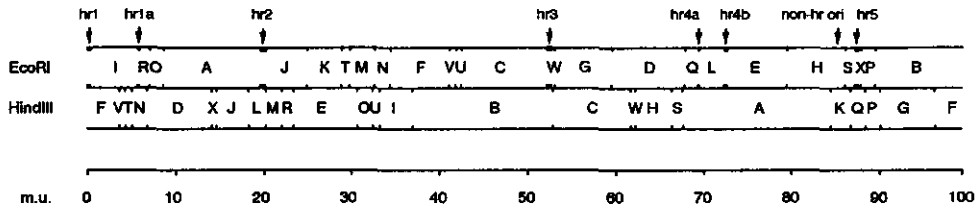


Figure 7.1. Linear map of *Eco*RI and *Hin*dIII restriction sites on AcMNPV DNA. Putative origins of replication including seven *hr* regions and one-*hr* region are shown above the map. m.u. = map unit.

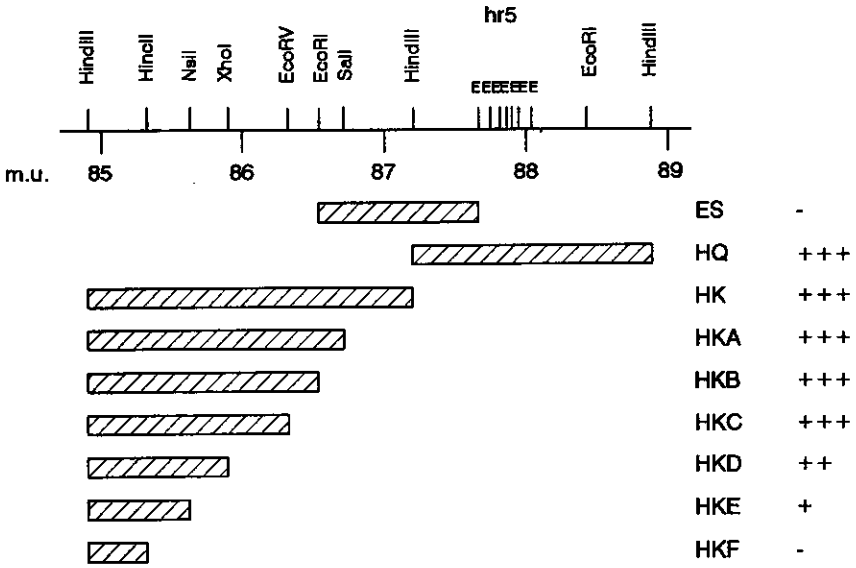
DNA analysis

The cells were harvested 48 h post infection with AcMNPV and total DNA was isolated from infected cells as described by Summers and Smith (1987) and resuspended in 64 μl TE. One aliquot (16 μl) of DNA was digested with the restriction enzyme *Hin*dIII to linearize plasmid DNA and one aliquot (16 μl) was digested with *Hin*dIII and *Dpn*I to determine if replication had occurred. After gel electrophoresis the DNA was transferred to membrane filters (Hybond-N) by the method of Southern (1975). Radioactively labeled (Rigby *et al.*, 1977) pUC19 was used as probe for hybridization in all the experiments.

RESULTS

In a previous report all six then known *hr* regions and adjacent sequences were tested for replication activity (Kool *et al.*, 1993b). It was shown that all six *hr* regions could function

A.



B.

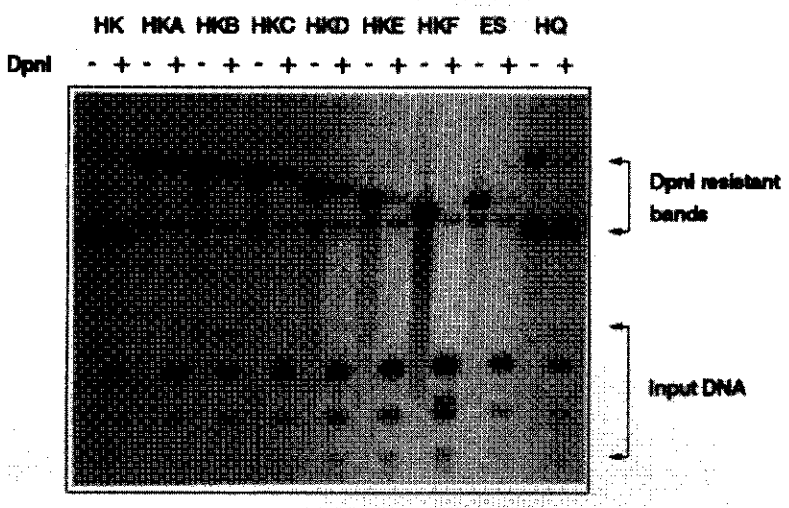


Figure 7.2. A. Schematic diagram showing the position of fragments *Eco*RI-S (ES), *Hin*dIII-Q (HQ), *Hin*dIII-K (HK), and subclones HKA-HKF. A plus (+) or a minus (-) indicates the replication ability of the various fragments in the replication assay. E = *Eco*RI; m.u. = map unit. **B.** Replication activity of the plasmids containing *Hin*dIII-K (HK), subclones HKA-HKF, *Eco*RI-S (ES), and *Hin*dIII-Q (HQ). *S. frugiperda* cells were transfected with 1 μg plasmid DNA and infected with AcMNPV (MOI = 1) 24 h after transfection. Cellular DNA was isolated 48 h p.i. and digested with *Hin*dIII with (+) or without (-) *Dpn*I. Southern hybridization was carried out using ^{32}P labeled pUC19 as probe.

as an origin of DNA replication in an infection-dependent replication assay. Recently, Leisy and Rohrmann (1993) identified another *hr* region, *hr*1a, located between the *Eco*RI-I and *Eco*RI-R fragment, that could also function as an origin of replication (Fig. 7.1). When the regions flanking *hr*5 were tested for replication activity, it was found that the *Hin*dIII-K fragment (map unit 84.9 - 87.2) also contained a putative origin of replication (Kool *et al.*, 1993b). However, sequence analysis of this fragment showed no obvious homology with any of the *hr* regions (Friesen and Miller, 1987; Guarino *et al.*, 1986), indicating that either different sequences can function as *ori* or that a common secondary structure and not the sequence itself, is recognized by the replication machinery. To locate this putative non-*hr ori* in the *Hin*dIII-K fragment more precisely, subclones were made and tested in the transient replication assay.

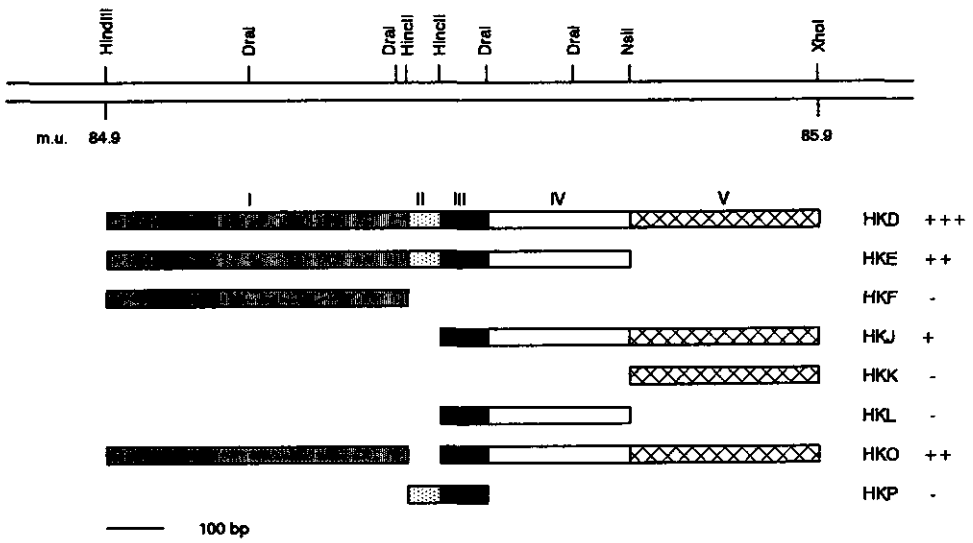


Figure 7.3A. Schematic diagram showing the position of various subclones of HKD. A plus (+) or a minus (-) indicates the replication activity of the various fragments in the replication assay. m.u. = map unit.

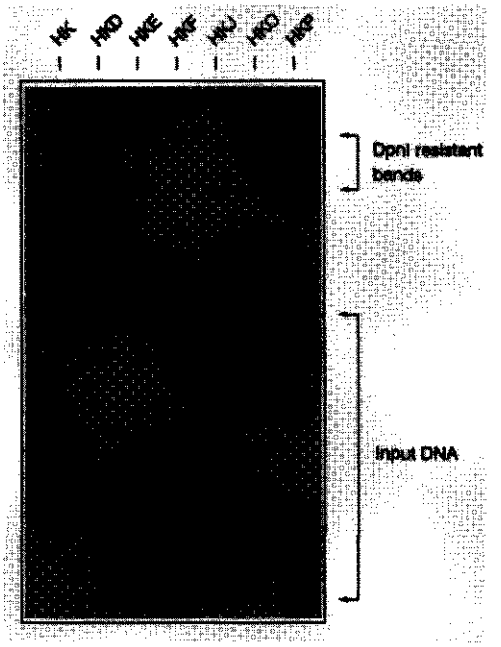


Figure 7.3B. Replication activity of the plasmids containing the fragments as indicated in A. *S. frugiperda* cells were transfected with 1 μg plasmid DNA and infected with AcMNPV (MOI = 1) 24 h after transfection. Cellular DNA was isolated 48 h p.i. and digested with *Eco*RI and *Dpn*I. Southern hybridization was carried out using ^{32}P labeled pUC19 as probe.

Subclones pAcHKA through pAcHKF were made using the internal *Sal*I, *Eco*RI, *Eco*RV, *Xho*I, *Nsi*I and *Hin*cII restriction sites, respectively, of the *Hin*dIII-K fragment (Fig. 7.2A). The complete *Hin*dIII-K fragment (pAcHK) and the subclones were tested in a replication assay along with pAcES and pAcHQ (containing the fragments *Eco*RI-S and *Hin*dIII-Q [*hr5*], respectively) as negative and positive controls. The results in Fig. 7.2B show that the relative efficiency of replication is slightly lower for pAcHK as compared to pAcHQ, which contains *hr5*. Subclones pAcHKA to pAcHKD replicated more or less at the same level as pAcHK. Subclone pAcHKE replicated significantly less than *Hin*dIII-K, whereas subclone pAcHKF did not replicate at all. This suggested that the *ori* activity of *Hin*dIII-K is located between the *Hin*dIII (m.u. 84.9) and *Xho*I sites (m.u. 85.9) of pAcHKD, and that essential *ori* sequences are located in that part of pAcHKE, that is lacking in pAcHKF (Fig. 7.2A).

To investigate these essential sequences further, several subclones were made of pAcHKD (Fig. 7.3A). To construct pAcHKJ, the *Hin*dIII-*Hin*cII fragment in pAcHKD was deleted, sites were filled up with Klenow enzyme and religated. Plasmid pAcHKK was also derived from pAcHKD by deleting the *Hin*dIII-*Nsi*I fragment. To make pAcHKL, pAcHKJ was digested with *Nsi*I and *Sma*I, using the *Sma*I-site in the vector, sites were filled up with Klenow enzyme and the remaining part was religated. Plasmid pAcHKO was made from pAcHKD by deleting the internal 61 bp of the *Hin*cII fragment in pAcHKD and religating the remaining part. Plasmid pAcHKP contains a 158 bp *Dra*I fragment cloned into the *Hin*cII site of pUC19. All these subclones were subsequently tested in a replication assay (Fig. 7.3B). Subclones pAcHKF and pAcHKP (Fig. 7.3B) as well as pAcHKK and pAcHKL (not shown) did not replicate. Subclone pAcHKO, that lacked the internal 61 bp *Hin*cII fragment, replicated, but significantly less efficient than the parental pAcHKD clone, indicating that these 61 bp are not essential for *ori* activity, but contain auxiliary sequences (Fig. 7.3B). Subclone pAcHKJ, that lacked in addition to the 61 bp *Hin*cII fragment also the *Hin*dIII-*Hin*cII fragment, was still able to replicate, although at a very low level (Fig. 7.3B). This suggests that pAcHKJ still contained all the essential *ori* sequences, but the lower replication efficiency of pAcHKJ compared to pAcHKO showed that the *Hin*dIII-*Hin*cII fragment also contained auxiliary sequences.

On the basis of these results 5 regions can be identified in the *Hin*dIII-*Xho*I fragment in pAcHKD, which contribute to the replication ability of this fragment to different extents (Fig. 7.3A). None of the regions alone is sufficient to allow replication as shown by the negative results of clones pAcHKF, pAcHKK, pAcHKL, and pAcHKP. The regions I, II, and V contain auxiliary *cis*-acting sequences, because deletion of any of these regions reduces the replication efficiency. Only regions III and/or IV seem to be essential as deletion of one or both of these regions abolishes the replication ability. However, the combination of regions III and IV (clone pAcHKL) or regions II and III (clone pAcHKP) are not sufficient for replication.

Sequence analysis of the HKD fragment

The sequence of the *Hin*dIII-*Xho*I fragment (pAcHKD) of *Hin*dIII-K is part of the C-terminal coding region of the *p94* gene (Friesen and Miller, 1987). The function of this early transcribed gene is unknown, but it is not essential for virus replication (Friesen and

A. Palindromes:

```
Ac HdK A                              TGCATTCAACGC TCTGTTTGCAATCCA

Ac HdK B              GTTGATAATAAATTTGTCTTTGCA TTCAAACGCTCTGTTTGCAATCCAC

Ac hr5                          GCTTTACGAGTAGAA TTCTACGTGTAAAAC

Bm hr4b                           AAACATAGTTCA TTGAACTTAATTT

Op HdN A      CAAGCTGCCCAGAGAATCGGGTTGGGCAGGC GGCGCGCCCAACACCAACTCATTCAGCTGG

Op HdN B                                GTTGCAGC GCTGCAAC
```

B. Repeats:

```
Rpt 1:      GGACGTGTCGTTTAAAACATGTT
            GGACATG-CGTTTAAA-CATGTT

Rpt 2:      GTTCGTCAAC
            GTTGGTCAAC

Rpt 3:      Ac hr cons          GATGATGTC-AT(3-5)GT(4-6)
            Bm hr cons          GATGATGTC-AT(2-6)GT(1-7)
            OpE5 cons           CAGG--GTC----GT(0-2)
            Op HdN cons         GNNGANGTCGAT(0-5)GT(2-5)
            Ac HdK cons         ANNTTNGTC-AT(1-3)GT(0-5)
            MLTF (AdML)               GGCC-AC-GTGACC
            Ig enh μE3 (μE3)          GGTC-AT-GTGGCA
```

Figure 7.4. Comparison of palindrome motifs (A) and other repeated motifs (B) identified in the AcMNPV (Ac) *Hin*dIII-K (HdK) region and other putative baculovirus origins of BmMNPV (Bm) and OpMNPV (Op). The *Eco*RI site, respectively *Eco*RI-like site, in the palindromes of Ac *hr5* and Ac HdK B are shown in bold. Repeats 3 were also aligned with the binding site for the major late transcription factor (MLTF) found in the adenovirus major late promoter (AdML) and in the immunoglobulin M heavy-chain enhancer (μE3).

Nissen, 1990). Sequence analysis of the 5 regions in pAcHKD showed no obvious homology with any of the *hr* regions. A closer inspection of the sequence in the essential regions III and IV, however, resulted in the identification of two overlapping, imperfect palindromes in region III (Fig. 7.4A). Palindromes are also found in the *hr* regions of AcMNPV and seem to be essential for *ori* activity (Pearson *et al.*, 1992). Characteristic for the *hr* palindromes

is that they all contain an *Eco*RI site at their core. Although the palindromes in region III of pAcHKD do not contain an *Eco*RI site, one (HdK-B) contains an *Eco*RI-like sequence at its core, differing in only one position. Besides this *Eco*RI-like motif there is almost no sequence homology with the *hr* palindromes. Recently, *hr* regions containing palindromic sequences similar to those in AcMNPV DNA were found in *Bombyx mori* MNPV DNA (Majima *et al.*, 1993). In addition, four repeats of a previously undescribed palindrome motif were identified in *hr*4b of BmMNPV (Fig. 7.4A). But there is no homology between this BmMNPV palindrome and the ones in *Hin*dIII-K. A recently identified putative *ori* in *Orgyia pseudotsugata* MNPV (OpMNPV) shows no obvious homology with the sequence of *Hin*dIII-K or the *hr* regions in AcMNPV or with other sequences in the OpMNPV genome (Pearson *et al.*, 1993). Two palindromes are present in the essential part of the OpMNPV *ori*, including a 16 bp perfect palindrome, and a 61 bp imperfect one with some homology to each other but with no homology with the palindromes in *Hin*dIII-K (Fig. 7.4A).

Other structures, besides palindromes, often found to be associated with replication origins are direct repeats and A/T-rich regions in proximity to palindromes (DePamphilis, 1993). The palindromes in the *hr* regions are also interspaced by many direct repeats and A/T-rich regions. Inspection of the sequence in *Hin*dIII-K around the palindromic sequences reveals that the region upstream of palindrome A and part of palindrome B is indeed A/T-rich. A search for direct repeats in the sequence of pAcHKD resulted in numerous repeated motifs throughout the sequence. Two copies of a 23 bp repeated motif (GGACG/ATGTCGTTTAAAACATGTT) were found at the borders of regions I and II and regions III and IV (Fig. 7.4B, Fig. 7.5). A second 10 bp repeated motif with the sequence GTTC/GGTCAAC was found at the borders of region II (Fig. 7.5). Another repeated motif with the consensus sequence $GATGATGTCAT_{1\text{-}7}GT_{1\text{-}7}$ was found in many copies in the *hr* regions of AcMNPV. This motif is also conserved in the *hr* regions of BmMNPV (Majima *et al.*, 1993), and present, though less conserved, in the *hr* region OpE 5 of OpMNPV (Theilmann and Stewart, 1992) (Fig. 7.4B). The nucleotides shown in bold (-**GTCA**- and -**G**-) are the most conserved within this motif. Several copies of this motif with only one or a few mismatches, mainly in the first 6 nucleotides, are also found in the sequence of pAcHKD and in the sequence of OpMNPV fragment *Hin*dIII-N, which contains a putative OpMNPV *ori* (Pearson *et al.*, 1993) (Fig. 7.4B). The function of this motif is unknown, but it resembles the -CANNTG- motif recognized by helix-loop-helix group proteins and in

particular the binding site for MLTF (USF) found in the adenovirus major late promoter (AdML) and in the immunoglobulin M heavy-chain enhancer (μE3) (Fig. 7.4B) (Gregor *et al.*, 1990).

DISCUSSION

The results in this paper demonstrate that the AcMNPV genome contains besides the seven previously identified putative *ori's* in the *hr* regions (Pearson *et al.*, 1992; Leisy and Rohrmann, 1993; Kool *et al.*, 1993a, b) another putative *ori* located in the *Hin*dIII-K fragment. The structure and sequence of this *ori* in *Hin*dIII-K is distinctly different from those of the *ori's* in the *hr* regions. Deletion analysis of the *Hin*dIII-K fragment showed that all the sequences involved in the replication ability of this fragment are located between the *Hin*dIII (m.u. 84.9) and the *Xho*I site (m.u. 85.9) (Fig. 7.3A). Further analysis of this subfragment showed that it can be divided in 5 regions of which only region III and/or IV are essential (Fig. 7.3A, B). The other regions are not essential, but contain auxiliary sequences that stimulate the replication efficiency of this *ori*. However, plasmids containing the regions III and IV failed to replicate (Fig. 7.3A, pAcHKL), suggesting that at least one of the auxiliary regions is necessary for the ori to replicate. Sequence analysis of these regions identified several structural features often found in other replication *ori's* of eukaryotic viruses (DePamphilis, 1993). Two imperfect palindromes, one with a *Eco*RI-like site at its core, were identified within region III, preceded by an A/T-rich region. The sequences of these palindromes do not exhibit homology to previously identified palindromes in other baculovirus replication *ori's*. Furthermore, several other repeated motifs were found in the *ori*-containing sequence of *Hin*dIII-K, some of which show homology with repeats found in other baculovirus replication *ori's*, but their relevance in replication is unclear.

Although the complex structure of the *ori* in *Hin*dIII-K is different from the structure in the *hr ori's*, the replication efficiency of *Hin*dIII-K is almost the same as that of *Hin*dIII-Q, which contains *hr*5. Pearson *et al.* (1992) suggested that the replication efficiency of the *hr* regions is correlated with the number of palindromes. However, our results with the *Hin*dIII-K *ori* suggest that other sequences are involved as well. Recently, an *ori* with also a complex structure has been identified in OpMNPV with no homology with the *hr* regions

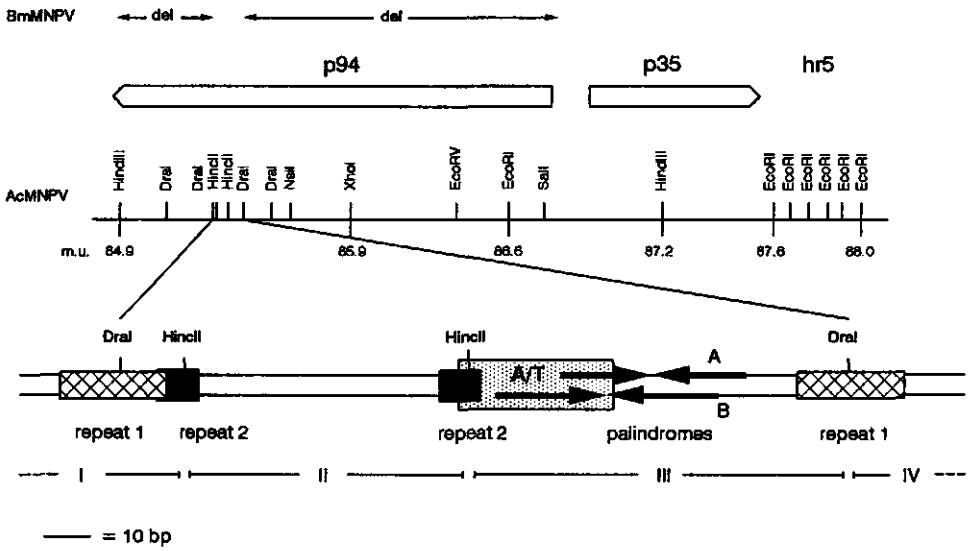


Figure 7.5. Comparison of the regions in BmMNPV and AcMNPV, spanning the *p94* and *p35* genes and *hr5*. *P35* and *hr5* are found in both genomes at the same location and orientation, whereas most of the *p94* sequences are deleted in the BmMNPV genome as compared to AcMNPV. Only 151 nucleotides of the *p94* gene were retained in BmMNPV, which map exactly with the this paper identified regions II and III of the *ori* in AcMNPV *Hin*dIII-K. The repeats found in these regions, the A/T rich region, and palindromic motifs are shown. m.u. = map unit.

in AcMNPV (Pearson *et al.*, 1993). Complex origins composed of multiple elements have been found in several other viral DNA genomes. The simian cytomegalovirus (SCMV) and the related human cytomegalovirus (HCMV), for instance, contain complex origins spanning 1.3 to 2.6 kbp, respectively (Anders and Punturieri, 1991; Anders *et al.*, 1992).

The question remains whether each of the seven *hr* regions and the *Hin*dIII-K region also function as an *ori in vivo*. Deletion of *hr5* from the AcMNPV or BmMNPV genome had no effect on the replication of these viruses (Rodems and Friesen, 1993; Majima *et al.*, 1993).

This suggests that not the complete set of identified *ori's* is necessary or that maybe none of the identified *hr ori's* are necessary for replication or function as an *ori in vivo*. It is not known whether the *ori* in *Hin*dIII-K can be deleted from the genome. Friesen and Nissen (1990) reported that the *p94* gene, containing the *ori* in its sequence, is not essential. However, the *p94* gene was not deleted, but only the open reading frame of *p94* was interrupted.

The importance of the putative *ori* in the AcMNPV *Hin*dIII-K fragment *in vivo* is supported by sequence data of the closely related BmMNPV (Kamita *et al.*, 1993). In this virus the region, spanning the *p94* and *p35* genes upstream of *hr5*, contains an almost identical *p35* gene, as compared to the AcMNPV *p35*, and a region homologous to *hr5* of AcMNPV. However, most sequences corresponding to the *p94* gene were missing. Only 151 nucleotides of the *p94* gene, with high degree (96.1%) of sequence homology to the corresponding sequence of AcMNPV *p94*, were found in BmMNPV. These 151 nucleotides map exactly with regions II and III in the AcMNPV *ori* of *Hin*dIII-K (Fig. 7.5). The repeated motifs, the A/T-rich region and the palindromes were retained in the BmMNPV genome, but flanking sequences (regions I, IV, and V) were deleted. This suggests that region III, containing the palindromes, is the most important part of this *ori* and can not be deleted from the genome. Region II is not essential, but deletion of this short region (61 bp) strongly reduces the replication efficiency (Fig. 7.3), and this might be the reason that this sequence is also retained in the BmMNPV genome. The flanking regions I, IV, and V have only auxiliary functions and are not essential.

These alignment data (Kamita *et al.*, 1993) and the observation that after prolonged serial passage of AcMNPV large replicating DNA molecules are found in which repeated sequences derived from the *Hin*dIII-K fragment accumulate (Lee and Krell, 1992), corroborate the importance of this region as genuine *ori in vivo*.

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CHAPTER 8

FUNCTIONAL MAPPING OF REGIONS OF THE *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRAL GENOME REQUIRED FOR DNA REPLICATION

SUMMARY

Previous results showed that plasmids containing one of the eight putative origins (*ori's*) of *Autographa californica* MNPV (AcMNPV) are replicated after transfection into *Spodoptera frugiperda* cells if essential *trans*-acting factors are supplied by AcMNPV infection (Kool *et al.*, 1993a, b; Leisy and Rohrmann, 1993). In this report a transient complementation assay is described in which four cotransfected cosmid clones, instead of AcMNPV infection, provided essential *trans*-acting factors for plasmid DNA replication. In this assay plasmid replication was found to be independent of the presence, in *cis*, of a viral *ori*. No replication of plasmids occurred when one of the cosmids was omitted from the transfection mixture. This result indicated that this assay is a valid approach to identify AcMNPV replication genes. We further used the assay to define essential regions in the four required cosmids. Six regions of the AcMNPV genome, *Eco*RI-I (map unit [m.u.] 0.3 - 5.8), *Eco*RI-O (m.u. 6.9 -8.7), *Sst*I-F (m.u. 38.9 - 45.0), *Eco*RI-D (m.u. 59.9 - 68.3), a *Bam*HI-*Sst*II fragment of *Bam*HI-B (m.u. 84.3 - 89.7), and *Eco*RI-B (m.u. 90.0 - 100), with at least seven genes, were found to be essential for plasmid DNA replication. These results show that it will now be possible to define the set of AcMNPV genes involved in DNA replication.

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INTRODUCTION

Autographa californica nuclear polyhedrosis virus (AcMNPV) is the type member of the insect virus family *Baculoviridae* (Francki *et al.*, 1991). It has a circular, double-stranded DNA genome of about 131 kbp and replicates in the nucleus of infected insect cells. The molecular genetics of AcMNPV has been studied in much detail (see Blissard and Rohrmann, 1990, for review) due to the great interest in this virus as a biological insecticide (Payne, 1988) and as expression vector (Luckow and Summers, 1988; Miller, 1988).

In contrast to other large DNA viruses such as adeno-, herpes- and vacciniavirus (see Fields and Knipe, 1990; Kornberg and Baker, 1992, for reviews), the process of viral DNA replication of baculoviruses is poorly understood. Central to the understanding of this process is the identification of *cis*-acting elements and *trans*-acting factors which play a role in DNA replication.

The location of the *cis*-acting elements has been inferred from the structure of defective viral genomes (Kool *et al.*, 1991; Lee and Krell, 1992). Specific segments of AcMNPV were maintained in defective DNA molecules. In AcMNPV eight *cis*-acting elements have been identified that can possibly function as origins (*ori's*) of DNA replication (Pearson *et al.*, 1992; Kool *et al.*, 1993a, 1993b; Leisy and Rohrmann, 1993). Plasmids containing these sequences are replicated in insect cells infected with AcMNPV. Seven of the eight putative *ori's* are located in the homologous regions (*hr*1, *hr*1a, *hr*2, *hr*3, *hr*4a, *hr*4b and *hr*5) of AcMNPV (Kool *et al.*, 1993b; Leisy and Rohrmann, 1993), which are interspersed along the genome (Cochran and Faulkner, 1983; Guarino *et al.*, 1986). In addition to the seven *hr's*, the *Hin*dIII-K fragment of AcMNPV also contains a putative *ori*, although this fragment does not contain an *hr* region (Kool *et al.*, 1993b, 1994b).

The viral genome of AcMNPV has the potential to encode for more than 100 proteins (see O'Reilly *et al.*, 1992; Kool and Vlak, 1993, for review). During infection, viral genes are expressed in a temporally regulated, sequential fashion and are classified according to their expression before or after viral DNA replication. Genes expressed prior to the initiation of viral DNA replication are called early genes, and appear to be involved in the regulation and expression of other genes and for the process of DNA synthesis.

To date only three AcMNPV genes have been identified which are thought to be involved in DNA synthesis. These include a putative DNA polymerase (Tomalski *et al.*, 1988), a

proliferative cell nuclear antigen-like protein (PCNA) (Crawford and Miller, 1988; O'Reilly *et al.*, 1989) and a helicase-like protein (Lu and Carstens, 1991). The involvement of the DNA polymerase and the PCNA-like protein in DNA replication has been inferred indirectly by amino acid similarities to genes known to be associated with DNA synthesis in other systems. Neither has been shown to be essential for DNA replication although deletion of a part of the PCNA-like protein coding region caused a delay of all late viral gene expression (Crawford and Miller, 1988). The involvement of the *helicase* gene in DNA replication was inferred from the characterization of a DNA replication defective, temperature-sensitive mutant in which the mutation was mapped within the *helicase* gene (Lu and Carstens, 1991).

By analogy to the similar-sized large DNA viruses such as herpesvirus it can be expected that AcMNPV codes for many more proteins involved in viral DNA replication than the three identified so far. Challberg (1986) described a protocol using transient complementation assays for the identification of genes involved in herpes simplex virus type 1 (HSV-1) replication. Using this approach seven *trans*-acting HSV-1 genes were identified as necessary and sufficient for HSV-1 *ori*-dependent DNA replication. In addition, four genes were also identified that stimulated DNA replication (Wu *et al.*, 1988). Recently, this same assay was used to identify several *trans*-acting factors, that were involved in DNA replication, in the human cytomegalovirus genome (HCMV) (Pari *et al.*, 1993).

In this report we demonstrate that a combination of four cosmids, collectively containing 96% of the AcMNPV genome, can supply all of the *trans*-acting factors required for transient plasmid replication. The replication assay is now a valuable tool to define AcMNPV genes which are involved in DNA replication. All four cosmids were found to contain one or more essential genes. Subclones were made and tested, individually and in combination, for their ability to substitute for the original cosmid. This approach allowed us to identify six regions within the AcMNPV genome encompassing at least seven genes that encode *trans*-acting factors necessary and sufficient for DNA replication.

MATERIALS AND METHODS

Cells and virus

Spodoptera frugiperda (*Sf*-AE-21) cells (Vaughn *et al.*, 1977) were cultured in TNM-FH

medium (Hink, 1970), supplemented with 10% fetal calf serum (FCS). The E2-strain of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) (Smith and Summers, 1978) was used as wild-type (wt) virus. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Summers and Smith, 1987).

Cosmid constructions

AcMNPV DNA was partially digested with the restriction enzyme *Eco*RI. Fragments varying in size from 35-45 kbp were isolated from sucrose gradients and ligated into cosmid pJB8 (Ish-Horowicz and Burke, 1981), which was digested with *Eco*RI and dephosphorylated (Sambrook *et al.*, 1989). After packaging the cosmids were propagated in HB101 and analyzed by restriction enzyme analysis. Cosmids 35, 50, 56 and 57 covered 96% of the AcMNPV genome.

Plasmid constructions

AcMNPV fragments were cloned into pBR322, pUC19 or pJDH119 (Hoheisel, 1989), and transformed to *Escherichia coli* JM101 using standard techniques (Sambrook *et al.*, 1989). DNA-isolation, purification in CsCl gradients, digestion with restriction enzymes, and agarose gel electrophoresis were carried out using standard procedures (Sambrook *et al.*, 1989).

Replication assays

S. frugiperda cells were plated onto 35-mm-diameter Petri dishes at a density of 2 x 10^6 cells per dish 24 h before transfection. Approximately 2 h prior to transfection the medium was removed and the cells were washed with Hink's medium without BSA and FCS. Transfections were carried out according to the method described by Groebe *et al.* (1990) with some modifications as described previously (Kool *et al.*, 1993b). Briefly, 1 μg of the *ori* (*hr2*) containing plasmid pAcHL and 0.5 μg of each of cosmids 35, 50, 56 and 57 were mixed with 35 μl H_2O and 15 μl of lipofectin (GIBCO BRL) in 1 ml of Hink's medium without BSA and FCS and added onto the cells. When a cosmid was substituted with one or more subclones, 0.5 μg of each of these subclones was used for transfection. After incubation for 6 h at 27°C, the lipofectin-containing supernatant was removed by washing the cells twice with complete Hink's medium. The cells were further incubated in 2 ml of complete Hink's

medium at 27°C for up to 72 h. As a positive control for these experiments a transfection with only pAcHL was done, followed by an infection with AcMNPV (MOI 1) 24 h after transfection.

DNA analysis

The cells were harvested 72 h post transfection and total DNA was isolated from infected cells as described by Summers and Smith (1987) and resuspended in 64 μl TE. One aliquot (16 μl) of DNA was digested with the restriction enzyme *Hin*dIII to linearize plasmid DNA, and one aliquot (16 μl) was digested with *Hin*dIII and *Dpn*I, to determine if replication had occurred. Test plasmids were amplified in *E. coli* JM101, which is Dam$^+$. The adenine residue within the GATC recognition sequence for *Dpn*I is thus methylated and hence sensitive to *Dpn*I digestion as *Dpn*I cleaves only methylated GATC sequences. DNA which is replicated in insect cells is not methylated at GATC sequences and therefore is resistant to *Dpn*I digestion. Hence, *Dpn*I can distinguish newly replicated from input plasmid DNA (Kool *et al.*, 1993a; Stow and McMonagle, 1983). After gel electrophoresis, the DNA was transferred to membrane filters (Hybond-N) by the method of Southern (1975). Radioactively labeled (Rigby *et al.*, 1977) pUC19 was used as the probe for hybridization in all these experiments.

RESULTS

In order to develop a transient complementation assay for AcMNPV DNA replication a set of overlapping genomic fragments was necessary. Therefore, a cosmid library of the AcMNPV genome was constructed containing overlapping fragments which together should cover the entire AcMNPV genome. Four cosmids were identified that contained the majority of the AcMNPV genome (Fig. 8.1). Two sets of the cosmids overlap; cosmid 35 and 50 both contain the *Eco*RI-U fragment and cosmid 56 and 57 overlap in the *Eco*RI-B fragment. The *hr*2 and *hr*4 regions, as well as the *Eco*RI-L fragment are not present in this set of cosmids.

These four cosmids were used in a transient complementation assay to test whether they could supply all the *trans*-acting factors necessary for replication of an *ori*-containing plasmid and thus could substitute for a virus infection. The reporter used for DNA replication was

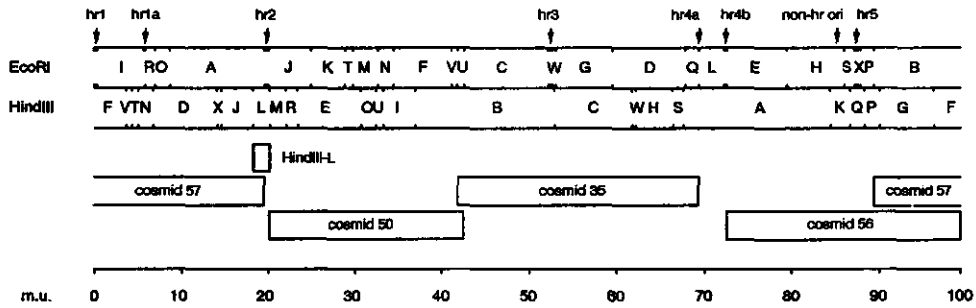


Figure 8.1. Linear AcMNPV restriction map for *Eco*RI and *Hin*dIII showing the location of the four cosmids and *Hin*dIII-L (*hr2*).

plasmid pAcHL (Fig. 8.1), which contains the HindIII-L fragment with *hr2* as an *ori* (Kool *et al.*, 1993b). Replication of pAcHL was monitored by treatment of transfected-cell DNA with *Dpn*I, which cleaves only unreplicated, *dam*-methylated input-DNA. In cotransfections of the four cosmids the reporter plasmid pAcHL became *Dpn*I-insensitive indicating that replication of pAcHL had occurred (Fig. 8.2, lane 3). The *Dpn*I-resistant signal was lower than that found when transfection of pAcHL is followed by an infection (Fig. 8.2, lane 1).

When cotransfections with the four cosmids were performed with pUC19 alone, it replicated (Fig. 8.2, lane 4). This result differed from when pUC19 is transfected followed by an infection with AcMNPV, when no replication of pUC19 occurred (Fig. 8.2, lane 2; Kool *et al.*, 1993a, 1993b). Replication of plasmids with or without an *ori* is also found when plasmids were cotransfected with complete viral DNA (Fig. 8.2, lanes 9 and 10), but the level of replication of pUC19 is much lower than that of the *ori*-containing plasmid. However, the finding that plasmids with or without an *ori* replicated, when cotransfected with the four cosmids alone, means that these cosmids expressed all *trans*-acting proteins necessary for plasmid DNA replication and could therefore substitute for an infection with virus. Also these results indicate that *Eco*RI-L, which is not present in any of the four cosmids, is not necessary, and thus does not contain a gene essential for DNA replication. In addition, the replication of pUC19 with complete viral DNA indicates that *Eco*RI-L is also not responsible for *ori*-dependent replication.

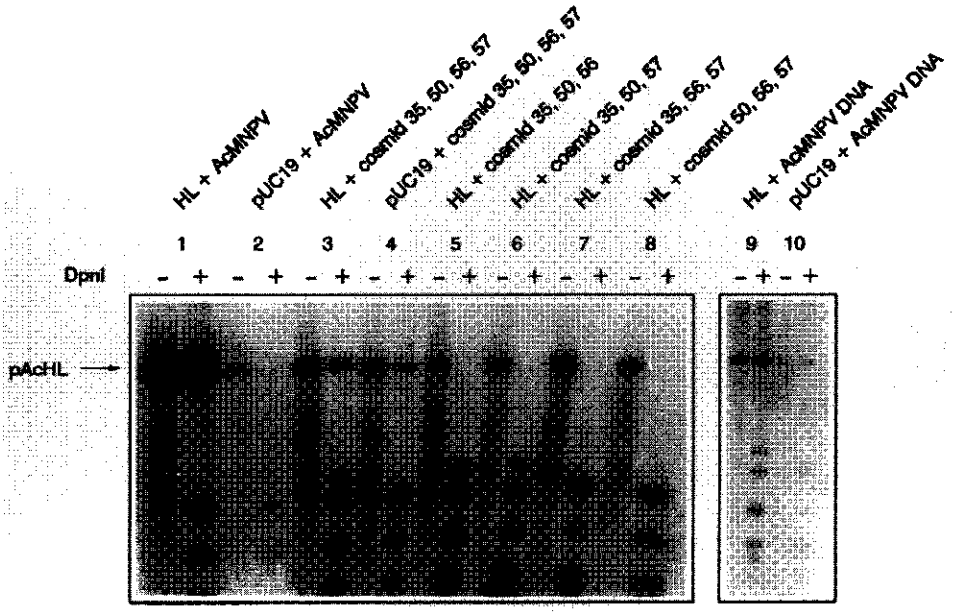
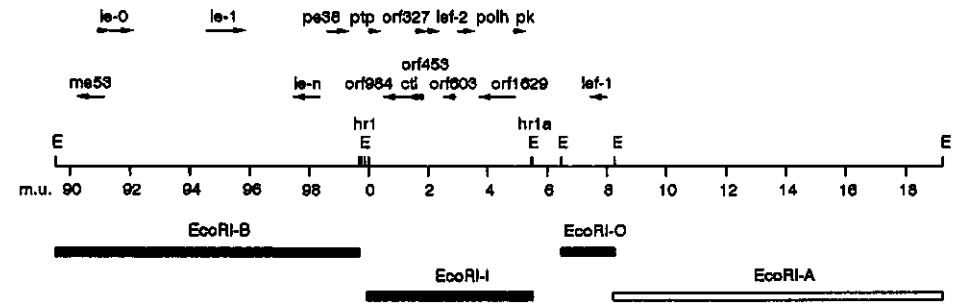


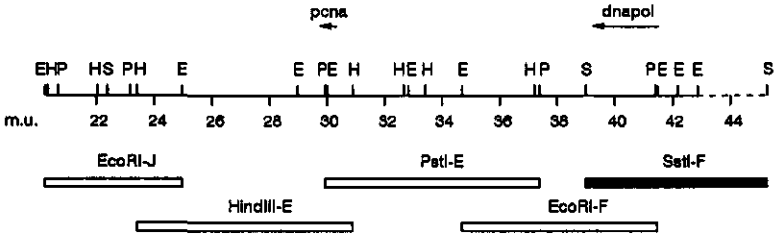
Figure 8.2. Replication activity of plasmid pAcHL (*Hin*dIII-L) with *hr2* as AcMNPV origin of DNA replication, or pUC19 without a viral origin. Southern blot of total cellular DNA from transfected *S. frugiperda* cells at 48 h post infection (lanes 1 and 2), or at 72 h post transfection (lanes 3-10). The DNA was digested with *Hin*dIII with (+) or without (-) *Dpn*I. Lanes 1 and 2 show replication activity of pAcHL and PUC19, transfected into *S. frugiperda* cells, followed by an AcMNPV infection (MOI 1), 24 h after transfection. Lanes 3-8 show replication activity of pAcHL and pUC19 cotransfected with either all four cosmids (lanes 3 and 4) or only three out of four (lanes 5-8). Lanes 9 and 10 show replication activity of pAcHL and pUC19 cotransfected with complete naked AcMNPV-DNA. Southern hybridization was carried out using ^{32}P-labeled pUC19 as a probe.

With the establishment that cotransfection of the four cosmids supported DNA replication, the next step was to identify which of the four cosmids contained genes essential for this replication. Therefore, cotransfections were carried out with combinations of three cosmids, by eliminating one cosmid at a time. No replication of pAcHL was detected when any of the four cosmids was omitted (Fig. 8.2, lanes 5-8). Since all four cosmids were needed to complement DNA replication in this transient assay, each must contain one or more genes

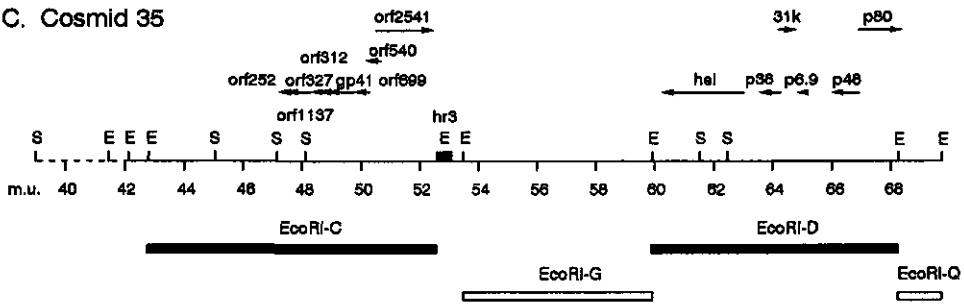
A. Cosmid 57



B. Cosmid 50



C. Cosmid 35



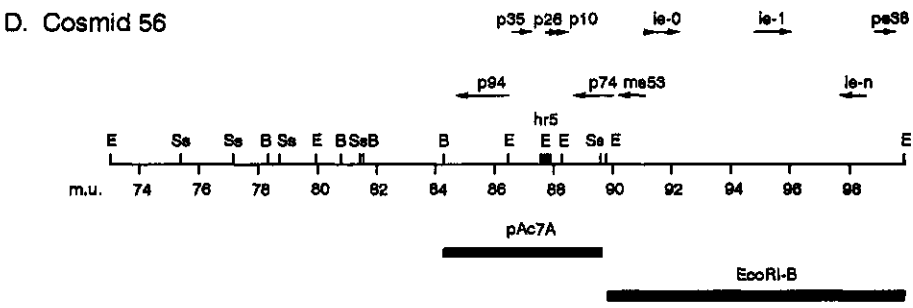


Figure 8.3A-8.3D. Four cosmid maps with subclones and putative essential genes indicated. Essential subclones are indicated as black rectangles. B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII, P = *Pst*I, S = *Sst*I, Ss = *Sst*II.

essential for DNA replication. To identify these genes, several subclones of the four cosmids were tested, together with the other 3 cosmids, for their ability to substitute for the respective cosmid. The results are summarized in table 8.1 and Fig. 8.3.

For cosmid 57 the fragments *Eco*RI-I, *Eco*RI-O and *Eco*RI-A were cloned and tested for their ability to substitute for cosmid 57 (Table 8.1, 1-6, Fig. 8.3A). Fragment *Eco*RI-B was not tested at this stage (see below). Individual subclones of cosmid 57 did not support replication of pAcHL, but a combination of *Eco*RI-I with *Eco*RI-O resulted in replication. Therefore, there must be at least two essential genes in cosmid 57, one or more on *Eco*RI-I and one or more on *Eco*RI-O.

To locate essential genes in cosmid 50 the fragments *Eco*RI-J, *Hin*dIII-E, *Pst*I-E, *Eco*RI-F and *Sst*I-F were cloned, and tested for their ability to substitute for cosmid 50 (Table 8.1, 7-11, Fig. 8.3B). *Sst*I-F alone appeared to be enough to substitute for cosmid 50. Since cosmid 50 contains only a part of *Sst*I-F (Fig. 8.3B), the essential gene(s) in *Sst*I-F must be located in that part that is present in cosmid 50. In this region only one gene, the DNA polymerase gene (*dna pol*), has been mapped (Tomalski *et al.*, 1988).

Subclones *Eco*RI-C, *Eco*RI-G, *Eco*RI-D and *Eco*RI-Q were tested for their ability to substitute for cosmid 35 (Table 8.1, 12-21, Fig. 8.3C). *Eco*RI-D contains a gene (*p143*) coding for a putative helicase. Since a temperature-sensitive mutation in this gene has been reported to cause a defect in the ability to synthesize viral DNA at the restrictive temperature (Lu and Carstens, 1991), we know that this gene is essential for DNA replication.

Table 8.1. Summary of results of the cotransfections with the four cosmids and their respective subclones.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
cosmid 57	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+								
cosmid 50	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+								
cosmid 35	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+								
cosmid 56	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-								
*Eco*RI-B	-	-	-	-	-	-																	+	-	+	+	+	+	+	+
*Eco*RI-I	+	-	-	+	+	-																	+	+	-	+	+	+	+	+
*Eco*RI-O	-	+	-	+	-	+																	+	+	+	-	+	+	+	+
*Eco*RI-A	-	-	+	-	+	+																								
*Eco*RI-J							+	-	-	-	-																			
*Hin*dIII-E							-	+	-	-	-																			
*Pst*I-E							-	-	+	-	-																			
*Eco*RI-F							-	-	-	+	-																			
*Sst*I-F							-	-	-	-	+												+	+	+	+	-	+	+	+
*Eco*RI-C												+	-	-	-	+	+	+	-	-	-		+	+	+	+	+	-	+	+
*Eco*RI-G												-	+	-	-	+	-	-	+	+	-									
*Eco*RI-D												-	-	+	-	-	+	-	+	-	+		+	+	+	+	+	+	-	+
*Eco*RI-Q												-	-	-	+	-	-	+	-	+	+									
pAc7A																						+	+	+	+	+	+	+	+	-
*Eco*RI-B																						-								
Replication	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	+	-	-

Note. A plus or minus at the bottom of the table indicates whether or not replication of the reporter plasmid pAcHL was detected with the combination of cosmids and/or plasmids indicated in the table.

Replication assays with the four subclones showed that *Eco*RI-G and -Q were not required for replication. Only a combination of *Eco*RI-C and *Eco*RI-D could substitute for cosmid 35, which means that cosmid 35 contains, besides the *helicase* gene in *Eco*RI-D, at least one other essential gene located in *Eco*RI-C. To date only a part of *Eco*RI-C has been sequenced (Kool *et al.*, 1994a), and eight open reading frames (ORFs) were identified. Except for *gp41* (Whitford and Faulkner, 1992), neither whether these genes are expressed or the function of the products they encode is known.

To identify the essential gene(s) in cosmid 56, we initially focused on the region surrounding *hr5* that contains several early genes that could encode proteins required for replication. Therefore, a *Bam*HI-*Sst*II subclone of *Bam*HI-B (Fig. 8.3D, pAc7A) was tested for its ability to substitute for cosmid 56 in the replication assays (Table 8.1, 22). Fragment *Eco*RI-B, which is also part of cosmid 56, was not investigated at this stage, because this fragment was also present in cosmid 57 (Fig. 8.3D). Any required *trans*-acting factor expressed by *Eco*RI-B would thus be supplied by cosmid 57. The result of the assay was that pAc7A was able to substitute for cosmid 56. Only three early genes, *p94*, *p35* (Friesen and Miller, 1987) and *p26* (Liu *et al.*, 1986; Rankin *et al.*, 1986) are located on this fragment, which means that either one or a combination of these genes is essential for replication.

To complete this analysis, we examined the *Eco*RI-B fragment. *Eco*RI-B contains several early genes (*me53*, *ie-1*, *ie-n* and *pe38*) and one or more of these genes may be essential for DNA replication, either directly or indirectly, because of their known *trans*-activation of delayed-early genes (Blissard and Rohrmann, 1991; Guarino and Summers, 1986; Nissen and Friesen, 1989; Lu and Carstens, 1993). To test the requirement of *Eco*RI-B, transient assays with the complete set of six plasmids required for replication (Fig. 8.3), with and without *Eco*RI-B (Table 8.1, 23, 24), were performed with pAcHL as the reporter plasmid. In addition, to show that all these regions are essential, transfections were carried out with mixtures of only five plasmids and *Eco*RI-B by eliminating one plasmid at a time. The results in Fig. 8.4 demonstrate that fragment *Eco*RI-B indeed contains (a) gene(s) essential for replication.

The requirement of regions *Eco*RI-I (map unit [m.u.] 0.3 - 5.8), *Eco*RI-O (m.u. 6.9 - 8.7), *Sst*I-F (m.u. 38.9 - 45.0), *Eco*RI-D (m.u. 59.9 - 68.3) and 7A (m.u. 84.3 - 89.7) was confirmed, using a similar plasmid depletion approach (Table 8.1, 25-30). When *Eco*RI-C was omitted from the transfection mixture, replication still occurred. This suggests that the

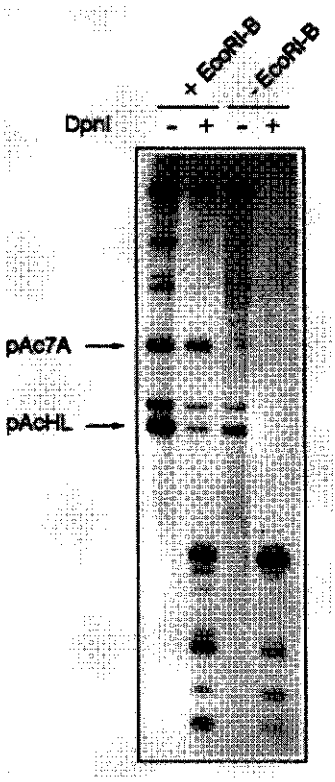


Figure 8.4. Southern blot with results of cotransfection with set of seven essential plasmids. *S. frugiperda* cells were transfected with equimolar amounts of plasmids containing the following fragments: *Eco*RI-I, *Eco*RI-O, *Sst*I-F, *Eco*RI-C, *Eco*RI-D, *Bam*HI-*Sst*II subfragment of *Bam*HI-B (7A), plus *Eco*RI-B (lane 1) or minus *Eco*RI-B (lane 2). Plasmid pAcHL (1 μg), that contains *hr*2 as an origin, was used as reporter plasmid. Total DNA from the transfected cells was isolated 72 h after transfection and digested with *Hin*dIII with (+) or without (-) *Dpn*I. Southern hybridization was carried out using ^{32}P-labeled pUC19 as a probe.

essential gene(s) within *Eco*RI-C must be located in the 3.0 kbp *Eco*RI-*Sst*I subfragment of *Eco*RI-C, which overlaps with *Sst*I-F (Fig. 8.3). This implies that fragment *Sst*I-F contains, in addition to the DNA polymerase gene, at least one other essential gene, located in the part that overlaps with *Eco*RI-C. Recently, the *lef-3* gene was identified within this region, shown to be essential for late gene expression (Li *et al.*, 1993), and this gene may also be essential for replication. By analogy with the results found for the cotransfections with cosmid DNA (Fig. 8.2, lanes 3-8) or with complete viral DNA (Fig. 8.2, lanes 9 and 10) replication of all plasmids, having a viral *ori* or not, is observed in the cotransfection experiment where all essential regions are present in the transfection mixture (Fig. 8.4). However, plasmid pAc7A, that contains two viral *ori's*, the non-*hr ori* in *Hin*dIII-K and *hr*5, replicates better than the plasmids without an *ori*, and also better than plasmid pAcHL, that contains *hr*2. Elimination of any plasmid from this mixture abolished replication of all other plasmids.

DISCUSSION

In this paper a transient complementation assay, based on the strategy described by Challberg (1986) for HSV-1, was used to identify regions of the AcMNPV genome that are involved in DNA replication. For this purpose, a cosmid library of AcMNPV was constructed and four cosmids were identified that lacked only the *hr*2 and *hr*4 regions and the *Eco*RI-L fragment. We demonstrated that these four cosmids can provide all of the functions required for transient replication of a plasmid containing an AcMNPV origin of DNA replication and have allowed us to locate and identify regions of the AcMNPV genome containing all the genes involved in DNA replication.

In contrast to the reported transient replication systems for herpes simplex virus 1 (HSV-1) and human cytomegalovirus (HCMV), in which plasmid replication was completely dependent on the presence, in *cis*, of an authentic *ori* (Challberg, 1986; Wu *et al.*, 1988; Pari *et al.*, 1993), it was found for AcMNPV that transient replication was not dependent on the presence of a putative *ori* when plasmids were cotransfected with either complete viral DNA, cosmid DNA, or plasmid DNA carrying all the required genes for DNA replication. Plasmids without a viral *ori*, e.g pUC19, were replicated as well as plasmids with an *ori*. This is also in contrast with experiments where intact virus was used to supply *trans*-acting factors for plasmid replication. In that case plasmid replication was completely dependent on the presence, in *cis*, of an AcMNPV origin of DNA replication (Kool *et al.*, 1993a, 1993b). Non-specific transient replication of plasmid DNA when cotransfected with naked viral DNA was previously reported by Guarino and Summers (1988).

Although integration of plasmid DNA into the viral genome during cotransfection may be a possible explanation for the *ori* independent replication, it is unlikely because DNA isolations always resulted in *Dpn*I resistant fragments with the size expected for the nonintegrated plasmids. An alternative explanation could be that proteins present in the virus particles are necessary to discriminate between plasmids with and without a viral *ori*. One of these proteins could be IE-1, since this protein has been found to be associated with budded virions (Theilmann and Stewart, 1993). It has also been demonstrated that transfected cells expressing IE-1 contained an *hr*5 enhancer-specific DNA-binding activity (Guarino and Dong, 1991). Furthermore, it has been shown that the IE-1 polypeptide contains a DNA-binding domain and is able to form complexes with *hr*5 (Kovacs *et al.*, 1992). In the absence

of IE-1 or any other virus-associated protein, i.e. when plasmids are cotransfected with naked viral DNA, the selectivity for the authentic *ori* may thus be lost and other sequences may be used as *ori*.

Although transient plasmid DNA replication in this system appears to be independent of the presence of a viral *ori*, the results obtained demonstrate that this system is a valid approach to identify AcMNPV replication genes. For instance, when the *helicase* gene, already known to be required for viral DNA replication (Lu and Carstens, 1991), or the DNA polymerase gene, predicted to be also required, were not present in the transfected DNA, no replication of plasmid DNA was detected.

Six regions of the AcMNPV genome were found to contain genes essential for transient plasmid DNA replication. These regions are: *Eco*RI-I (m.u. 0.3 - 5.8), *Eco*RI-O (m.u. 6.9 - 8.7), *Sst*I-F (m.u. 38.9 - 45.0), *Eco*RI-D (m.u. 59.9 - 68.3), a *Bam*HI-*Sst*II subfragment of *Bam*HI-B (7A) (m.u. 84.3 - 89.7) and *Eco*RI-B (m.u. 90.0 - 100). It is concluded that *Sst*I-F contains, besides the DNA polymerase gene, at least one other essential gene, while *Eco*RI-D contains the *helicase* gene. Thus, in this system, replication depends on the simultaneous expression of at least 7 genes, located on six different cotransfected plasmids.

*Eco*RI-I contains several ORFs (Fig. 8.3A); (Possee *et al.*, 1991). Since AcMNPV DNA replication is an early event (Tjia *et al.*, 1979) and transient plasmid replication proceeds along with viral DNA replication (Kool *et al.*, 1993b), plasmid replication requires the expression of early genes. Possible candidates for essential replication genes within *Eco*RI-I are ORF 984 and ORF 453, because they are expressed early (Hardin and Weaver, 1990; Tilakaratne *et al.*, 1991). The function of the proteins coded for by these ORFs is not known. Other possible candidates in this region are ORF 327 and *lef-2* for which early transcripts have also been reported (Mainprize *et al.*, 1986). Furthermore, Passarelli and Miller (1993a) showed that *lef-2* is an early gene required for late and very late gene expression. Another gene required for late and very late gene expression, called *lef-1* (Passarelli and Miller, 1993b), maps to the *Eco*RI-O fragment of AcMNPV. This region was also found to be essential for transient plasmid replication as demonstrated in this paper. Since late and very late gene expression is dependent on viral DNA replication *in vivo* (Rice and Miller, 1986), *lef-1* and *lef-2* may be directly involved in DNA replication and, as a consequence also, but indirectly, be involved in late and very late gene expression.

Fragment *Sst*I-F was able to substitute for cosmid 50 in the complementation assay.

Sequence analysis revealed one ORF in this region coding for a putative DNA polymerase (Tomalski *et al.*, 1988). Further subcloning of the *dna pol* gene from *Sst*I-F and subsequent testing must confirm that *dna pol* is the only essential gene within cosmid 50 or that other genes in this region are required. The *pcna*-like protein gene (Crawford and Miller, 1988), also located within cosmid 50 (Fig. 8.3B) was not essential and we observed no stimulation of transient plasmid replication. Since interruption of the *pcna*-like protein gene is known to cause a delay in the expression of late and very late genes (Crawford and Miller, 1988), it may be directly involved in the expression of these genes, but not in DNA replication.

When cosmid 35 was analyzed, two regions were found to contain one or more essential genes. In one region, *Eco*RI-D, the *helicase* gene is likely to be the only essential gene, but other genes in this region can not be ruled out. The identity of the essential gene in the other region, *Eco*RI-C, is still unknown. The observation that *Sst*I-F could substitute for *Eco*RI-C, however, means that there is an additional essential gene on *Sst*I-F, which is located between the left *Eco*RI-site of *Eco*RI-C and the right *Sst*I-site of *Sst*I-F. Recently, *lef-3* was identified within this region, shown to be essential for late gene expression (Li *et al.*, 1993), and that may also be essential for DNA replication.

The fifth essential region is located within a *Bam*HI-*Sst*II subfragment of *Bam*HI-B. This region contains four intact ORFs, corresponding with *p94*, *p35* (Friesen and Miller, 1987), *p26* (Liu *et al.*, 1986; Rankin *et al.*, 1986) and *p10* (Kuzio *et al.*, 1984). The *p10* gene is a very late gene non-essential for viral replication (Vlak *et al.*, 1988). The other three genes are early genes and therefore may be involved in DNA replication. However, insertion mutations in the *p94* gene (Friesen and Nissen, 1990) were found to have no effect on the replication of the mutant virus. Also the fact that most of *p94* is absent in *Bombyx mori* MNPV (BmMNPV), a baculovirus highly homologous to AcMNPV (Kamita *et al.*, 1993), makes it unlikely that *p94* is essential for replication. The function of *p26* is not known, but Rodems and Friesen (1993) recently showed that *p26* can be deleted from the genome. This recent data does suggest that *p35* is involved in virus DNA replication, although other coding regions within this portion of the virus genome my have a role. The *p35* gene was found to prevent virus-induced apoptosis in *S. frugiperda* 21 cells (Clem *et al.*, 1991). *P35* deletion mutants of AcMNPV caused premature death of the cells. In addition, a reduction in virus yield from 200- to 15,000-fold and a very low level of late and very late gene expression was observed (Hershberger *et al.*, 1992). This suggests that *p35* is not essential for viral DNA

replication, but functions as a stimulatory gene. The reason that this portion of the genome containing the *p35* gene in this paper was identified as being essential for transient plasmid replication can be that (a) the replication signal obtained without *p35* was below detection level or (b) that *p35* is essential for this transient plasmid replication in contrary to viral DNA replication.

The last essential region for transient plasmid replication is *Eco*RI-B. Several immediate-early genes were identified within this region: *me53*, *ie-0*, *ie-1*, *ie-n* and *pe38* (Knebel-Mörsdorf, *et al.*, 1993; Chisholm and Henner, 1988; Guarino and Summers, 1987; Carson *et al.*, 1991; Krappa and Knebel-Mörsdorf, 1991). At least one of these genes is probably essential for DNA replication. The products of these early genes appear to activate other early genes (Blissard and Rohrmann, 1991; Guarino and Summers, 1986; Guarino and Summers, 1987; Nissen and Friesen, 1989; Lu and Carstens, 1993). Furthermore, IE-1, but not IE-0, was found to be required for the expression of late and very late genes (Passarelli and Miller, 1993a). The requirement of IE-1 in activating late promoters, and the augmentation of late gene expression by IE-N, is probably indirect, involving the expression of other early *trans* regulators or gene products involved in DNA replication. Based on this it may be assumed that at least *ie-1*, and possibly also *ie-n*, are essential for DNA replication. This idea is also supported by the observation that various attempts to delete the *ie-1* gene from the AcMNPV genome have been failed (Kovacs *et al.*, 1992).

The products of the other two putative regulatory genes, *me53* (Knebel-Mörsdorf *et al.*, 1993) and *pe38* (Krappa and Knebel-Mörsdorf, 1991), contain both zinc finger-like motifs, while in addition, PE38 contains a leucine zipper motif. These motifs are also found in several other regulatory DNA binding proteins. Recently, Lu and Carstens (1993) showed that in addition to IE-1, PE38 is also able to *trans*-activate the promoter of the *helicase*-like gene, which is augmented by IE-N. This suggests that *pe38* is, although may be not essential, at least involved in DNA replication. For *me53*, a direct involvement in gene regulation or DNA replication has not been reported.

In conclusion, six regions of the AcMNPV genome, containing at least seven genes have been found to be essential for transient plasmid DNA replication. For HSV-1 also seven genes were identified as essential for transient plasmid replication. In addition, four genes were identified which stimulated DNA replication (Challberg, 1986; Wu *et al.*, 1988). Three of these stimulatory genes, *ie175*, *ie110* and *ie63* are immediate early genes and are known

to stimulate the expression of several HSV genes in transient expression systems (see Fields and Knipe, 1990, for review). Thus, it seems likely that these immediate-early genes function indirectly in transient replication assays by stimulating the expression of other genes, the products of which are, in turn, directly involved in DNA synthesis. However, in contrast to both the HCMV system, in which at least three *ie* transcriptional regulators are essential for transient plasmid replication (Pari *et al.*, 1993), and the AcMNPV system, with at least one essential *ie*-gene, the virus-encoded *trans*-activators in HSV-1 are not essential (Wu *et al.*, 1988). An explanation for this could be that in AcMNPV at least the *ie-1* gene may be bifunctional. Besides its function as a *trans*-activator, it may affect or regulate initiation of replication, based on its putative binding capacity to *hr* regions.

The seven HSV-1 genes necessary and sufficient for origin-dependent plasmid DNA replication in a transient complementation assay, comprise genes for a DNA polymerase (UL30) and an associated polymerase accessory protein (UL42), a single-stranded-DNA-binding protein (UL29), a heterotrimeric helicase-primase complex (UL5, UL8 and UL52) and an origin-binding protein (UL9) (see Challberg and Kelly, 1989; Kornberg and Baker, 1992, for reviews). For AcMNPV only a *helicase* gene has been identified as being essential for DNA replication (Lu and Carstens, 1991).

The experiments described in this paper illustrate that the transient complementation assay is a powerful tool for the identification of genes essential or auxiliary for AcMNPV DNA replication. The identification and availability of these genes has now opened the way to study the baculovirus DNA replication machinery in detail.

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CHAPTER 9

IDENTIFICATION OF GENES INVOLVED IN DNA REPLICATION OF THE *AUTOGRAPHA CALIFORNICA* BACULOVIRUS

SUMMARY

Using a transient replication assay, nine genes involved in DNA replication were identified in the genome of the *Autographa californica* baculovirus. Six genes specifying *helicase*, *dna pol*, *ie-1*, *lef-1*, *lef-2*, and *lef-3* are essential for DNA replication while three genes, *p35*, *ie-2*, and *pe38* stimulate DNA replication. No stimulation by the *pcna*-like protein gene was observed. At high copy numbers of transfected replication genes origin-independent replication of plasmids occurred in the transient complementation assays, while at lower copy numbers this process became origin specific.

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INTRODUCTION

The *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV) is the type species of the *Baculoviridae*, a large family of insect viruses. It has a circular, supercoiled DNA genome of approximately 131 kilo basepairs (see Blissard and Rohrmann, 1990; O'Reilly *et al.*, 1992; Kool and Vlak, 1993). It has been extensively exploited for the overexpression of eukaryotic genes and is being engineered for possible use as a viral insecticide. Despite its widespread use, little is known about the mechanism by which AcMNPV DNA replicates. Eight regions distributed around the genome have been identified as putative origins (*ori's*) of DNA replication (Pearson *et al.*, 1992; Kool *et al.*, 1993a, b; Leisy and Rohrmann, 1993). Seven of these *ori's* are located within homologous regions (*hr's*) (Fig. 9.1) which contain repeats of closely related imperfect palindromes (Guarino *et al.*, 1986). One *ori* is located within the *Hin*dIII-K fragment, which does not contain an *hr* region (Kool *et al.*, 1994b).

To date only a putative helicase (Lu and Carstens, 1991) and DNA polymerase gene (Pearson *et al.*, 1993) have been identified as essential for baculovirus DNA replication. In addition, a gene encoding a protein resembling a proliferating cell nuclear antigen (PCNA), which is a DNA polymerase processivity factor in other systems, has been identified in AcMNPV (O'Reilly *et al.*, 1989), but its role in DNA replication has not yet been determined.

Using a transient replication assay, six large regions of the AcMNPV genome were identified that contain one or more genes involved in DNA replication (Kool *et al.*, 1994a). In this report, we demonstrate that these six regions contain six genes that are essential for DNA replication: *helicase* (Lu and Carstens, 1991), *dna pol* (Tomalski *et al.*, 1988), *lef-1* (Passarelli and Miller, 1993b), *lef-2* (Possee *et al.*, 1991; Passarelli and Miller, 1993a), *lef-3* (Li *et al.*, 1993), and *ie-1* (Guarino and Summers, 1987). Furthermore, three additional genes were found that were not essential but stimulated DNA replication at different rates: *p35* (Friesen and Miller, 1987) and two genes known as transcriptional transactivators, *ie-2* (formerly called *ie-n*) (Carson *et al.*, 1988, 1991) and *pe38* (Krappa and Knebel-Mörsdorf, 1991).

MATERIALS AND METHODS

Cells and virus

Spodoptera frugiperda (*Sf*-9) cells (Vaughn *et al.*, 1977) were cultured in TNM-FH medium (Hink, 1970), supplemented with 10% fetal calf serum (FCS). The E2-strain of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) (Smith and Summers, 1978) was used as wild-type (wt) virus. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Summers and Smith, 1987).

Plasmid constructions

The nine replication genes were identified within six regions previously shown to be essential for DNA replication (Kool *et al.*, 1994a; see also Fig. 9.1). Subclones of each region were tested for their ability to substitute for the larger parental clone. The following clones were constructed: *Lef-1* is located in *Eco*RI-O and was cloned as an *Nru*I-*Eco*RI fragment (map units [m.u.] 7.5 to 8.7 [Kool and Vlak, 1993]) into pUC19. *Lef-2* is in *Eco*RI-I and was cloned as *Mlu*I fragment (m.u.1.9 to 2.6) with *Mlu*I-*Bgl*II linkers into the *Bam*HI-site of pUC19. *Dna pol* and *lef-3* are in *Sst*I-F. *Dna pol* was cloned as a *Sst*I-*Eco*RV fragment (m.u. 38.9 to 41.6) into pBKS-, and *lef-3* was cloned as an *Eco*RI-*Apa*I (m.u. 42.8 to 44.5) fragment into pJDH119 (Hoheisel, 1989). The *helicase* gene (p143) in *Eco*RI-D was cloned as an *Eco*RI-*Ssp*I (m.u. 59.9 to 63.5) fragment into pBKS-. Region 7A contains the *p35* gene on the *Eco*RI-S fragment (m.u. 85.8 to 87.7). It was cloned into pUC19. *Ie-1*, *ie-2*, and *pe38* are present on *Eco*RI-B. *Ie-1* was cloned as a *Cla*I-*Hin*dIII fragment (m.u. 94.7 to 96.9) into pUC8. *Ie-2* as a *Pst*I-N fragment (m.u. 96.9 to 98.9) and *pe38* as a *Pst*I-*Eco*RI fragment (m.u. 98.9 to 100.0) were cloned into pUC19. The *pcna*-like protein gene was cloned as an *Eco*RI-*Hin*dIII fragment, containing *Eco*RI-T and part of *Eco*RI-M (m.u. 29.0 to 30.9) into pBKS-. The reporter plasmid (pAcHL) contained *hr2* in the HindIII-L fragment cloned into pBKS-. All the plasmids were transformed into *Escherichia coli* JM101 or DH5α using standard techniques (Sambrook *et al.*, 1989). DNA isolation, purification in CsCl gradients or Qiagen columns, digestion with restriction enzymes, and agarose gel electrophoresis was carried out according to standard procedures (Sambrook *et al.*, 1989) or the manufacturer's instructions.

Replication assays

Transient replication assays were carried out as described previously (Kool *et al.*, 1994a), with minor modifications. *S. frugiperda* (*Sf*-9) cells were plated onto 6-well plates at a density of 2 x 10^6 cells per well in TNM-FH medium (Hink, 1970), supplemented with 10% fetal calf serum (FCS). After 24 h, the medium was removed and 1 μg of the *ori*-containing plasmid pAcHL (*hr*2) was mixed with equimolar amounts of plasmids containing the replication genes, with 0.5 μg DNA of a 5 kbp plasmid as standard. The DNA was mixed with 200 μl transfection buffer (25 mM HEPES, 140 mM NaCl, 125 mM $CaCl_2.2H_2O$, pH 7.1) and 200 μl Grace's medium plus 10% FCS and added onto the cells. After incubation for 4 h at 27°C, the transfection medium was removed and 1 ml of fresh TNM-FH medium, supplemented with 10% FCS was added. After 72 h at 27°C, total DNA was isolated from the cells (Summers and Smith, 1987) and resuspended in 64 μl TE (10 mM Tris-HCl, 1mM EDTA, pH 8.0). An aliquot (16 μl) was digested with *Hin*dIII to linearize the plasmids, and with *Dpn*I to determine if replication had occurred. After agarose gel electrophoresis, the DNA was transferred to membrane filters (GeneScreen, New England Nuclear) by the method of Koetsier *et al.* (1993). Radioactively labeled pBKS- was used as probe for hybridization.

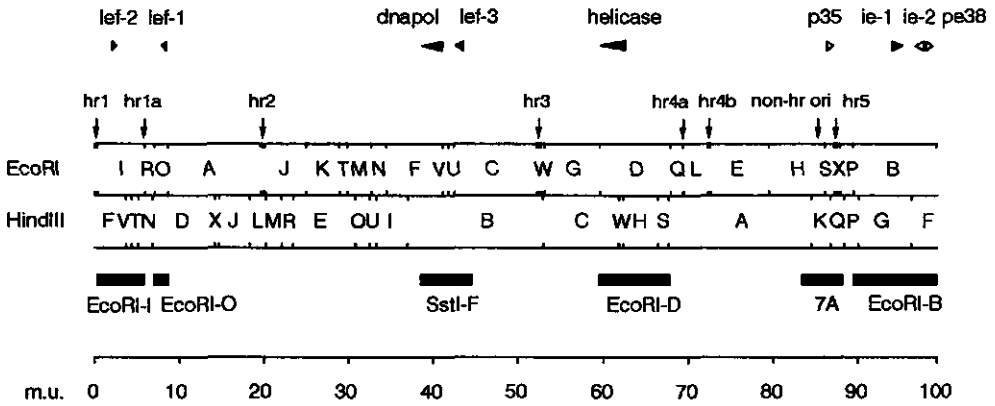

Figure 9.1. Linear map of *Eco*RI and *Hin*dIII restriction sites on AcMNPV DNA. Six regions previously shown to be required for DNA replication (Kool *et al.*, 1994a) include *Eco*RI-I, *Eco*RI-O, *Sst*I-F, *Eco*RI-D, 7A, and *Eco*RI-B. Putative origins of replication including seven *hr* regions and one non-*hr* region are shown above the map together with the location of the essential (black arrows) and stimulating (open arrows) replication genes. m.u. = map unit.

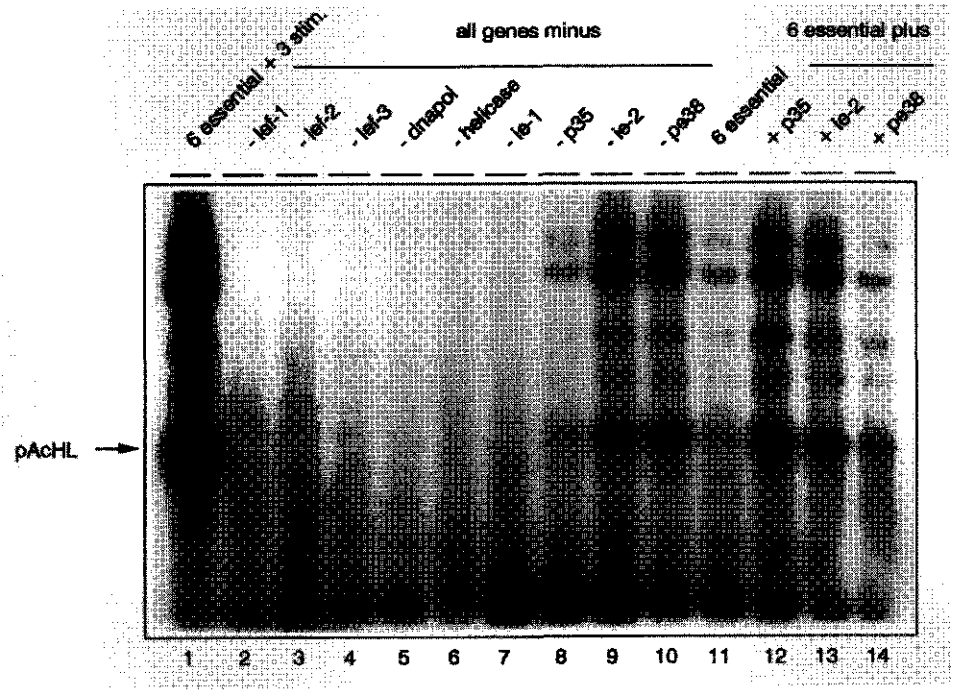

Figure 9.2. Transient replication assay of nine AcMNPV replication genes. These were identified within six regions previously shown to be essential for DNA replication (Kool *et al.*, 1994a; see also Fig. 9.1). *S. frugiperda* cells were cotransfected with equimolar amounts of plasmids containing the AcMNPV replication genes and 1 μg pAcHL (*Hin*dIII-L) containing *hr*2 as AcMNPV origin of DNA replication. Total cellular DNA was isolated 72 h after transfection and digested with *Hin*dIII and *Dpn*I. Southern hybridization was carried out using ^{32}P labeled pBKS- as probe.

RESULTS

Identification of the AcMNPV replication genes

The six regions of the AcMNPV genome, previously reported to be involved in DNA replication, are *Eco*RI-I, *Eco*RI-O, *Sst*I-F, *Eco*RI-D, a *Bam*HI-*Sst*II subfragment of *Bam*HI-B (7A), and *Eco*RI-B (Fig. 9.1) (Kool *et al.*, 1994a). In order to locate the genes on these fragments that influenced DNA replication, subclones of each of these six regions were tested

in a *Dpn*I-based replication assay (Kool *et al.*, 1994a) for their ability to substitute for the original clone. From minimal subclones, nine genes were identified including six that were essential for DNA replication, and three which stimulated replication (Fig. 9.1, black and open arrows, respectively). When nine plasmids containing each of these genes were transfected into uninfected *Spodoptera frugiperda* (*Sf*-9) cells, a high level of plasmid replication was observed (Fig. 9.2, lane 1). When either *lef-1*, *lef-2*, *lef-3*, *dna pol*, *helicase* or *ie-1* was omitted from the transfection mix, no replication was detected (lanes 2-7) indicating that each of these genes is essential. When one stimulating gene, either *p35*, *ie-2* or *pe38* was omitted, lower levels of replication were observed (lanes 8-10). Lane 11 shows that the six essential genes alone were sufficient for replication, although the *Dpn*I resistant signal is weak. The addition of one stimulatory gene to the minimal set of replication genes resulted in differing levels of signal intensity for each gene. *P35* stimulated replication to much higher level than *ie-2* or *pe38*, the latter giving the lowest increase (lanes 12-14). *Ie-2* and *pe38* are known transactivators of early gene transcription (Carson *et al.*, 1988; Lu and Carstens, 1993), and *p35* is known as an inhibitor of virus-induced apoptosis in *S. frugiperda* cells (Clem *et al.*, 1991).

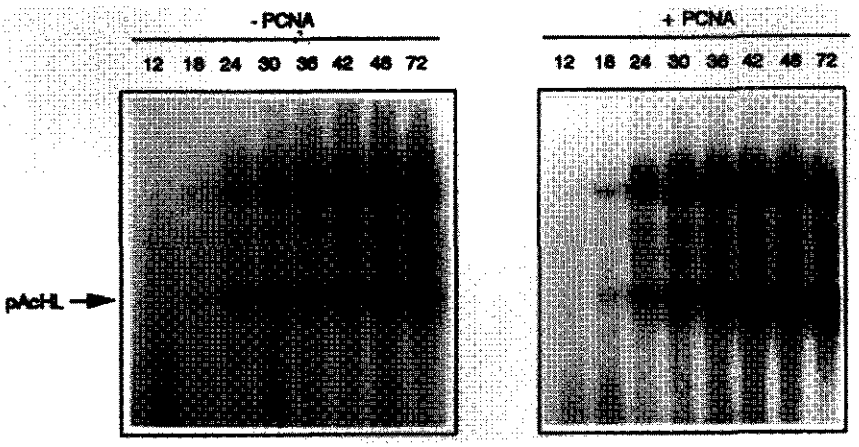

Figure 9.3. Time course of transient plasmid DNA replication. *S. frugiperda* cells were cotransfected with pAcHL (*Hin*dIII-L, *hr*2) as reporter plasmid and equimolar amounts of nine plasmids containing the AcMNPV replication genes with or without *pcna*. Total cellular DNA was isolated at the indicated times (in hours) post transfection and digested with *Hin*dIII and *Dpn*I. Southern hybridization was carried out using ^{32}P labeled pBKS- as probe.

Another gene of the AcMNPV genome that has been suggested to play a role in DNA replication is the gene encoding a PCNA-like protein (Crawford and Miller, 1988; O'Reilly *et al.*, 1989). Homologues of this gene code for DNA polymerase processivity factors and are often found to be essential in other DNA replication systems (Kornberg and Baker, 1992). It was already known that the *pcna*-like protein gene is not essential in AcMNPV for DNA replication, since deletion of this gene from the viral genome still yielded viable virus, though exhibiting a delay in late gene expression (Crawford and Miller, 1988). However, no significant stimulation was detected when varying concentrations of the AcMNPV *pcna*-like protein gene were included in the transient complementation assays (data not shown). To test whether the absence of *pcna* could only cause a delay in DNA replication *S. frugiperda* cells were transfected with the complete set of replication genes and pAcHL, with and without *pcna*. Cellular DNA was harvested at different time intervals after transfection. In both cases newly replicated plasmid DNA was first detected 18 h after transfection and no significant difference was found in the levels or timecourse of plasmid replication (Fig. 9.3).

Origin-dependent plasmid replication

The results in Fig. 9.2 demonstrate that plasmid replication in the transient complementation assay is viral origin-independent. Not only the reporter plasmid pAcHL (containing *hr*2 as viral origin), but all added plasmids are replicating when all essential genes are present. This agrees with previous results which showed that plasmid replication becomes viral origin-independent when naked DNA instead of intact virions are used to supply essential *trans*-acting factors (Kool *et al.*, 1994a). The difference in gene copy number may play a role in this phenomenon, since cells infected with virus at a multiplicity of infection (MOI) of 1 contain only one copy of each gene, while in cells transfected with cloned viral replication genes the gene dose is much higher, potentially resulting in an overproduction of replication proteins. Replication origins may thus become saturated with origin-recognizing protein(s) and the excess of protein(s) may bind non-specifically to other sequences. To test this hypothesis the amount of plasmid DNA containing the nine replication genes and the amount of reporter plasmid were reduced to 10%, 1%, 0.1% or 0.01% of the original amount used in Fig. 9.1. This did not result in specific replication of the *ori*-containing plasmid, but led to a dramatically decreased replication signal for all plasmids (Fig. 9.4A). However, when only the amount of the replication genes was reduced, with the

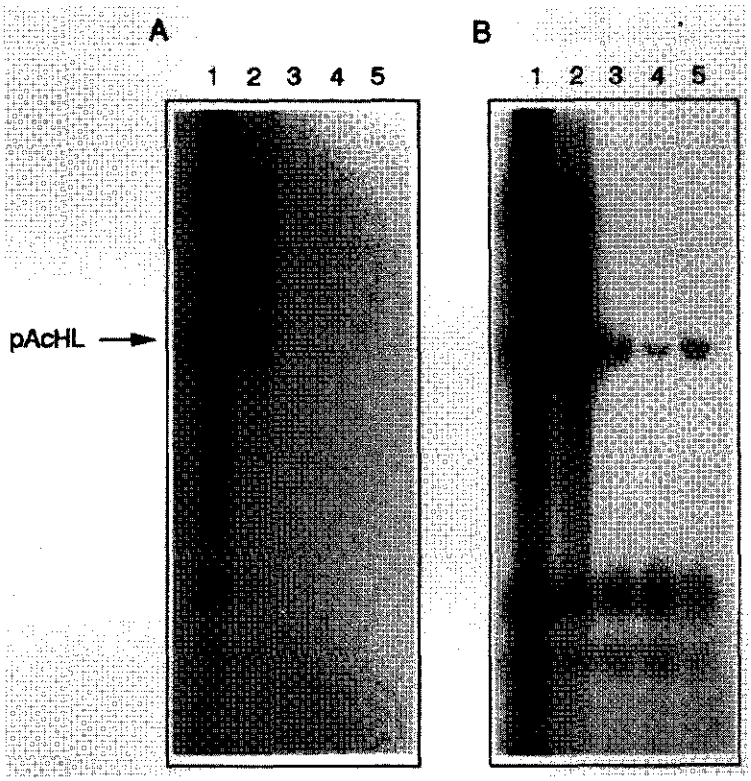

Figure 9.4. Origin-dependent plasmid DNA replication. *S. frugiperda* cells were cotransfected with pAcHL (*Hin*dIII-L, *hr*2) as reporter plasmid and equimolar, but for each lane different total amounts (including [A] the reporter plasmid or not [B]) of the nine plasmids containing the AcMNPV replication genes. The total amount of plasmid DNA containing the replication genes was such that in lane 1 the total amount was as described in the Materials and Methods section, in lane 2 only 10% of this amount was used, 1% in lane 3, 0.1% in lane 4, and 0.01% in lane 5. The amount of reporter plasmid pAcHL used for transfection was stepwise decreased the same way as for the replication genes in panel A, whereas in panel B the amount of reporter plasmid was kept constant and 1 μg was used in all lanes. Total cellular DNA was isolated 72 h after transfection and digested with *Hin*dIII and *Dpn*I. Southern hybridization was carried out using ^{32}P labeled pBKS- as probe.

amount of the *ori*-containing plasmid kept constant at 1 μg, specific replication occurred and the level of this replication signal was even slightly higher when only 10% of the original input of plasmid DNA for the nine genes was used (Fig. 9.4B, lanes 1 and 2). Even lower amounts of input DNA resulted in replication of the *ori*-containing plasmid, although the signal became much weaker (Fig. 9.4B, lanes 3-5).

DISCUSSION

Six genes, representing the *helicase*, *dna pol*, *ie-1*, *lef-1*, *lef-2*, and *lef-3* genes, were identified in the baculovirus genome of AcMNPV as essential for transient DNA replication. The genes *p35*, *ie-2*, and *pe38* were found to stimulate this replication. No significant stimulation was detected from the *pcna*-like protein gene, nor did the absence of this protein cause a delay in plasmid DNA replication in the transient replication assay. These results, together with the fact that a *pcna*-like protein gene is absent from the genomes of the closely related *Bombyx mori* MNPV (S. Maeda, pers. comm.) and OpMNPV (data not shown), suggest that *pcna* is not directly involved in baculovirus DNA replication, but only has some role in regulating late gene expression in AcMNPV (Crawford and Miller, 1988).

Two of the three identified stimulatory genes, *ie-2* and *pe38*, are known as transactivators of transcription (Carson *et al.*, 1988; Lu and Carstens, 1993). Under the assay conditions used here they are not essential for DNA replication. In herpes simplex virus 1 transient replication assays, the four identified transactivators are also not essential for replication of *ori*-containing plasmids (Wu *et al.*, 1988). In contrast, in replication assays containing genes from the herpesviruses Epstein-Barr virus and human cytomegalovirus, the three identified transactivators for these viruses are essential (Fixman *et al.*, 1992; Pari *et al.*, 1993; Pari and Anders, 1993).

The third stimulatory gene identified in AcMNPV, *p35*, has previously been identified as inhibitor of virus-induced apoptosis in *S. frugiperda* cells (Clem *et al.*, 1991). Cells infected with AcMNPV *p35* deletion mutants showed an accelerated apoptotic death, a 200 to 15,000-fold reduction in virus yield, and low levels of late and very late gene expression (Hershberger *et al.*, 1992). This already suggested that *p35* is not essential for viral DNA replication, but most likely functions as a stimulatory gene. However, it is known that infection with a *p35* deletion mutant in *Trichoplusia ni* 368 cells does not result in a reduction of virus production (Clem *et al.*, 1991). Also the fact that *p35* is absent in the OpMNPV genome (Gombart *et al.*, 1989) suggests that *p35* does not have an essential function in addition to a role in inhibiting apoptosis, a process which occurs only in certain cell lines. Along this line, the stimulating effect of *p35* in the transient replication assays is probably not based on activation of the replication process, but due to inhibiting apoptosis, possibly induced by the expression of one or more of the replication genes. This phenomenon

may be similar to what has been described for adenoviruses where the expression of two genes (13S and 12S E1A) induces apoptosis, a process also found to be cell-line specific, and which is normally inhibited by the E1B protein (White and Stillman, 1987).

Previous results (Kool *et al.*, 1994a) showed transient DNA replication to be *ori*-independent, when naked DNA was used instead of complete virus to supply essential *trans*-acting factors. Data from Fig. 9.4 suggest that this phenomenon resulted from employing high gene copy numbers in the transfections. Under these conditions, a relative abundance of replication proteins may be produced, which may cause saturation of specific origin sequences with these proteins. The excess of protein molecules thus may bind to other origin-like structures, even when the affinities are low, and hence cause replication of any plasmid.

A review of well-characterized replication systems from a variety of organisms including *E. coli*, bacteriophages T7 and T4, or the animal viruses SV40 and herpesvirus indicates a common theme in the number and function of the components required for DNA replication by these systems (Kornberg and Baker, 1992). All these systems are composed of a helicase, DNA polymerase, DNA polymerase accessory protein, primase, and single stranded DNA binding protein components. In addition, there are often requirements for a topoisomerase and origin binding proteins.

Until now, possible functions of the six essential replication proteins of AcMNPV are only known for the helicase and the DNA polymerase, based on their homology with other systems. The three *lef* genes are known to be essential for late gene expression (Passarelli and Miller, 1993a, b; Li *et al.*, 1993), but since it is known that late gene expression is DNA replication dependent (Rice and Miller, 1986), their role in this process is probably indirect. IE-1 is known as a strong transactivator of early genes (Guarino and Summers, 1986). A direct search in databases for homology with any of these four replication proteins had no result, but a intensive search in the replication proteins of herpesviruses using computer software and visual alignments proved more successful. These comparisons are shown in Chapter 10. The possibility of an evolutionary link between the *Baculoviridae* and the *Herpesviridae* is appealing because the two viral families share a number of features in DNA structure and replication. Both replicate in the nucleus and their genomes are circular (baculovirus) or become circular (herpesvirus) during replication. Their genomes may also replicate in a similar manner as transfection of *ori*-containing plasmids into infected cells results in large concatemers of input plasmid DNA (Leisy and Rohrmann, 1993;

Hammerschmidt and Mankertz, 1991) suggesting a rolling circle mode of replication.

Whereas the identification of six essential genes and three genes that stimulate replication will provide the foundation for further investigations of baculovirus DNA replication, a variety of other genes may be involved in this process. The replication assay we employed is limited by the fact that it does not result in the production of functional viral genomes. If the viral genome replicates in a manner similar to *ori*-containing plasmid DNA, with the production of long concatemers of the genome, then other genes required for the resolution of these structures are likely present. Furthermore, the involvement of host genes in the replication process cannot be ruled out.

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CHAPTER 10

SEQUENCE COMPARISONS OF FOUR BACULOVIRUS REPLICATION PROTEINS WITH PUTATIVE HOMOLOGUES OF THE *HERPESVIRIDAE*

SUMMARY

Chapter 9 of this thesis describes the identification of nine genes in the baculovirus genome of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) that are involved in DNA replication. Six of them were found to be essential in a transient replication assay, whereas three genes were found to stimulate DNA replication. Two of the six essential replication proteins, the helicase and the DNA polymerase, have previously been shown to be related to homologues of the *Herpesviridae*. For the other four essential AcMNPV replication proteins, IE-1, LEF-1, LEF-2, and LEF-3 it is now shown that they are possibly also related to replication proteins of the *Herpesviridae*. These four AcMNPV replication proteins were aligned with their homologues of another baculovirus, *Orgyia pseudotsugata* MNPV, and replication proteins of five different herpesviruses. Based on the alignments with the herpesvirus proteins it is suggested that *ie-1* codes for a single stranded DNA binding protein, *lef-1* for a primase-associated protein, *lef-2* for a DNA polymerase processivity factor and *lef-3* for a primase.

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INTRODUCTION

Nine genes involved in DNA replication were identified in the baculovirus genome of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) (Kool *et al.*, 1994; Chapter 9). Six of them were found to be essential in a transient replication assay. These genes are *helicase*, *dna pol*, *ie-1*, *lef-1*, *lef-2*, and *lef-3*, whereas three additional genes, *p35*, *ie-2*, and *pe38* were found to stimulate DNA replication. Putative functions for the essential replication proteins are only known for the helicase and the DNA polymerase, based on the presence of several conserved helicase and DNA polymerase motifs in these proteins (Lu and Carstens, 1991; Tomalski *et al.*, 1988). *Ie-1* is known as a strong transactivator of several early baculovirus genes (Guarino and Summers, 1986), and the three *lef* genes were originally identified as genes essential for late gene expression (Passarelli and Miller, 1993a, b; Li *et al.*, 1993). For the three *lef* genes no homology was found so far with other known proteins in databases.

Tomalski *et al.* (1988) and Lu and Carstens (1991) already showed for the AcMNPV DNA polymerase and the helicase, respectively, that they are related to DNA polymerase and helicase encoded by herpesviruses, based on the finding of several conserved motifs in the proteins. However, baculoviruses and herpesviruses show more similarities. Both have enveloped nucleocapsids that replicate in the nucleus. Their genomes are circular (baculovirus) or become circular (herpesvirus) during replication and they may also replicate in a similar manner as transfection of origin-containing plasmids into infected cells results in large concatemers of input plasmid DNA (Leisy and Rohrmann, 1993; Hammerschmidt and Mankertz, 1991) suggesting a rolling circle mode of replication. This led to the examination of the possible relatedness of the other four, hitherto unassigned, baculovirus replication genes, *ie-1*, *lef-1*, *lef-2*, and *lef-3*, with genes involved in herpesvirus replication.

For a number of herpesviruses the genes involved in DNA replication have been identified and sequenced, including herpes simplex virus type 1 (HSV-1) (Challberg, 1986; Wu *et al.*, 1988; McGeoch *et al.*, 1988), human cytomegalovirus (HCMV) (Chee *et al.*, 1990; Pari *et al.*, 1993; Pari and Anders, 1993), and Epstein-Barr virus (EBV) (Baer *et al.*, 1984; Fixman *et al.*, 1992) (Table 10.1). Although the number of replication genes differs, a core set of six genes was found in all three herpesviruses. Homologues of these six replication genes are also found in two other completely sequenced herpesvirus genomes, varicella zoster virus

Table 10.1. Summary of AcMNPV replication genes, OpMNPV homologues, and possible herpesvirus counterparts.

AcMNPV	HSV-1	VZV	EHV	EBV	HCMV	CCV	Function
Helicase	UL5	55	57	BBLF4	UL105	ORF25	helicase
dnapol	UL30	28	30	BALF5	UL54	ORF57	DNA polymerase
ie-1	UL29	29	31	BALF2	UL57	ORF56	single stranded DNA-binding protein
lef-1	UL8	52	54	BBLF2/3	UL102	?	primase associated protein
lef-2	UL42	16	18	BMRF1	UL44	?	DNA polymerase processivity factor
lef-3	UL52	6	7	BSLF1	UL70	?	primase
-	UL9	51	53	-	-	?	origin binding protein
p35							apoptosis inhibitor (non-essential)
ie-2[a]	IE175[a]			BZLF1 (Zta)[a]	UL36-38[a]		regulatory protein[b]
pe38[a]	IE110[a]			BRLF1 (Rta)[a]	IRS1[a]		regulatory protein[b]
	IE63[a]			BMLF1 (Mta)[a]	IE1/IE2[a]		regulatory protein[b]
	TIF[a]						regulatory protein[b]
					UL84		unknown early protein
					UL112-113		unknown early protein

[a] The HSV, EBV, and HCMV *trans*-activators show no obvious homology to each other or to the AcMNPV or OpMNPV *trans*-activators.

[b] for AcMNPV, OpMNPV, and HSV, the regulatory proteins are not essential for replication.

(VZV) (Davison and Scott, 1986) and equine herpesvirus type 1 (EHV-1) (Telford *et al.*, 1992). No homologues for HSV-1 UL9, the origin-binding protein, were found in HCMV or EBV. The genome of the more distantly related herpesvirus, channel catfish virus (CCV), has also been completely sequenced and contains homologues of the helicase (ORF 25) and the DNA polymerase (ORF 57) (Davison, 1992). The gene encoding the ssDNA-binding protein (ORF 56) was identified in this study (Table 10.1).

To investigate the relation between baculovirus and herpesvirus replication proteins firstly computer-assisted alignments of the proteins coded by HSV UL8, UL9, UL29, UL42, and UL52 were made with their homologues in the other herpesviruses. This allowed the identification of patterns of conserved motifs within each protein family. The consensus sequences of these conserved motifs were then used to search the sequences of the four AcMNPV replication proteins for patterns of homology. Using the BESTFIT program of GCG, this search for conserved motifs will always result in a "best fit" in any kind of protein. However, when the conserved motifs of one herpes protein family were compared with the four unassigned baculovirus replication proteins, only one of the four showed a similar linear spatial organization of "best fits" of these motifs, except for the origin-binding proteins (HSV UL9 and homologues). Using these "best fits" all four AcMNPV baculovirus proteins and their homologues in *Orgyia pseudotsugata* (OpMNPV) were aligned entirely with their putative herpesvirus homologues (Table 10.1). However, the overall homology between the four baculovirus replication proteins and their putative herpesvirus homologues is relatively low and, based on available computer software, the significance of the protein alignments was difficult to assess.

Still, all these similarities between baculoviruses and herpesviruses, and some remarkable aspects about the alignments of the baculovirus replication proteins with their putative herpesvirus homologues, as described in this chapter, suggests that the alignments are significant enough to indicate that *ie-1* codes for a single stranded DNA binding protein, *lef-1* for a primase-associated protein, *lef-2* for a DNA polymerase processivity factor, and *lef-3* for a primase.

MATERIALS AND METHODS

Protein sequences

The protein sequences of the baculovirus replication proteins IE-1, LEF-1, LEF-2, LEF-3, and herpesvirus homologues were obtained by translation of sequences available in the GenBank/EMBL database: AcMNPV IE-1 (Guarino and Summers, 1987), OpMNPV IE-1 (Theilmann and Stewart, 1991), CfMNPV IE-1 (Kuzio *et al.*, 1992), BmMNPV IE-1 (Huybrechts *et al.*, 1992), HzSNPV IE-1 (Goodge *et al.*, 1994), SeMNPV IE-1 (E.A. van Strien, pers. comm.), AcMNPV LEF-1 (Passarelli *et al.*, 1993b), OpMNPV LEF-1 (Pearson *et al.*, 1993), AcMNPV LEF-2 (Possee *et al.*, 1991), OpMNPV LEF-2 (Ahrens and Rohrmann, unpublished), AcMNPV LEF-3 (Li *et al.*, 1993), OpMNPV LEF-3 (Ahrens and Rohrmann, unpublished), HSV-1 proteins (McGeoch *et al.*, 1988), VZV proteins (Davison and Scott, 1986), EHV proteins (Telford *et al.*, 1992), EBV proteins (Baer *et al.*, 1984), HCMV proteins (Chee *et al.*, 1990), CCV proteins (Davison, 1992), and MCMV SSB (Messerle *et al.*, 1992).

Protein alignments

Protein sequences were aligned by use of the GAP and BESTFIT programs of GCG (Genetics Computer Group, University of Wisconsin, Madison) and by visual inspection and valuation of putative motifs. Shaded areas in the alignments indicate conserved amino acids based on the algorithm of Needleman and Wunsch (1970) and on the following groupings: DE, KR, ST, CS, FYW, ILVM. Gaps in the sequences are introduced for optimal alignment.

RESULTS

IE-1: a putative single stranded DNA binding protein

Computer-assisted alignment of the amino acid sequences of six herpesvirus homologues of the HSV-1 single stranded DNA binding protein (SSB), UL29, resulted in the identification of 20 conserved domains (Fig. 10.1). Short sequence motifs of 16 of these domains were also recognized in the same order in the IE-1 genes of two baculoviruses, AcMNPV (Guarino and Summers, 1987) and OpMNPV (Theilmann and Stewart, 1991). One

internal domain (XII) and three conserved domains located at the C-terminus of UL29 and homologues were not present in IE-1. The N-terminal 145 amino acids of AcMNPV IE-1 are essential for *trans*-activation of early baculovirus gene expression (Kovacs *et al.*, 1992). This region encompasses domains I, II, III, and IV (Fig. 10.1). Domains I and IV are not conserved in the IE-1 sequence of AcMNPV but are present in OpMNPV IE-1. In addition, domain III, which is conserved in AcMNPV IE-1, is absent in OpMNPV IE-1 (Fig. 10.1). This may be similar to the SSBs of adenovirus type 2 and 5 in which residues 1-173 are poorly conserved and play a role in controlling the host range of the virus (Brough *et al.*, 1985).

The C-terminal 437 amino acids of IE-1, which includes the domains V-XVII (Fig. 10.1), have been shown to be involved in DNA binding (Kovacs *et al.*, 1992). Wang and Hall (1990) identified a major DNA binding site in UL29 located between amino acid residues 300 and 849 which encompasses the same conserved domains (VI-XVI) as the IE-1 binding domain (Fig. 10.1). One of the motifs in this region that have been suggested to be involved in DNA binding is the zinc finger motif in domain IX (Gao *et al.*, 1988), present in most of the herpesvirus sequences, but absent in CCV and the corresponding part of the IE-1 sequences (Fig. 10.1). Although zinc fingers participate in DNA binding in several proteins (Struhl, 1989), including the SSB of adenovirus (Eagle and Klessig, 1992), the absence of this motif in the CCV and the IE-1 sequences questions whether a zinc finger is also here involved in DNA binding.

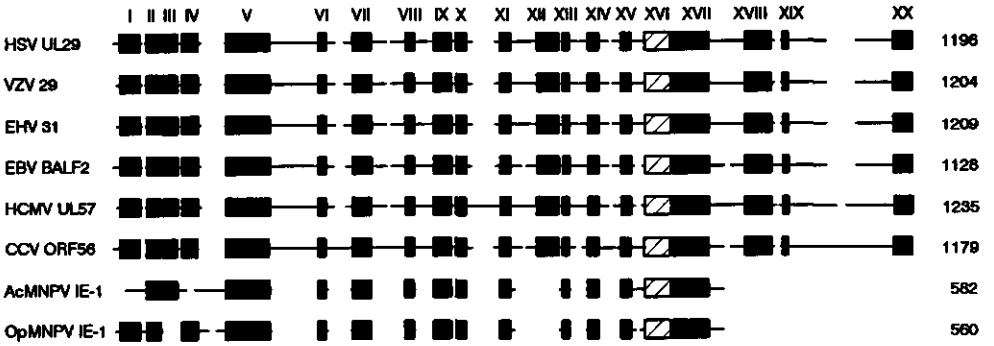
A second potential DNA-binding motif is located in domain XVI. This motif, shown in Fig. 10.2, was originally identified from alignments of HSV-1 UL29 with the gene 5 SSB from fd bacteriophage (Wang and Hall, 1990). It consists of a conserved set of basic and aromatic amino acids separated by nonconserved residues. It occurs in all SSBs examined from both prokaryotic and eukaryotic organisms, and is highly conserved in all the herpesvirus SSBs as well as in IE-1 proteins from six different baculoviruses (Fig. 10.2), suggesting that this pattern of amino acids is functionally important.

The last domain for which a putative function has been determined is domain XX (Fig. 10.1). Gao and Knipe (1992) demonstrated that the C-terminal 28 amino acids of ICP8 function as a nuclear localization signal (NLS). This domain seems not to be conserved in the IE-1 sequences, but the C-terminal ends of the IE-1 proteins are also rich in arginine and lysine residues, a feature often found in NLS, and it is therefore possible that they also

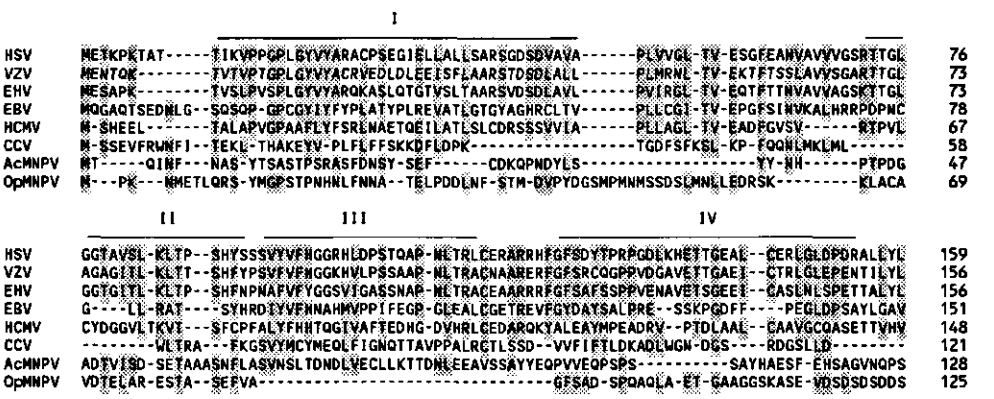
function as *NLS*.

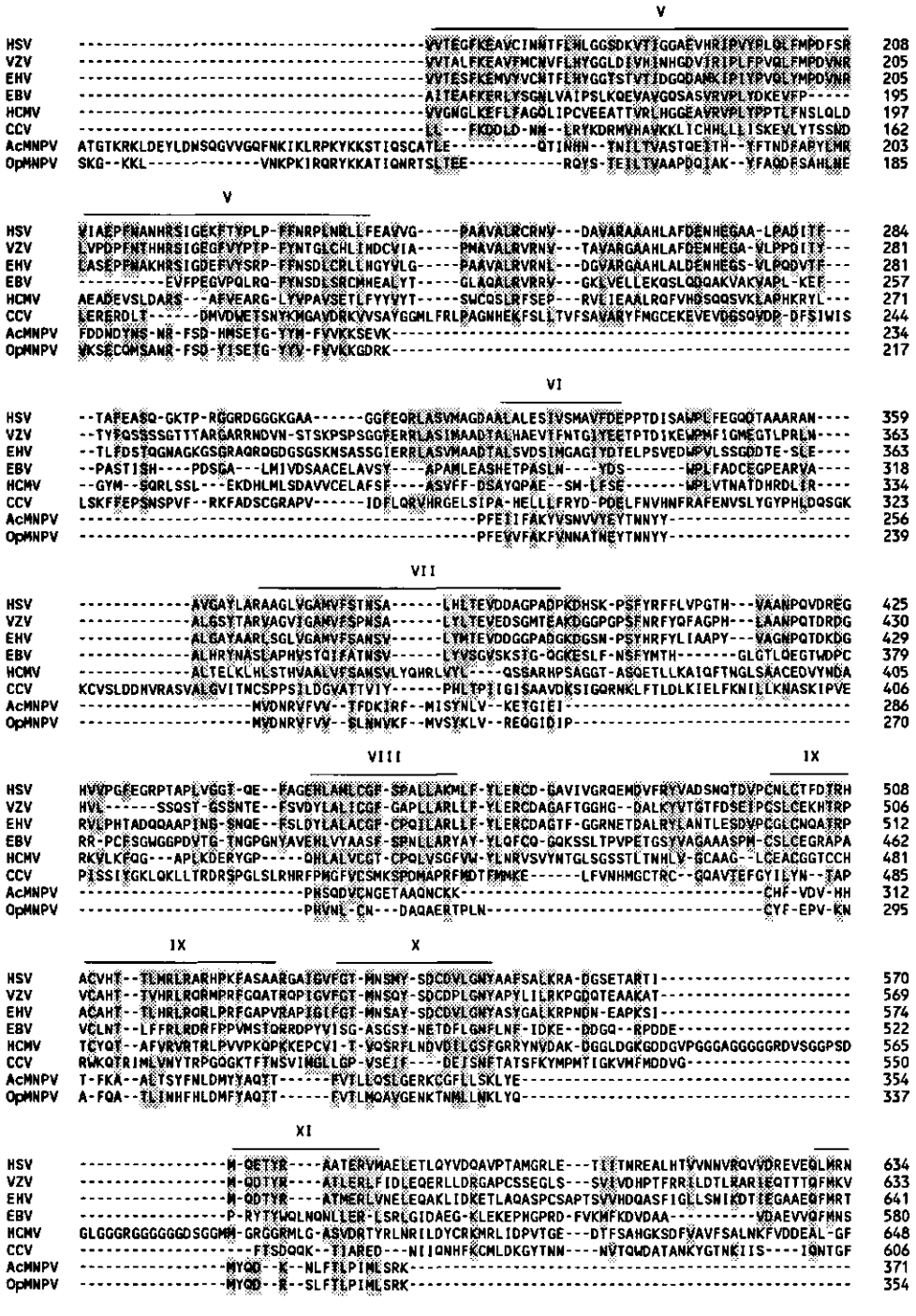
SSBs are presumably required for DNA replication because they stabilize single stranded regions at the replication fork. This could also explain why IE-1 acts as a strong *trans*-activator of several baculovirus early genes (Guarino and Summers, 1986). IE-1 may stabilize ssDNA of promoters thereby keeping them open and accessible for transcriptional initiation. The ability of an SSB to activate transcription is not unique to IE-1. HSV-1 UL29, the adenovirus SSB, the gene 32 protein of bacteriophage T4, and the SSB protein of *Escherichia coli* exhibit similar roles in activation of transcription (Gao and Knipe, 1991; Chang and Shenk, 1990; Gauss *et al.*, 1987; Haynes and Rothman-Denes, 1985).

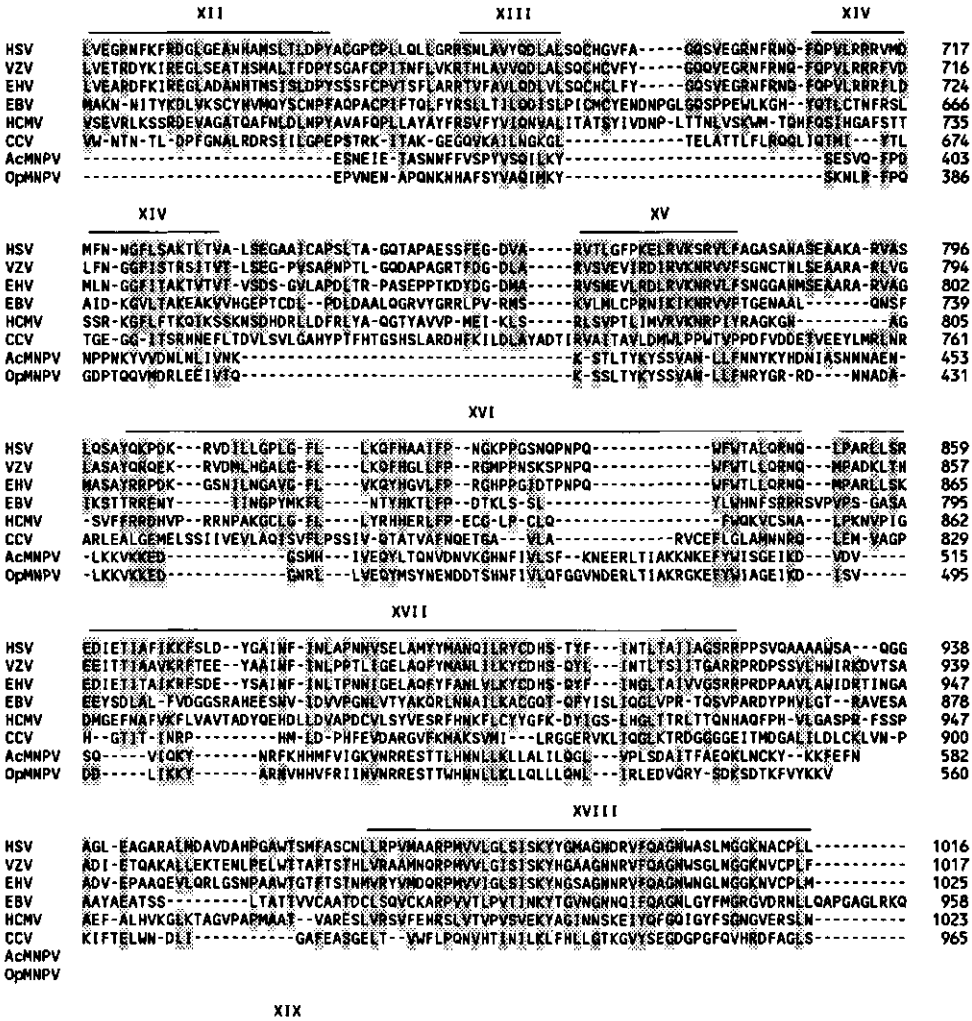
A.



B. Alignment of two baculovirus IE-1 proteins with six herpesvirus homologues






XII
XIII
XIV
HSV
VZV
EHV
EBV
HCMV
CCV
AcMNPV
OpMNPV
XIV
XV
XVI
XVII
XVIII
XIX

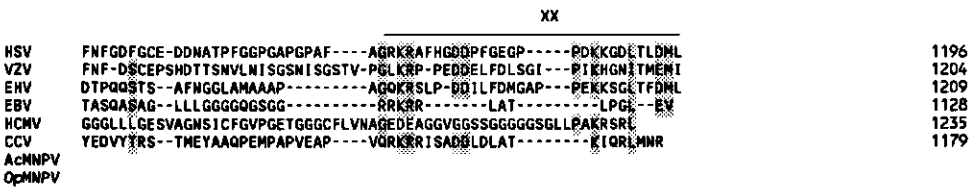


Figure 10.1. Schematic (A) and complete sequence (B) alignments of IE-1 proteins of AcMNPV and OpMNPV with HSV-1 UL29 and homologues. The solid rectangles show the location of conserved sequence domains connected by diverse regions. The crosshatched rectangle shows the location of a putative DNA binding domain.

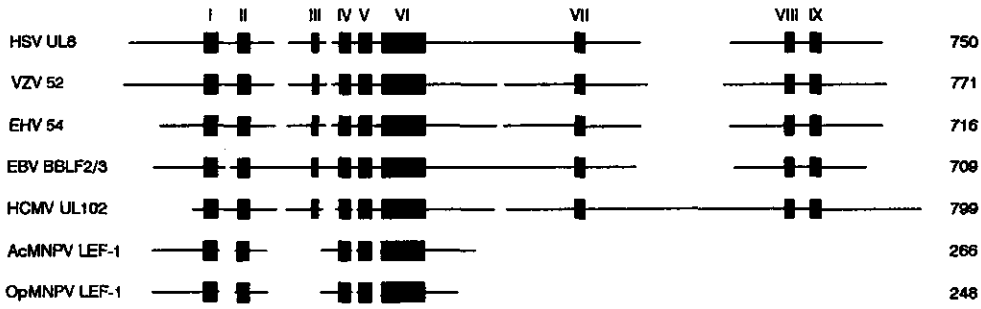
GP5	16	R-X4-R-X04-Y-X07-Y-X06-Y-X04-K-X26-F-X06-R	80
GP32	67	K-X3-K-X12-Y-X14-Y-X06-Y-X03-K-X04-Y-X07-K	123
SSB	43	K-X5-K-X04-W-X05-F-X09-Y-X02-K-X04-W-X07-R	86
PIKE	16	R-X5-K-X04-Y-X07-Y-X06-Y-X19-K-X12-F-X06-R	81
Ad-5	410	R-X3-K-X03-F-X36-Y-X13-F-----K-X16-W-X11-R	499
HSV-1	803	K-X3-R-X09-F-X04-F-X04-F-X03-K-X10-W-X06-R	849
VZV	801	R-X3-R-X09-F-X04-F-X04-F-X01-R-X12-W-X06-R	847
EHV	808	R-X3-K-X10-F-X04-Y-X04-F-X01-R-X12-W-X06-R	855
EBV	741	K-X3-R-X12-F-X03-Y-X04-F-X03-K-X06-W-X06-R	771
CCV	758	R-X4-R-X22-F-X12-F-X04---X05-R-X03-F-X07-R	821
HCMV	810	R-X5-R-X09-F-X02-Y-X06-F-X01---X09-W-X09-K	857
MCMV	791	R-X4-K-X04-F-X02-F-X06-F-X01-R-X08-W-X09-R	831
AcIE-1	455	K-X3-K-X10-Y-X12-F-X04-F-X04-R-X11-W-X05-K	511
BmIE-1	459	K-X3-K-X10-Y-X12-F-X04-F-X04-R-X11-W-X05-K	516
OpIE-1	433	K-X3-K-X10-Y-X12-F-X04-F-X06-R-X11-W-X05-K	491
CfIE-1	433	K-X3-K-X10-Y-X12-F-X04-F-X06-R-X11-W-X05-K	491
HzIE-1	529	K-X2-R-X10-Y-X12-F-X04---X05-R-X11-W-X05-K	584
SeIE-1	580	K-X2-K-X10-Y-X07-F-X04-F-X09-R-X11-W-X05-K	635

Figure 10.2. Single-stranded DNA-binding sequence motif. Wang and Hall (1990) aligned partial protein sequences from various SSBs with that portion of the gene 5 protein (fd bacteriophage) involved in DNA binding. This analysis yielded a region in all the sequences which consists of conserved aromatic and basic amino acids separated by variable numbers of unrelated residues. These spacer regions are designated by X with a subscript indicating the number of residues in each case. Wang and Hall (1990) identified this motif in gene 5 protein of bacteriophage fd (GP5), gene 32 protein of bacteriophage T4, SSB of *E. coli*, and DNA-binding proteins from bacteriophage ike (PIKE), HSV-1, VZV, EBV, and adenovirus type 5 (AD-5). Here it is shown that it is also present in herpesvirus DNA-binding proteins of EHV (Telford *et al.*, 1992), HCMV (Chee *et al.*, 1990), MCMV (Murine cytomegalovirus, Messerle *et al.*, 1992), CCV (Davison, 1992), and IE-1 proteins of the baculoviruses AcMNPV (Guarino and Summers, 1987), BmMNPV (*Bombyx mori* MNPV, Huybrechts *et al.*, 1992), OpMNPV (Theilmann and Stewart, 1991), CfMNPV (*Choristoneura fumiferana* MNPV, Kuzio *et al.*, 1992), HzSNPV (*Helicoverpa zea* SNPV, Goodge *et al.*, 1994), and SeMNPV (*Spodoptera exigua* MNPV, E.A. van Strien, pers. comm.). Numbers indicate the residue numbers for each sequence shown in the alignment.

LEF-1: a putative primase-associated protein

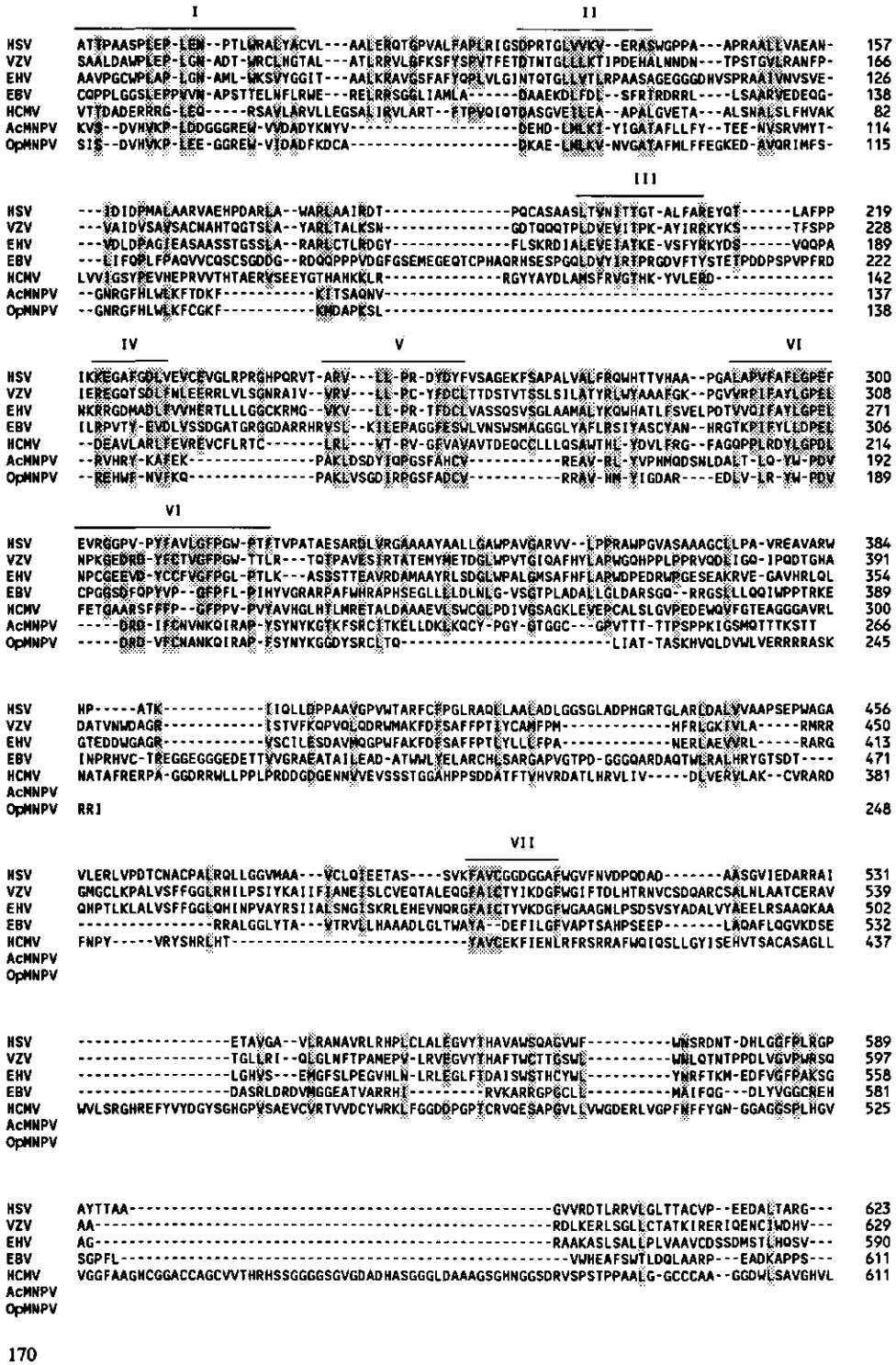
The initial alignments of the HSV-1 primase-associated protein, UL8, to BBLF2/3 and UL102, the possible UL8 counterparts in EBV and HCMV respectively, were not obvious. Their relatedness to UL8 was initially based on their corresponding genomic locations, and was strengthened by the finding that they all share a similar region of 55 amino acids (Fig. 10.3, domain VI; Fixman *et al.*, 1992). This region of similarity is also found in VZV gene 52, and EHV gene 54, and our analyses suggest it is also present in the LEF-1 sequences of AcMNPV (Passarelli and Miller, 1993b) and OpMNPV (Pearson *et al.*, 1993) (Fig. 10.3). Besides this region, eight other similar regions were identified in the herpesvirus homologues. The highest homology is found in the N-terminal 320 amino acids whereas the C-terminal 430 residues are very poorly conserved. The much shorter LEF-1 proteins show only distant homology with the conserved N-terminal region. Five of the 9 regions (I, II, IV-VI), identified in the herpesvirus homologues, showed similarity to domains in the LEF-1 proteins in a similar order (Fig. 10.3).

A.



B. Alignments of two baculovirus LEF-1 proteins with five herpesvirus homologues

```
HSV      MDTADIVWVEES--VSAITLYAVWLPPR--AREYFHALVYFVCRNAAGEGRARFAEVSVTATELRDFY-GSADVSVQA----VVAARA    80
VZV      MDATQITLVRESGHICAASIYTSWTQSGQLTQNGLSVLYYLLCKNSCGKYVPKFAELTVQQEDLCRYSRHGGSVSAAT--FASICRAAS   87
EHV      M----------------------------------------LCRRGSD-YTAEFCHVPVSGELLKRGARDASLVTPAR----VASAAQT   44
EBV      MMETP-------------------------------------AESVRARVSSVTEYNVTQTAGRWWAIW-VVGIVPIKREDVETLIVVQA   66
HCMV     ^----------------------------------------------------------------------------GALASA    6
AcMNPV   MLVCNYTQKRVDMMWDAIAYNDSRK-----------------------YAFMTVNA-RWIN-ADRYFDTSAQL----YSYIVQN   55
OpMNPV   MAPCKYTPERVQMMWDAIAYNDSRR-----------------------LAFMIDRP-RWVH-AHNFFDSAAQL----FAYIVKN   55
```


I
II
III
IV
V
VI
VI
VII
HSV
VZV
EHV
EBV
HCMV
AcMNPV
OpMNPV

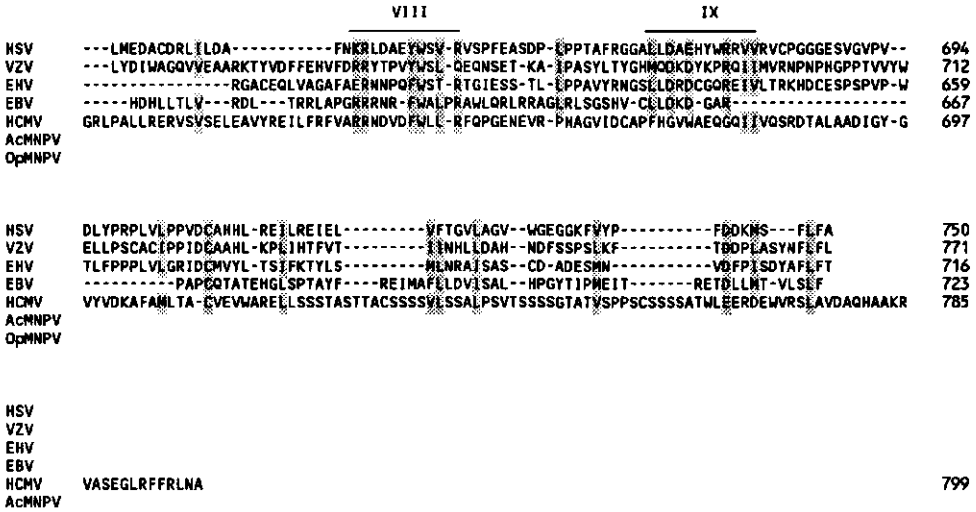


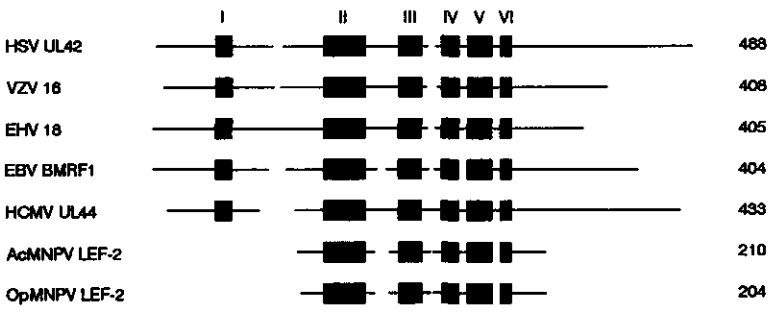
Figure 10.3. Schematic (A) and complete sequence (B) alignments of LEF-1 proteins of AcMNPV and OpMNPV with HSV-1 UL8 and homologues. The solid rectangles show the location of conserved sequence domains connected by diverse regions.

LEF-2: a putative DNA polymerase processivity factor

Alignment of HSV UL42 which encodes a DNA polymerase processivity factor with homologues from five other herpesviruses revealed a significant amount of sequence conservation in the N-terminal two-thirds of the proteins, but a marked lack of homology for the C-terminal region distal to amino acid 314 (Fig. 10.4). In the N-terminal region six conserved domains were located (Fig. 10.4). A search for these domains in the baculovirus replication proteins revealed that domains II-VI were partially conserved in the same order in LEF-2 suggesting that this protein is a homologue of UL42. Complete alignment of the LEF-2 sequences of AcMNPV (Possee *et al.*, 1991) and OpMNPV (C.H. Ahrens and G.F. Rohrmann, unpublished) with the herpesvirus UL42 homologues showed only limited amino acid similarity (13 to 21% identity, 27 to 37% similarity). The highest similarity was found between AcMNPV LEF-2 and HCMV UL44. This protein alignment showed that the 90 N-terminal amino acids as well as the 138 C-terminal amino acids of HCMV UL44 are absent in the much shorter LEF-2 protein. For HSV-1, Tenney *et al.* (1993) determined that

deletion of the C-terminal 172 amino acids of UL42 did not affect UL42 function. This region, which is almost completely absent in LEF-2, is also not conserved between different herpesvirus UL42 homologues. Furthermore, Monahan *et al.* (1993) identified two regions in UL42 (amino acids 129 to 163 and 202 to 337), which are required for UL42 to enhance the activity of DNA polymerase. These regions encompass a sequence that aligns precisely with LEF-2. Information about the requirements of the N-terminal part of UL42 is poor. Digard *et al.* (1993) demonstrated that the N-terminal 18 amino acids are dispensable, but that deletion up to amino acid 132 destroyed the function of UL42.

A.



B. Alignment of two baculovirus LEF-2 proteins with five herpesvirus homologues

```
                                                                                                                I
HSV     MTD-----SPGGVAPASPVEDASDASLGQPEEGAPCQVVLQGAELNGILQA-----FAPLRT-SLLD--SLLVMGDRGILI--HNTIFG    74
VZV     M-Q----LRSRT---DDALDMELHAGFDAPE]---ARAVLTEKTLTGLISS-----ISPLVN-RLRD--SILIFSQEGLII--HCSLET    68
EHV     M-ALPRAMRPGGSHPNNFLFNTL-PVIDNPVERQRAMAVFERESLRDAFEM-----LTPIAP-SLKN--AFLIFNEDGLLI--HTSVGG    77
EBV     M-ETTQTLRFKTKALAVLSKCYDHAQTHL------KGGVLQN-LLSVNYGGPRLAAVANAGT-AGLI--SFEVSPDAVAEWQNHQSPEE    79
HCMV    M-D-----R-KTRLSEPPTLALRL-----------KPYKTAIQQLRSVIRA-----LKENTTVTFLPTPSLILQTVRSHCVSK-ITF--    63
AcMNPV
OpMNPV

HSV     EQVFLPLEHSQFSRYRW-RGPTAAFLSLVDQKRSLLSVFR-ANQYPDLRRVEL-AETGQAPF-RTLVQR-IWTTTSDGE----------   148
VZV     EQLYIPIPANMFDQYNWT-GPRMVVLAATEGRSSLIDAFRHTKDPSTPTRLYF-KFTGQPPE-RSIIQTMVWQRPGDCGPD--------   146
EHV     EQVYIPIQTNNMESYSWKKAPPAVFLANVDGRRGLLDAFK-AKTQTNVSKVVF-EIENYSPS-RILTQT-VFSARDQTEEDTEMGSDAE   162
EBV     APAAVSFRNLATGR-TCVLGKEL-FGSAVEQA--SLQFYKR----PQGSRPEFVKLTMEYDD-K--VSK--SHNTCALMP---------   147
HCMV    ------------NSSCLYITDKSFQPKTINNSTPLLGNF-----------HY-LTSSKDLTKFYVQ-----DISDLSAKI--------   114
AcMNPV                                                    MA--NASYNVVSPLL-----RASCLDKKATY-----    24
OpMNPV                                                    M-----ER-VWNPAA------GIDGLKRSETY-----    20

                                  II                                                          III
HSV     -AVELASETLNKRE-LTSFVVLVPQGTPDVQLRLTRPQLTKVLNATGADSATPTTFELGVN-GKFSVFTTSTCVTFAAR---EEGVSSS   231
VZV     -DQVQCYKQVVKRE-LACYTNNFPNLTPDISICLKRDQFTR-LQRLLKTFGFTTCFILTAT-DMYIQTAGGGFISF---NVSLD-INGS   227
EHV     GATQTVSSRLVKHE-FNNYALNLPTRQPDVSNSLSKAQLNKTL-GVCKQAGDPITFQCLFD-DTLQVRSGDRQVVFSVDYQHADNGGVE   248
EBV     -YNPPASDRL-RNEQNIGQVLLMPKTASSLQ-KWAROQGSGGVKVTLNPDLYVTTYTSGEACLTLDYKPLSVGPYEAFTGP--------   225
HCMV    -SN-CAPQFNN--EFSSACVHGQ-----DI-VRESENSAV-----------HVDLDFGVVADLL--KWI-GPHTRVKRNVKKAPC---   175
AcMNPV  -LID--PDQFIDKLTLTPYTVFYNGG---VLVKISGLRLYMLLTA-------PPTINEIKN-SNE--KKRS------KRNICMKECVEG   91
OpMNPV  -LVD--PHQFVGVLTLSPYTVFERG----LFVRMSGMRLLALLAA-------PKPQEPQPAVRRE--PQRS------RRNVCLKACADG   87
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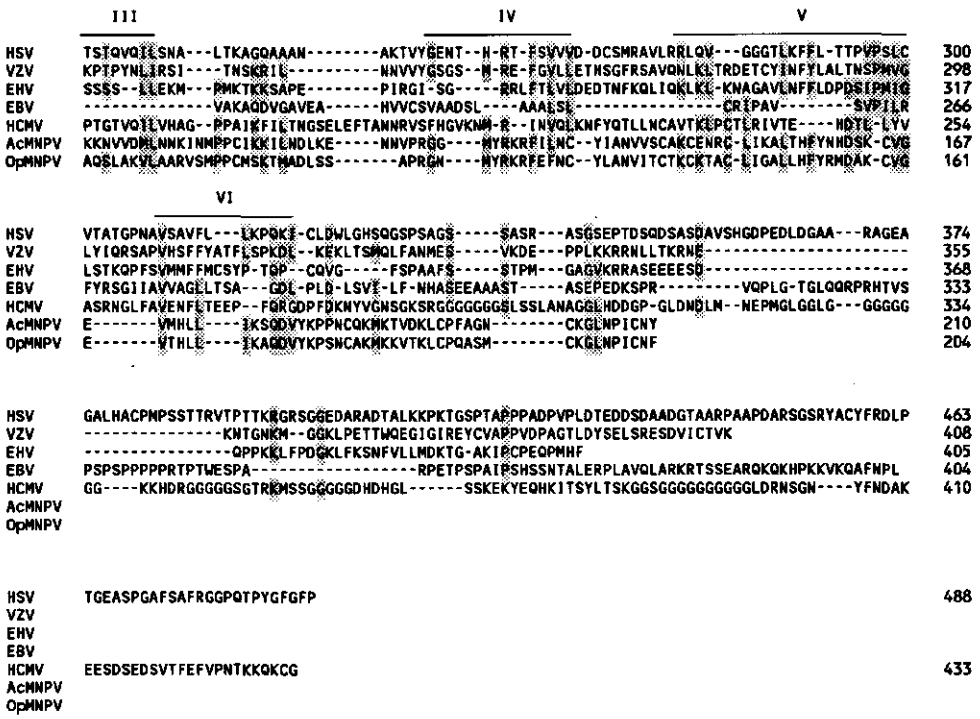


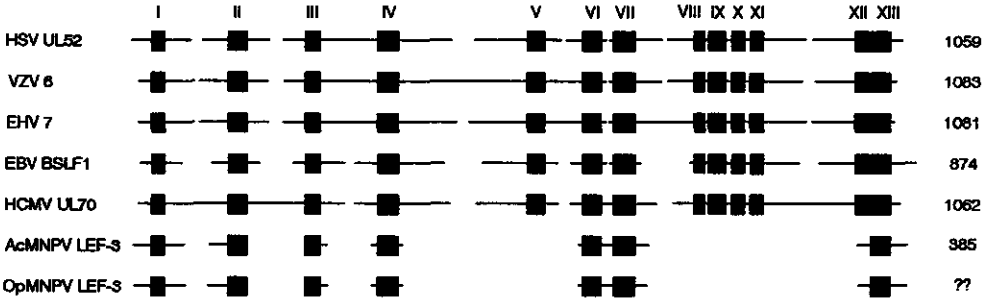
Figure 10.4. Schematic (A) and complete sequence (B) alignments of LEF-2 proteins of AcMNPV and OpMNPV with HSV-1 UL42 and homologues. The solid rectangles show the location of conserved sequence domains connected by diverse regions.

LEF-3: a putative primase

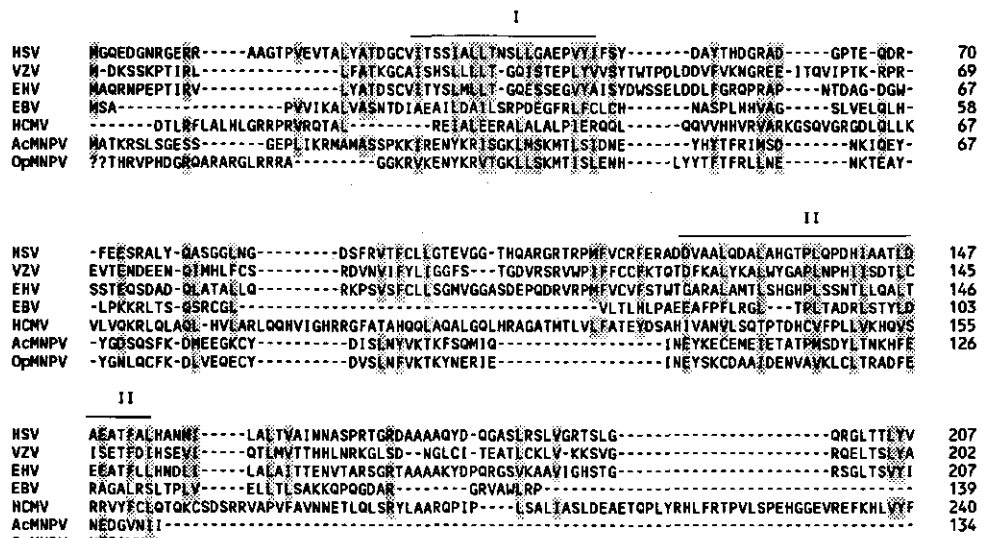
Analysis of LEF-3 suggests that it is homologous to HSV UL52, which encodes a putative primase (Dodson and Lehman, 1991). Alignment of HSV UL52 with its homologues from four other herpesviruses revealed 13 conserved domains (Fig. 10.5). Sequences with homology and the same spatial organization of seven of these domains (I-IV, VI, VII, and XII) were found in both AcMNPV LEF-3 (Li *et al.*, 1993) and its OpMNPV homologue (C.H. Ahrens and G.F. Rohrmann, unpublished) (Fig. 10.5). Remarkably, the large region between domains IV and VI with the exception of domain V, that is very poorly conserved between the herpesvirus proteins, is absent in the LEF-3 sequences (Fig. 10.5), indicating that this region may not be important for the primary function of the protein. In contrast, the

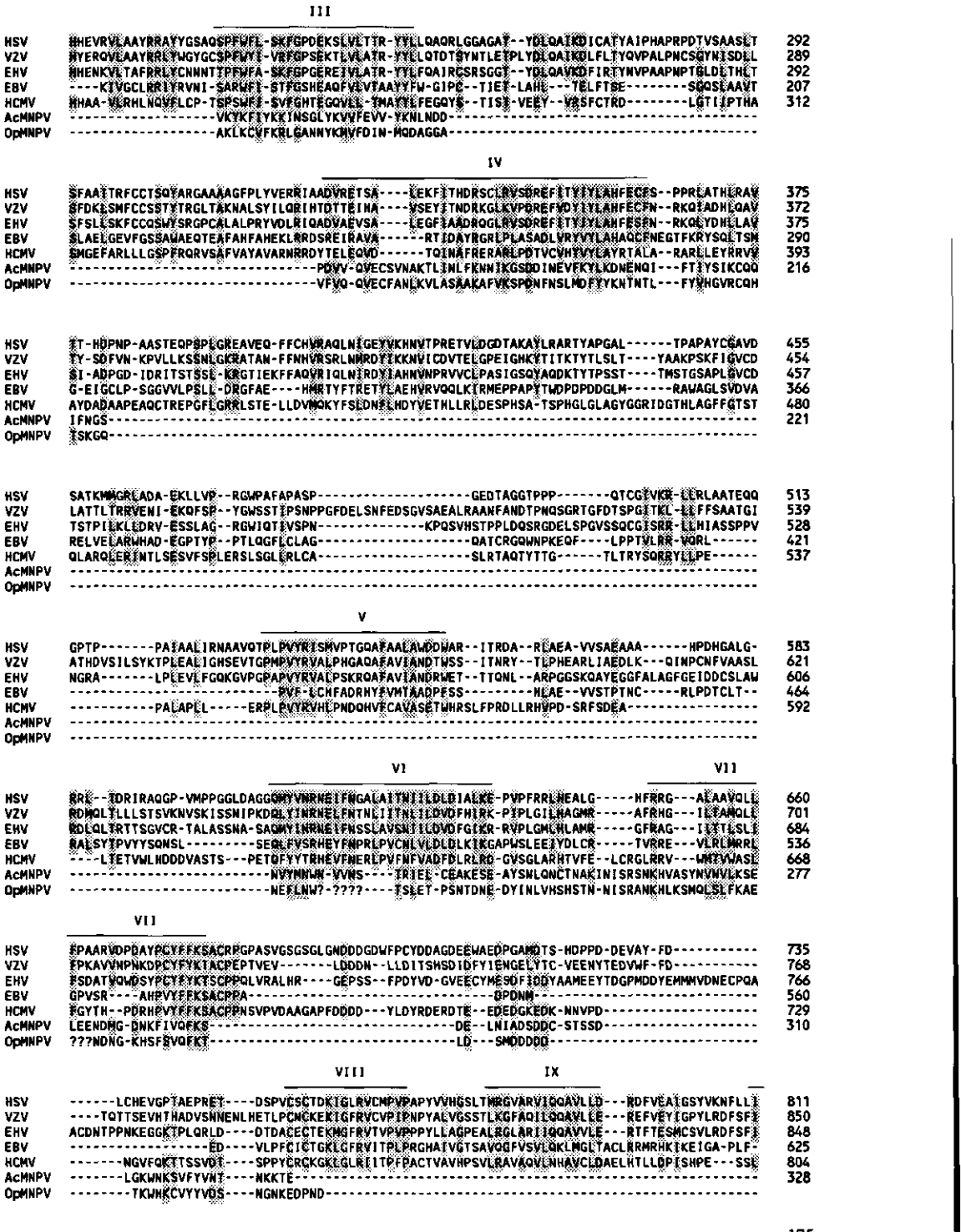
region between domains VII and XIII, which is also absent in the LEF-3 sequences, is strictly conserved between the different herpesvirus proteins. This region therefore may have a function specific for herpesvirus replication.

A.



B. Alignments of two baculovirus LEF-3 proteins with five herpesvirus homologues




III
IV
V
VI
VII
VII
VIII
IX
HSV
VZV
EHV
EBV
HCMV
AcMNPV
OpMNPV

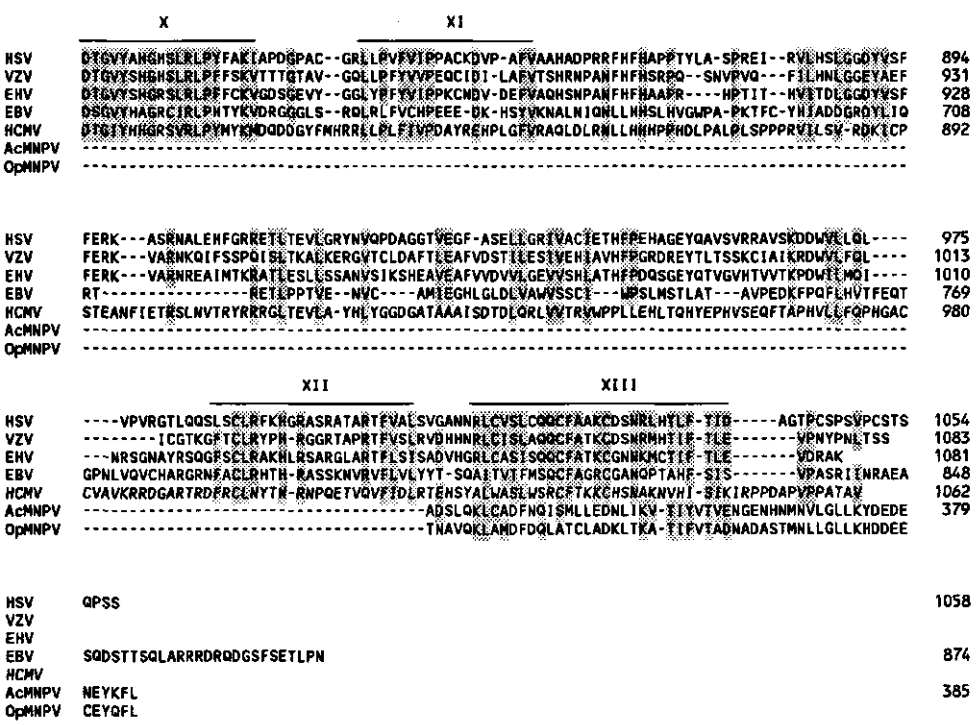


Figure 10.5. Schematic (A) and complete sequence (B) alignments of LEF-3 proteins of AcMNPV and OpMNPV with HSV-1 UL52 and homologues. The solid rectangles show the location of conserved sequence domains connected by diverse regions.

DISCUSSION

Traditionally the viral families of the *Baculoviridae* and *Herpesviridae* have been separated based on different morphology and host specificity, but they share a number of striking similarities. In addition to enveloped nucleocapsids and replication in the host cell nucleus, they both have similar sized, large double stranded DNA genomes that have a circular form during at least one stage of their replication cycle. Evidence from origin-containing plasmid replication in infected cells suggests that they also may both replicate via a rolling circle intermediate (Leisy and Rohrmann, 1993; Hammerschmidt and Mankertz, 1991). Furthermore, recent evidence suggests that baculoviruses may form latent

infections in host insects (Hughes *et al.*, 1993). Both viral families code for a core set of six proteins essential for DNA replication.

Two of the baculovirus replication proteins, helicase and DNA polymerase have previously been shown to be related to herpesvirus homologues (Lu and Carstens, 1991; Tomalski *et al.*, 1988). The other four baculovirus replication proteins, LEF-1, LEF-2, LEF-3, and IE-1 now also appear to be related to herpesvirus homologues, based on the presence of several sequence motifs in these proteins, with homology to and the same linear spatial organization as conserved domains in the replication proteins of herpesviruses. It is interesting is that only the baculovirus helicase is considerably larger in size than the herpesvirus homologues, whereas the other five baculovirus replication proteins are all smaller, containing 14% (DNA polymerase) to 66% (LEF-1) less amino acids than their herpesvirus homologues.

The present alignments of the four baculovirus replication proteins with their putative herpesvirus homologues suggest that *ie-1* codes for a SSB, *lef-1* for a primase-associated protein, *lef-2* for a DNA polymerase processivity factor, and *lef-3* for a primase. The assignment to *ie-1* to code for a SSB is supported by the finding of a conserved ssDNA binding sequence motif in six baculovirus IE-1 proteins, also found in many other prokaryotic and eukaryotic SSBs (Fig. 10.2).

The overall homology between the four baculovirus replication proteins IE-1, LEF-1, LEF-2, LEF-3, and their putative herpes homologues is very low. However, even between the different herpesviruses the homology between homologous genes is sometimes very low. The SSB of CCV showed only limited amino acid similarity with homologues of other herpesviruses. It is, possibly due to the low homology, even not known whether the CCV genome contains homologues of HSV UL8, UL42, or UL52. For the DNA polymerase processivity factors it was also shown that the homology between the different herpesviruses was not very high, ranging from 15 to 25% identity, except when HCMV was compared with the homologue in human herpesvirus 6 (44% identity) (Agulnick *et al.*, 1993). The finding of 21% identity between AcMNPV LEF-2 and HCMV UL44 is within the same range and provides enough evidence that AcMNPV LEF-2 is related not only to HCMV UL44 but also to the other herpesvirus homologues. The herpesvirus primase-associated proteins (UL8 and homologues) are very poorly conserved between the different herpesviruses, especially when the C-terminal 400-500 amino acids were compared. LEF-1,

the much shorter putative baculovirus homologue (266 amino acids) showed, however, distant homology with the more conserved N-terminal 300 amino acids of the herpesvirus homologues. LEF-3, a putative homologue of the herpesvirus primases also showed only distant homology with the different herpesvirus homologues. Therefore, based on existing computer software, it is not possible to make firm conclusions concerning the significance of these protein alignments. Functional biochemical analysis of the baculovirus replication proteins and the identified conserved motifs should shed more light on the functions of these proteins.

All six essential replication genes identified in AcMNPV thus probably have homologues in herpesviruses. In addition, each herpesvirus contains one or more additional essential genes. The alpha-herpesviruses HSV, VZV, and EHV, for example, contain an essential gene encoding an origin-binding protein and HCMV and EBV employ 11 and 9 essential genes, respectively (Wu *et al.*, 1988; Pari and Anders, 1993; Fixman *et al.*, 1992) (Table 10.1). It is possible that if additional components are required for AcMNPV DNA replication, one or more of the AcMNPV replication genes are multifunctional or that additional essential functions are supplied by the insect host cell. Since the *Baculoviridae* are a large and diverse family with viruses containing genome sizes ranging from 90 to over 160 kbp, additional essential genes may be required for replication by different baculoviruses.

The similarity between *Baculoviridae* and *Herpesviridae* in DNA structure and mechanism of DNA replication, added to the employment of an identical kind and amount of essential replication genes, poses the question whether these two groups of viruses share a common lineage. On the basis of the mutation rate of the conserved baculovirus polyhedrin genes as compared to the insect species in which they occur (Vlak and Rohrmann, 1985) it has been postulated that baculoviruses are ancient viruses that have evolved along with the insects. The relationship among replication genes could imply that herpesviruses have evolved from baculoviruses along with their invertebrate hosts towards vertebrates. Alternatively, the emergence of herpesviruses may be the result of an independent, parallel evolutionary event in ancient vertebrates. Since viral DNA replication in nuclear environments is a conserved process, conserved host replication genes may have been independently transduced into different ancient viral genomes.

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CHAPTER 11

SUMMARY AND CONCLUDING REMARKS

Baculoviruses are attractive biological agents for the control of insect pests. They are highly specific for insects and cause a fatal disease (Granados and Federici, 1986). In addition, baculoviruses are successfully exploited as expression vectors for the production of heterologous proteins for various applications (Luckow and Summers, 1988; Luckow, 1991). In both cases large-scale systems for the production of baculoviruses are important. Production in insect larvae is difficult to scale up and to control. Insect-cell cultures offer an attractive alternative. Moreover, in the case of pharmaceuticals and diagnostics in human and veterinary medicine insect-cell systems have to be applied since such systems are well defined.

Due to the great interest in baculoviruses as biological insecticides and expression vectors for foreign genes, the molecular genetic aspects of especially the *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV), the type member of the *Baculoviridae*, have been studied in much detail (Blissard and Rohrmann, 1990). Chapter 2 of this thesis presents, as of March 1994, an overview of the structural and functional organization of the AcMNPV genome. The genomes of AcMNPV (R.D. Possee, pers. comm.) and *Bombyx mori* MNPV (BmMNPV) (S. Maeda, pers. comm.) have been completely sequenced but are awaiting publication. In contrast to other large DNA viruses such as adenovirus, herpesviruses, and vacciniavirus (Fields and Knipe, 1990), the process of baculovirus DNA replication of AcMNPV is poorly understood. At the start of this study a few genes were found which were thought to be involved in AcMNPV DNA replication such as a helicase and a DNA polymerase. Sequences representing the origin of AcMNPV DNA replication were not known.

Baculoviruses can be produced on a large scale in insect-cell cultures using batch (Maiorella *et al.*, 1988), semicontinuous (Hink and Strauss, 1980) and continuous reactors (Kompier *et al.*, 1988). Continuous production of wild-type (wt) AcMNPV and recombinants thereof was achieved in a system consisting of one bioreactor producing insect cells in series with a second bioreactor for virus infection and protein production (Kompier *et al.*, 1988; Van Lier *et al.*, 1992). After a few weeks of continuous operation, however, the productivity decreased to a low level. In the case of wt AcMNPV, the number of polyhedra per cell, the fraction of cells containing polyhedra, and the concentration of extracellular virus were found to be decreased (Kompier *et al.*, 1988). Continuous production of an AcMNPV recombinant where the polyhedrin gene was replaced by the lacZ gene of *Escherichia coli* essentially gave the same results (Van Lier *et al.*, 1992). The decrease of virus production was ascribed to a phenomenon known as passage effect (Tramper and Vlak, 1986), but the underlying mechanism remained unknown.

Analysis of samples obtained from continuous bioreactor systems (Chapter 3) showed that with ongoing production a mutant AcMNPV became dominant. This mutant lacked about 43% of the original genome. The deleted DNA included the polyhedrin gene and several genes essential for DNA replication. The replication of the mutant appeared to be dependent on the presence of an intact helper AcMNPV. The passage effect in the continuous system is thus thought to be the result of interference between the deletion mutant and helper virus. These so-called defective interfering particles (DIPs) can only accumulate when the concentration of the intact virus is high enough to support the replication of these DIPs. Thus, for a successful continuous production of baculoviruses low multiplicities of infection should be used to avoid the accumulation of DIPs.

One of the regions of the AcMNPV genome putatively involved in the generation of the DIPs is located in the *Eco*RI-C fragment of AcMNPV. Deletion mutants often lacked a considerable portion of EcoRI-C, but also maintained a consistent segment of this fragment that may be essential for replication and/or encapsidation. To investigate the genetic functions of the *Eco*RI-C fragment in the defective genomes and their possible role in the generation of these genomes, the nucleotide sequence of a 7.3 kilobase pair region of the right part of the *Eco*RI-C fragment was determined (Chapter 4). Eight putative open reading frames (ORFs) were identified and their respective amino acid sequences compared with a number of data libraries. The product of ORF 1227 corresponded with GP41, a virion protein, and

its predicted protein sequence was found to be 55 amino acids longer at its C-terminus than reported previously (Whitford and Faulkner, 1992). The majority of ORF 1227, including the additional 55 amino acids, moreover, showed a high degree of homology with protein P40 of *Helicoverpa zea* SNPV, also a structural virion protein (Ma *et al.*, 1993). Three other ORFs in the analyzed AcMNPV region showed homology with ORFs in the HzSNPV sequence, indicating that the general organization of this region is similar in both viruses, and possibly between MNPVs and SNPVs. However, no sequences have yet been identified within this region that may play a role in the generation and/or encapsidation of the DIPs.

The generation and characterization of DIPs was further investigated in Chapter 5. Three small separate regions, representing only 5% of the original AcMNPV genome, were found to be retained in DNA of defective genomes after 40 serial passages in insect cells with undiluted inocula. Independently, Lee and Krell (1992) showed that after 80 serial passages of AcMNPV, DIPs were found which contained tandem repeats of DNA, mainly derived from a small region of the AcMNPV genome, located in the *Hin*dIII-K fragment. Since all these defective genomes were still able to replicate in insect cells, although only with the help of intact virus, they must have retained essential *cis*-acting elements necessary for DNA replication. Therefore, a replication assay was developed to study whether these regions, retained in the defective genomes, contained *cis*-acting elements such as an origin (*ori*) of DNA replication. Transfection of *Spodoptera frugiperda* cells with plasmids containing these sequences followed by superinfection with intact helper AcMNPV resulted in amplification of these plasmids, as demonstrated by the *Dpn*I sensitivity assay. In order to demonstrate replicating activity of these plasmids, it appeared essential to transfect the cells well (24 h) before superinfection with helper virus, and for an optimal replication result the multiplicity used for superinfection had to be 1 or lower (Chapters 5 and 6). Using this assay seven putative origins of DNA replication were identified in the AcMNPV genome (Chapters 5, 6, and 7).

Six of the seven putative *ori's* were found in the homologous regions *hr*1, *hr*2, *hr*3, *hr*4a, *hr*4b, and *hr*5 of AcMNPV (Chapter 6), which are interspersed along the genome (Cochran and Faulkner, 1983; Guarino *et al.*, 1986). Recently, another *hr* region, *hr*1a, has been identified in the AcMNPV genome, that could also serve as *ori* in a replication assay (Leisy and Rohrmann, 1993). Initial studies demonstrated that the *hr* regions function as enhancers for transcription, when placed in *cis* to the promoter of early baculovirus genes (Guarino *et*

al., 1986; Guarino and Summers, 1986). Rodems and Friesen (1993) demonstrated that *hr* regions also function as enhancers *in vivo*. These results together with the data of this thesis imply that all *hr's* in AcMNPV may be bifunctional *in vivo*, i.e. have both enhancer and *ori* activity. Sequence analysis has shown that *hr's* contain two to eight 30 bp imperfect palindromes, interspaced by other repeated sequences, and that each palindrome contains a naturally occurring *Eco*RI site at its core (Guarino *et al.*, 1986; Guarino and Summers, 1986). One copy of such a palindrome appeared to be sufficient for either enhancer function or *ori* activity (Guarino *et al.*, 1986; Pearson *et al.*, 1992).

In addition to the seven *hr's*, the *Hin*dIII-K fragment of AcMNPV was also found to carry a putative *ori*, although this fragment does not contain an *hr* region (Chapter 6). The *Hin*dIII-K *ori* had a complex structure (Chapter 7), resembling those of other large DNA viruses. This *ori* contained several regions, some of which were found to be essential for its activity, whereas others contain auxiliary sequences, that enhance *ori* activity. Sequence analysis of these regions identified several structures often found in other viral replication *ori's*, such as palindromes and other repeated motifs (DePamphilis, 1993). Recently an *ori*, also with a complex structure, but different from AcMNPV *hr's*, has been identified in another baculovirus, *Orgyia pseudotsugata* MNPV (OpMNPV) (Pearson *et al.*, 1993).

The individual role of all these *ori's* during viral DNA replication, and whether they are all active simultaneously *in vivo*, is unclear. Deletion of *hr*5 from the AcMNPV genome or the closely related *Bombyx mori* MNPV (BmMNPV) genome had no effect on the replication of these viruses (Rodems and Friesen, 1993; Majima *et al.*, 1993). Also from the experiments with DIPs generated by serial passaging it can be deduced that not all the *ori's* are necessary for replication of the genome. After 40 serial, undiluted passages three small segments of the genome were predominantly found to be retained, harbouring only the *hr*1, *hr*3, and *hr*5 regions (Chapter 5). Deletion of all *hr's* would indicate the importance of these regions for virus replication *in vivo*.

The importance of the *ori* in the *Hin*dIII-K fragment is supported by sequence data of the corresponding region in the closely related BmMNPV (Kamita *et al.*, 1993). Although most of the auxiliary sequences of this *ori* were found to be deleted in the BmMNPV genome, the essential part of this *ori*, containing the palindromes and the A/T rich region, was retained suggesting that these elements could not be deleted. These sequence data and the observation that after prolonged serial passage of AcMNPV (80 passages) large replicating DNA

molecules are found in which repeated sequences from the *Hin*dIII-K fragment accumulate (Lee and Krell, 1992), may be a reflection of the importance of this region as genuine *ori in vivo* (Chapter 7).

The occurrence of multiple *ori's* is not unique for baculoviruses, but has also been reported for herpesviruses and Chilo iridescent virus (CIV). The genome of herpes simplex virus 1 (HSV-1) contains three *ori's*, ori_L and two copies of ori_S (for review, see Fields and Knipe, 1990) and it has been shown that the presence of a single *ori*, independent which one, is sufficient for replication (Longnecker and Roizman, 1986; Polvino-Bodnar *et al.*, 1987; Igarashi *et al.*, 1993). In CIV at least six putative *ori's* have been identified (Handermann *et al.*, 1992). It remains to be seen whether in the case of baculoviruses each of the eight putative *ori's* is necessary for viral replication. When the *ori's* are indeed functionally redundant, the presence of multiple *ori's* in the viral genome may increase the frequency of initiation and thus increase the speed of DNA replication. Analysis of intermediates of DNA replication may shed more light on the nature of *in vivo ori's*.

The experiments in Chapter 6 also supported the view that a circular topology is a prerequisite for replication of *ori*-containing plasmids. Linear DNA, even if it contained an *ori*, did not replicate. These results are in line with the circular nature of baculovirus DNA and suggest a model for baculovirus replication involving a theta structure or a rolling circle. The latter model is supported by data of Leisy and Rohrmann (1993), who demonstrated that replicating plasmids form large concatemeric molecules. In addition, the finding of defective genomes with many reiterations (concatemers) of a 2.8 kbp segment, mainly mapping in the AcMNPV *Hin*dIII-K fragment (Lee and Krell, 1992), supported also a rolling circle as model for DNA replication.

Not only *cis*-acting elements, but also *trans*-acting factors are important for DNA replication. Chapters 8 and 9 describe the functional mapping of AcMNPV genes required for DNA replication. A transient complementation assay was employed, in which, instead of AcMNPV infection, four co-transfected cosmid clones, encompassing almost the entire genome, provided all the essential *trans*-acting factors for plasmid DNA replication. No replication of plasmids occurred when one of the cosmids was omitted from the transfection mixture. This result indicated that this assay was a valid and powerful approach to identify the AcMNPV replication genes. The assay was first used to define essential regions in the four cosmids (Chapter 8). Six essential regions were retrieved and these were further

subcloned and tested (Chapter 9). Initially in this assay, plasmid replication appeared to be independent of the presence, in *cis*, of a viral *ori*, when cloned genes or viral DNA were used instead of complete virus to supply essential *trans*-acting factors (Chapter 8). However, this was caused by employing high gene copy numbers in the transfections (Chapter 9). As a consequence, a relative abundance of proteins is produced, which may lead to a saturation of specific origins with these proteins. The excess of proteins thus can bind to other origin-like structures, even when the affinities are low, and hence cause replication of any plasmid.

Nine genes involved in DNA replication were identified in the AcMNPV genome (Chapter 9). Six genes, specifying *helicase*, *dna pol*, *ie-1*, *lef-1*, *lef-2*, and *lef-3*, were found to be essential, while three genes, *p35*, *ie-2*, and *pe38*, stimulated DNA replication. No stimulation was observed by the *pcna*-like protein gene. Two of the three identified stimulatory genes, *ie-2* and *pe38*, are known as transactivators for transcription (Carson *et al.*, 1988; Lu and Carstens, 1993), whereas the third stimulating gene, *p35*, has previously been identified as inhibitor of virus-induced apoptosis in *S. frugiperda* cells (Clem *et al.*, 1991). However, the observation that infection with a *p35* deletion mutant in *Trichoplusia ni* cells did not result in a reduction of virus production (Clem *et al.*, 1991) suggests that the stimulating effect of *p35* in the transient replication assays is not based on activation of the replication process, but is due to inhibiting apoptosis, which may be induced by the expression of one or more of the replication genes.

Of the six essential AcMNPV DNA replication proteins, putative functions could only be attributed for the helicase and DNA polymerase, based on their homology with other known helicases and DNA polymerases (Lu and Carstens, 1991; Tomalski *et al.*, 1988). Studying other viral systems, a number of striking similarities was noticed between *Baculoviridae* and *Herpesviridae*. Although these two viral families have traditionally been separated based on their different morphology and host specificity, they both have a large double stranded DNA genome, which replicates in the host cell nucleus, and has a circular form in at least one stage of their replication cycle. Their genomes may also replicate in a similar manner as transfection of origin-containing plasmids into infected cells resulted in large concatemers of input plasmid DNA (Leisy and Rohrmann, 1993; Hammerschmidt and Mankertz, 1991). Most strikingly, the number of essential replication genes is similar for both baculoviruses and herpesviruses. An attempt was made to relate the other four, hitherto unassigned, baculovirus replication proteins, IE-1, LEF-1, LEF-2, and LEF-3 with proteins involved in

herpesvirus DNA replication (Chapter 10).

Firstly, the sequences of replication proteins of five different herpesviruses were aligned, which resulted in the identification of a number of conserved motifs in these proteins. Many of these conserved motifs showed (distant) homology with the four baculovirus replication proteins and, most importantly, in the same linear spatial organization as in their putative herpesvirus homologues. Using these conserved motifs as markers the four replication proteins IE-1, LEF-1, LEF-2, and LEF-3 of AcMNPV were aligned with herpesvirus homologues (Chapter 10). These alignments suggest that *ie-1* codes for a single stranded DNA binding protein (SSB), *lef-1* for a primase-associated protein, *lef-2* for a DNA polymerase processivity factor, and *lef-3* for a primase. The assignment to *ie-1* to code for a SSB was further supported by the finding of a conserved known single stranded DNA binding sequence motif in six baculovirus IE-1 proteins, which is also found in many other prokaryotic and eukaryotic SSBs (Chapter 10). Further computer-assisted examination and biochemical analysis has to be done to confirm the suggested functions for the four baculovirus replication proteins.

The similarity between *Baculoviridae* and *Herpesviridae* in DNA structure and mechanism of DNA replication, added to the employment of an identical kind and amount of essential replication genes, poses the question whether these two groups of viruses share a common lineage. On the basis of the mutation rate of the conserved baculovirus polyhedrin genes as compared to the insect species in which they occur (Vlak and Rohrmann, 1985) it has been postulated that baculoviruses are ancient viruses that have evolved along with the insects. The relationship among replication genes could imply that herpesviruses have evolved from baculoviruses along with their invertebrate hosts towards vertebrates. Alternatively, the emergence of herpesviruses may be the result of an independent, parallel evolutionary event in ancient vertebrates. Since viral DNA replication in nuclear environments is a conserved process, conserved host replication genes may have been independently transduced into different ancient viral genomes.

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SAMENVATTING

Baculovirussen worden toegepast als biologisch bestrijdingsmiddel bij de bestrijding van insektenplagen. Daarnaast worden deze virussen zeer succesvol geëxploiteerd als expressievector voor de produktie van heterologe eiwitten. In beide gevallen is het nodig om over grootschalige produktiesystemen te beschikken. Produktie van baculovirussen in rupsen is mogelijk maar moeilijk op te schalen en onder controle te houden. Insektecelculturen vormen derhalve een aantrekkelijk alternatief. In samenwerking met de Vakgroep Levensmiddelentechnologie, sectie Proceskunde, van de Landbouwuniversiteit is onderzocht of een grootschalige produktie van baculovirussen mogelijk is in continu operende bioreactoren.

Voor de produktie van het wilde type baculovirus *Autographa californica* kernpolyeder virus (AcMNPV) en daarvan afgeleide recombinanten is een continu produktiesysteem ontwikkeld bestaande uit twee in serie geschakelde reactoren, waarbij in de eerste de produktie van gezonde cellen plaatsvindt en in de tweede de produktie van virus en (recombinante) eiwitten. Echter na een paar weken van continu produktie blijkt de produktiviteit sterk af te nemen. In het geval van het wilde type AcMNPV neemt het aantal polyeders per cel, het aantal cellen met polyeders als ook de concentratie van extracellulair virus sterk af. Deze daling van virus- en polyederproduktie in de tijd werd toegeschreven aan het passage effect, maar het mechanisme was onbekend. Aan het begin van het in dit proefschrift beschreven onderzoek was er, in tegenstelling tot andere grote DNA-virussen, zoals adenovirus, vacciniavirus en herpesvirus, slechts weinig bekend over de replicatie van baculovirussen en het ontstaan van het passage effect.

Vanwege de grote belangstelling voor baculovirussen als biologisch bestrijdingsmiddel en als expressievector voor heterologe eiwitten, zijn de laatste jaren de moleculair-genetische

aspecten van met name AcMNPV, het voorbeeld van de familie van de *Baculoviridae*, uitvoerig onderzocht. Een actueel overzicht van de structurele en functionele organisatie van het AcMNPV genoom is te vinden in Hoofdstuk 2 van dit proefschrift.

Analyses van virusmonsters uit een continue produktie in insektecellen lieten zien dat er gedurende dit proces deletiemutanten gevormd werden met steeds grotere deleties in het oorspronkelijke virale genoom. Deze defectieve virusdeeltjes of "defective interfering particles" (DIPs) kunnen niet zelfstandig repliceren, maar zijn afhankelijk van de aanwezigheid van intakt virus dat als helper fungeert. De interferentie die optreedt tussen de deletiemutanten en het intakte virus, met als gevolg een dalende produktie van intakt virus, wordt nu als verklaring gezien voor het passage effect (Hoofdstuk 3).

Verder onderzoek aan deze deletiemutanten, nu gegenereerd door middel van seriële, onverdunde passage in insektecellen, liet zien dat na 40 passages mutanten ontstonden die nog slechts 5% van het oorspronkelijke virale genoom bevatten (Hoofdstuk 5). Daar deze mutanten nog steeds in staat waren te repliceren, moesten de noodzakelijke *cis*-elementen, nodig voor DNA replicatie, zich in deze 5% bevinden.

Eén van de gebieden van het AcMNPV genoom, dat veelvuldig betrokken was bij de vorming van de deletiemutanten, bleek een deel van het *Eco*RI-C fragment te zijn. Analyse van de nucleotidenvolgorde van een deel van dit fragment gaf acht mogelijke open leesramen (ORFs) aan. Eén ervan, ORF 1227, correspondeerde met het eerder gepubliceerde *gp41*, dat codeert voor een structureel viruseiwit. Echter de thans verkregen sequentie toonde aan dat dit eiwit 55 aminozuren langer moest zijn dan gepubliceerd. De mogelijke functies van de andere ORFs zijn nog onbekend. Ook werden geen sequenties gevonden die mogelijk als *cis*-elementen bij de DNA replicatie konden fungeren (Hoofdstuk 4).

Om te onderzoeken of de gebieden van het virale genoom die behouden bleven in de deletiemutanten inderdaad essentiële *cis*-elementen bevatten, zoals een oorsprong of "origin" van DNA replicatie (*ori*), werd een replicatietest ontwikkeld. Via deze test werden zeven mogelijke *ori's* in het AcMNPV genoom opgespoord en nader geanalyseerd (Hoofdstukken 5, 6 en 7). Transfectie van *Spodoptera frugiperda* insektecellen met plasmiden die een dergelijke, mogelijke *ori* bevatten, gevolgd door een infektie met intakt helper virus, resulteerde in replicatie van deze plasmiden.

Zes van de zeven mogelijke *ori's* werden aldus gelocaliseerd in de gebieden met homologe nucleotidenvolgorden: *hr*1, *hr*2, *hr*3, *hr*4a, *hr*4b en *hr*5, die verspreid over het

genoom liggen (Hoofdstukken 5 en 6). Deze *hr* gebieden bevatten, naast vele andere repeterende sequenties, ook 2 tot 8 kopieën van een palindroomsequentie ter grootte van 30 baseparen. Eerdere studies hebben aangetoond dat deze *hr* gebieden ook een stimulerende functie hebben bij het transcriptieproces.

Een zevende mogelijke *ori* is aangetoond in het AcMNPV *Hin*dIII-K fragment (Hoofdstukken 6 en 7). Nucleotidenvolgordebepaling toonde aan dat dit fragment geen homologie vertoont met één van de *hr* gebieden, maar dat er wel verscheidene structuren aanwezig zijn die kenmerkend zijn voor eukaryote *ori's*, zoals palindroomsequenties en een A/T-rijk gebied.

De individuele rol van al deze *ori's* gedurende de virale DNA replicatie en hun daadwerkelijke activiteit *in vivo* is echter nog niet bekend. Deletie van *hr5* uit het virale genoom had geen enkel effect op de replicatie. Ook het feit dat DIPs na 40 seriële passages alleen de *hr* gebieden *hr1*, *hr3* en *hr5* bevatten, doet vermoeden dat niet alle zeven *ori's* tegelijk nodig zijn. Twee aspecten dragen bij aan de belangrijkheid van de *ori* in het *Hin*dIII-K fragment. Vergelijking met het nauw verwante baculovirus *Bombyx mori* MNPV liet zien dat in dit virus de niet-essentiële delen van deze *ori* gedeleteerd waren, maar dat het essentiële deel, dat de palindooom sequenties en het A/T-rijke gebied bevat, behouden was. Daarnaast hebben andere studies laten zien dat na 80 seriële passages van AcMNPV in celcultuur, grote replicerende DNA moleculen werden aangetroffen, die bijna uitsluitend sequenties afkomstig van het *Hin*dIII-K fragment bleken te bevatten (Hoofdstuk 7).

Naast de *cis*-elementen zijn ook de betrokken *trans*-factoren voor DNA replicatie geïdentificeerd (Hoofdstukken 8 en 9). Met behulp van een transiënte complementatietest zijn negen genen gevonden in het AcMNPV genoom die direct of indirect betrokken zijn bij de DNA replicatie. Zes genen, te weten *helicase*, *dna pol*, *ie-1*, *lef-1*, *lef-2* en *lef-3*, bleken essentieel te zijn voor transiënte DNA replicatie, en drie genen, *p35*, *ie-2* en *pe38*, bleken een stimulerende werking te hebben op de DNA replicatie. Het *pcna* gen, waarvan men vermoedde dat het betrokken was bij de DNA replicatie, bleek echter geen rol te spelen bij de transiënte DNA replicatie. Van twee van de stimulerende genen, *ie-2* en *pe38*, is bekend dat ze een *trans*-activerende werking hebben op de transcriptie, terwijl *p35* bekend is als remmer van geprogrammeerde celdood (apoptosis). Het is niet bekend of de stimulerende werking van *p35* in de transiënte DNA replicatietest komt door een directe activatie van het replicatieproces, of doordat expressie van één der replicatiegenen apoptosis veroorzaakt, die

vervolgens geremd wordt door *p35* (Hoofdstuk 9).

Slechts voor twee van de essentiële DNA replicatiegenen, ***helicase*** en ***dna pol***, is de mogelijke functie bekend op basis van hun homologie met andere bekende helicase en DNA polymerase genen. Voor de produkten van de andere vier genen kon niet direct homologie gevonden worden in de bestaande databanken. In vergelijking met andere virussen, viel echter een aantal opvallende overeenkomsten waar te nemen tussen de *Baculoviridae* en de *Herpesviridae*. Hoewel deze beide virusfamilies gescheiden zijn op basis van verschillende morfologie en gastheerbereik, worden beide virusgroepen gekenmerkt door het bezit van een groot dubbelstrengig DNA genoom. Zowel baculo- als herpesvirussen repliceren in de kern van de cel en hebben, al of niet tijdelijk, een circulair DNA genoom. Verder is ook het aantal essentiële replicatiegenen voor beide virussen vergelijkbaar. Daarom werd nader onderzocht of de overige vier baculovirus replicatie-eiwitten IE-1, LEF-1, LEF-2 en LEF-3 wellicht verwant zijn met de replicatie-eiwitten van de herpesvirussen (Hoofdstuk 10).

Op basis van homologie met een aantal geconserveerde motieven in de herpesviruseiwitten is de hypothese nu dat *ie-1* wellicht codeert voor een enkelstrengig DNA-bindend eiwit, *lef-1* voor een primase-geassocieerd eiwit, *lef-2* voor een DNA polymerase stimulerend eiwit en *lef-3* voor een primase. De mogelijke functie van IE-1 wordt verder ondersteund door de identificatie van een geconserveerd enkelstrengig DNA-bindend sequentie motief in zes verschillende baculovirus IE-1 eiwitten. Biochemische analyses zullen deze voorgestelde functies moeten bevestigen.

De overeenkomst tussen baculovirussen en herpesvirussen wat betreft DNA structuur, DNA replicatie mechanisme en de daarbij betrokken replicatiegenen, doet de vraag rijzen in hoeverre deze virussen evolutionair met elkaar verwant zijn. Algemeen wordt aangenomen dat baculovirussen "oude" virussen zijn die zijn meegeëvolueerd met de insekten. De verwantschap met de herpesvirussen, die alleen infektieus zijn voor vertebraten, zou kunnen suggereren dat deze zijn ontstaan uit de baculovirussen, tegelijk met de evolutie van invertebraten naar vertebraten. Het alternatief is dat het ontstaan van herpesvirussen een evolutionaire gebeurtenis is geweest, die onafhankelijk heeft plaatsgevonden.

CURRICULUM VITAE

Op 16 november 1964 ben ik, Marcel Kool, in Haarlem geboren. Via Amstelveen, Geleen, wederom Amstelveen en Badhoevedorp, belandde ik uiteindelijk in Alphen aan den Rijn alwaar ik in 1983 op het Christelijk Lyceum mijn Gymnasium B diploma haalde. In datzelfde jaar begon ik met mijn studie Moleculaire Wetenschappen aan de toenmalige Landbouwhogeschool te Wageningen. Na afstudeeronderzoek te hebben gedaan in de Moleculaire Biologie (dr. Rene Klein Lankhorst en dr. Rommert van den Bos) en de Virologie (dr. Jan van Lent en dr. Just Vlak), en een praktijktijd Virologie / Proceskunde aan het Chemical Engineering Department van Cornell University te Ithaca in de Verenigde Staten (Prof. Michael Shuler), behaalde ik op 27 januari 1989 het ingenieursdiploma. Van februari 1989 tot en met juni 1994 was ik als Assistent In Opleiding werkzaam bij de Vakgroep Virologie van de Landbouwuniversiteit Wageningen onder begeleiding van dr. Just Vlak en Prof. Rob Goldbach. Het daar uitgevoerde onderzoek, waarvan de resultaten beschreven staan in dit proefschrift, was in het kader van een samenwerkingsproject tussen de Vakgroepen Virologie en Levensmiddelentechnologie, sectie Proceskunde (Prof. Hans Tramper), met financiële ondersteuning van de Programmacommissie Landbouw-biotechnologie (PcLb). Een deel van dit onderzoek is verder uitgevoerd in samenwerking met Prof. George Rohrmann van het Agricultural Chemistry Department van Oregon State University te Corvallis in de Verenigde Staten. Van juli tot en met oktober 1993 heb ik daar een aantal experimenten, beschreven in dit proefschrift, uitgevoerd.

Vanaf oktober 1994 zal ik als post-doc gaan werken aan meervoudige medicijn resistentie in kankercellen bij Prof. Piet Borst van het Nederlands Kanker Instituut in Amsterdam.

ACCOUNT

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