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AcMNPV As A Model for Baculovirus DNA Replication*

Eric B. Carstens**

(Department of Microbiology and Immunology, Queen's University at Kingston, Kingston, ON K7L 3N6，Canada)

Abstracts: Baculoviruses were first identified as insect-specific pathogens, and it was this specificity that lead to their use as safe, target specific biological pesticides. For the past 30 years, AcMNPV has served as the subject of intense basic molecular research into the baculovirus infectious cycle including the interaction of the virus with a continuous insect cell line derived from *Spodoptera frugiperda*. The studies on baculoviruese have led to an in-depth understanding of the physical organization of the viral genomes including many complete genomic sequences, the time course of gene expression, and the application of this basic research to the use of baculoviruses not only as insecticides, but also as a universal eukaryotic protein expression system, and a potential vector in gene therapy. A great deal has also been discovered about the viral genes required for the replication of the baculovirus genome, while much remains to be learned about the mechanism of viral DNA replication. This report outlines the current knowledge of the factors involved in baculovirus DNA replication, using data on AcMNPV as a model for most members of the *Baculoviridae*.

Key words: Baculovirus; DNA replication; AcMNPV; Molecular virology; Review

Throughout the ages, economic pressures have helped to establish the direction of basic research. Early research in virology was directed towards identifying etiological agents for specific diseases. These included not only human diseases but also diseases of biologic products of economic importance to humans. For example, following the arrival of silk production in Europe in the 12th century, most likely as a result of smuggling silkworms out of China, the rearing of silkworms became a huge industry, especially in Italy. Outbreaks of disease in silkworms were financially devastating to the silk industry. Early accounts of silkworm diseases are found in both ancient Asian and Western literature. Marco Vida of Cremona, an Italian bishop of the 16th century, described in poetic form an infection of silkworm that is reminiscent of baculovirus pathogenesis. In 1856, A. Maestra and E Cornalia independently observed polyhedral bodies in silkworm larvae and made the connection between the presence of polyhedra and the symptoms in the diseased silkworms (26, 94).

The use of induction of pathogenesis as an assay system for infectious agents led to the identification of the first virus, now known as Tobacco mosaic virus, which is responsible for tobacco mosaic disease (27).

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** Corresponding author.
Phone: +1-613-533-2463, Fax: +1-613-533-6796,
E-mail: carstens@queensu.ca

Von Prowzek showed in the early 1900's that jaundice in the silkworm is caused by a filterable agent, but he believed the agent to be a bacterium and the occlusion bodies to be by-products of the disease (155). Shortly thereafter, Glaser and Chapman (in gypsy moths) and C. Acqua (in silkworms) independently established the viral etiology of polyhedrosis (1, 45). In 1943, Glaser and Stanley concluded, based on biochemical analysis, that polyhedra contain nucleoproteins (nucleic acid and protein) (46). In 1947, von Bergold identified virus-like rod-shaped particles from polyhedral bodies by electron microscopy (5), and subsequently found that the virions contained nucleic acids and proteins (6). This group of rod-shaped enveloped viruses, containing a double stranded DNA genome, and often occluded in protein crystals was named baculovirus because of their shape (baculo: from *baculum* "stick" or "rod") (157).

TAXONOMY OF BACULOVIRUSES

The taxonomy of the family *Baculoviridae*, first described in 1976 (38), has recently been divided into four genera, based on phylogenetic, biological and morphological characteristics (ICTVonline: http:// www.ictvonline.org/virusTaxonomy.asp?version=2008) (14a). The *Alphabaculoviruses* include all lepidopteranspecific former *Nucleopolyhedroviruses*. The *Betabaculoviruses* include the former *Granuloviruses*. The *Gammabaculoviruses* comprise the Hymenopteran specific baculoviruses while the *Deltabaculoviruses* include the Dipteran-specific baculoviruses. These family members share common features including a rod-shaped enveloped nucleocapid 30-60 nm in diameter and 25-300 nm in length, carrying a double stranded circular DNA genome varying from about 80 to 180 kb in size. Typically, two different phenotypes of virions, the budded virions (BV) and the occlusionderived virions (ODV), are produced during the virus replication cycle. BV are produced immediately after initial infection and bud from the cytoplasmic membrane of infected cells. In insect infections, the BV, initially released from primary infected midgut cells, are responsible for systemic infection of tissues throughout the insect. The ODV are assembled and enveloped in the nucleus of infected cells at a late stage of the infection cycle. These virions eventually become occluded within a protein matrix called occlusion bodies (OBs). The ODV are eventually responsible for virus spread to other uninfected insects (39). All current representative baculovirus isolates form OBs as part of their replication cycle but the size, and appearance of the OBs varies between the genera. The *Alphabaculovirus, Deltabaculovirus* and *Gammabaculovirus* OBs are polyhedra in shape, vary in size from 0.15 to 15 μm and contain many enveloped virions while the *Betabaculovirus* OBs are ovoid in shape, generally smaller (0.13 - 0.5 μm) and usually contain a single ODV.

Baculovirus isolates have traditionally been named after the host insect from which they were first isolated, For example, the first baculovirus isolated from the alfalfa looper *Autographa californica* was called Autographa californica multiple nucleopolyhedrovirus (AcMNPV). It was later discovered that AcMNPV has a broad host range so other viruses that had been isolated and named after other host insects (AnfaMNPV from *Anagrapha falcifera*, GmMNPV from *Galleria mellonella*, RoMNPV from *Rachiplusia ou*, SpexMNPV from *Spodoptera exempta* and TnMNPV from *Trichoplusia ni*) are now considered

variants of the original AcMNPV isolate. All of these variants are classified as isolates within the type species *Autographa californica multiple nucleopolyhedrovirus*. According to the taxonomic code of the International Committee on Taxonomy of Viruses, all virus species names are italicized and not abbreviated while all vernacular or isolate virus names are non-italicized and can be abbreviated (37).

SEQUENCED BACULOVIRUS GENOMES

The first complete baculovirus genome sequence, from an isolate of the *Alphabaculovirus* type species called AcMNPV C6, was determined in 1994 (4). The AcMNPV genome consists of 133,894 bp, coding for about 152 predicted open reading frames (ORFs). Currently (Dec 2008), there are 48 reference baculovirus genome sequences available at NCBI Genomes (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome&orig_db=Genome&term=baculoviridae&cmd=search&cmd_current=&title=Viruses&list_uids=438782). These are complete genomic sequences of isolates that represent different baculovirus species. In some cases, there are other genomic sequences available but these additional genome sequences represent different isolates of the same species. This wealth of genomic sequence information including gene sequence and organization has led to the phylogenic analysis of the genetic diversity of baculoviruses (150). The richness of this genetic diversity can be seen by the total number of unique open reading frames identified (about 840) and the low number of core genes (about 29), identified in all baculovirus genomes sequenced to date. An excellent resource for up-to-date genomic and proteomic data and analysis of baculovirus genomes can be reviewed at the Viral Bioinformatics Resource Centre web site (http://athena.bioc.uvic.ca/ organisms/Baculoviridae) (32). The identification of genes conserved in all baculoviruses has provided some insight into the systems that are required by all baculoviruses and are related to processes of DNA replication, gene transcription, packaging and assembly of virions, cell cycle arrest, infectivity and host range (58). The 29 predicted baculovirus core genes, conserved in all sequenced baculovirus isolates, along with 14 other genes which are present in all but the *Deltabaculovirus* and *Gammabaculovirus* isolates are listed in Table 1.

TIME COURSE OF BACULOVIRUS REPLICATION

Basic baculovirus research was stimulated in the early 1970s by the establishment of insect cell cultures that supported the replication of AcMNPV, particularly continuous cell lines derived from the alfalfa looper *Spodoptera frugiperda* and the cabbage looper *Trichoplusia ni* (47, 149). The availability of these permissive cell lines led to the development of plaque assay protocols, which were used for both the quantification of virus titres and the preparation of genotypically pure virus stocks (59, 126). The preparation of plaque-purified virus stocks led to precise characterization of the baculovirus infectious cycle including the process of DNA replication.

One of the first studies of baculovirus DNA synthesis in infected cell cultures suggested that baculovirus infection resulted in the inhibition of cellular DNA synthesis, and that by 12 hr post infection, only 12% of the newly synthesized DNA was cellular (68). The study of the kinetics of viral DNA replication demonstrated that viral DNA replication

Table 1. Core Baculovirus Genes

AcMNPV ORF	Ortholog Group Name	No. of viruses	Missing in virus
Ac6	Late expression factor 2 (*lef-2*)*	53	
Ac14	Late expression factor 1 (*lef-1*)*	53	
Ac22	Per os infectivity factor 2 (*pif-2*)	53	
Ac40	p47 late expression factor (*p47*)	53	
Ac50	Late expression factor 8 (*lef-8*)	52	AsGV-CHN
Ac54	vp1054 capsid associated protein	53	
Ac62	Late expression factor 9 (*lef-9*)	53	
Ac65	DNA polymerase (*dnapol*)*	52	AsGV-CHN
Ac68	Unknown (ac68)	53	
Ac77	Very late expression factor 1 (*vlf-1*)	53	
Ac80	Tegument protein (*gp41*)	53	
Ac81	Unknown (*ac81*)	53	
Ac83	Capsid associated Protein (*vp91/p95*)	53	
Ac89	Major capsid protein (*vp39*)	53	
Ac90	Late expression factor 4 (*lef-4*)	53	
Ac92	p33 (*ac92*)	53	
Ac95	Helicase (*p143*)*	52	AsGV-CHN
Ac96	19kda (*ac96*)	53	
Ac98	38K (*ac98*)	53	
Ac99	Late expression factor 5 (*lef-5*)	53	
Ac109	Unknown (*ac109*)	52	AsGV-CHN
Ac115	Per os infectivity factor 3 (*pif-3*)	53	
Ac119	Per os infectivity factor 1 (*pif-1*)	52	AsGV-CHN
Ac133	Alkaline exonuclease (*an*)	53	
Ac138	ODV envelope protein (*p74*)	53	
Ac142	p49 (*ac142*)	53	
Ac143	ODV-E18 (*odv-e18*)	53	
Ac144	ODV-EC27 (*odv-ec27*)	53	
Ac148	ODV-E56 (*odv-e56*)	53	
Ac25	DNA binding protein (*dbp1*)	52	CuniNPV-FL1997
Ac8	Polyhedrin/granulin (*polh*)	52	CuniNPV-FL1997
Ac145	Unknown (*ac145*)	52	CuniNPV-FL1997
Ac106	Unknown (*ac106*)	52	CuniNPV-FL1997
Ac103	p45 (*ac103*)	52	CuniNPV-FL1997
Ac101	p40 (*ac101*)	52	CuniNPV-FL1997
Ac75	Unknown (*ac75*)	52	CuniNPV-FL1997
Ac53	Unknown (*ac53*)	52	CuniNPV-FL1997
Ac37	Late expression factor 11 (*lef-11*)*	52	CuniNPV-FL1997
Ac66	Desmoplakin (*ac66*)	49	CuniNPV-FL1997, NeseNPV-USA, NeleNPV-CDN, NeabNPV-CDN1997
Ac67	Late expression factor 3 (*lef-3*)*	49	CuniNPV-FL1997, NeseNPV-USA, NeleNPV-CDN, NeabNPV-CDN1997
Ac147	Immediate early transactivator 1 (*ie-1*)*	49	CuniNPV-FL1997, NeseNPV-USA, NeleNPV-CDN, NeabNPV-CDN1997
Ac100	Basic protein (*p6.9*)	48	AgipMNPV-Illinois, PsunGV-Hawaiian, AdorNPV-English, HaMNPV-CHN, SpliNPVII-CHN
Ac102	p12 (*ac102*)	49	CuniNPV-FL1997, NeseNPV-USA, NeleNPV-CDN, NeabNPV-CDN1997

The AcMNPV open reading frame numbers are shown in the first column. The ortholog group names are shown in the second column. The number of sequenced virus genomes which have been annotated to include the genes are indicated in the third column, while the names of the viruses which are reported to be missing particular genes is shown in the fourth column. A horizontal line separates the core ORFs present in all baculoviruses. It is possible that the degree of amino acid homology is too low to recognize some of the other genes such as *lef-3* (Ac67*)*, *p6.9* (Ac100) and *p12* (Ac102) in the *Gamma*- and *Deltabaculovirus* species. Genes marked with an asterisk are predicted to be essential for AcMNPV DNA replication. The lack of some core genes in AsGV-CHN may be related to annotation or sequencing errors. Other genes thought to be stimulatory for AcMNPV DNA replication including homologues of *ie-2*, *pe38*, *me53*, *lef-7* and *p35* are present in fewer than 22 sequenced genomes (see text for details).

initiated 5-6 hr post infection and reached a maximum rate by 18 hr post infection (145). This correlated well with the stages of protein synthesis seen in Ac-MNPV-infected Sf21 cells (18, 31). Different virus-specific proteins are detected before (early) and after the time of initiation of DNA replication (late), while host protein synthesis is inhibited during the virus replication cycle. The occlusion of virus in polyhedra correlates with the synthesis of the occlusion body protein (polyhedrin) at very late times after infection.

This cascade of baculovirus gene expression is primarily regulated at the level of transcription and facilitated by early gene expression (41). Early genes are transcribed by the host RNA polymerase II while late genes are transcribed by an alpha-amanitin resistant viral-encoded RNA polymerase activity (42, 49). The early phase can be functionally divided into two stages; the immediate early phase and the delayed early phase. The immediate early genes, transcribed in the absentce of any viral gene products, code for transcription factors that in turn are responsible for inducing the transcription of the delayed early genes. The delayed early genes are involved in stimulating viral DNA replication and late gene transcription. This differential regulation of transcription is largely directed by DNA promoter elements in the viral genome. Many of the early genes carry transcription start sites that mimic normal host insect gene promoters. This includes TATA-box-like sequences upstream of a consensus transcription initiation start sequence ATCA(G/T)T(C/T). The major immediate early gene is *immediate-early gene 1* (*ie-1*) (51) and its RNA-spliced partner *ie-0* (23). There are at least four other important immediate early regulatory genes, (*ie-2, pe38, me53 and he65*) identified on their ability to transactivate other viral genes or on their time of expression in infected cells (13, 67, 75, 120). No newly synthesized viral products are required for the production of these immediate early proteins, but these virus-specific proteins are required for further delayed early gene expression, prior to the initiation of viral DNA replication. In AcMNPV infected cells, immediate and delayed early gene expression occurs immediately after virion penetration, uncoating and presentation of the parental genome to the host transcription system in the nucleus to about 6 hr post infection when the first copies of newly synthesized DNA are produced (112). The concept that the immediate early genes are expressed in the absence of any other viral proteins was clearly shown by the demonstration that purified viral DNA, free of all viral proteins, was infectious and capable of stimulating a complete virus replication cycle when transfected into cell cultures (11, 19). This process of introducing purified naked DNA into insect cells is called transfection, a process that has developed into an important application of nanotechnology to a wide variety of biological applications (137). The ability to transfect purified baculovirus DNA into insect cells to initiate an authentic virus replication cycle was an important development leading to a new era in baculovirus research including discoveries in baculovirus genes, gene function, regulatory processes, DNA replication and the development of baculovirus as an efficient eukaryotic expression system (143). An excellent resource on the molecular biology of baculoviruses has recently been published on-line (http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=bacvir).

ORIGINS OF BACULOVIRUS REPLICATION

Complete and accurate DNA replication of a genome is a fundamental process of life, integral to the maintenance of the genetic integrity of all organisms. The presence of DNA genomes throughout living organisms suggests that the process of DNA replication must be very well conserved in evolution. Thus, the advances made in understanding this process in eubacterial systems have been applicable to eukaryotic systems including their viruses. Indeed, the study of replication in viruses has lead to a greater understanding of how this process occurs in eukaryotes. Many of these studies have been based on the replicon theory put forward by Jacob, Brenner and Cuzin in 1963 (65). The replicon was proposed to be a functional unit with two components: a cis-acting sequence called the replicator, and a trans-acting protein called the initiator. The replicon model has stimulated research into what constitutes an origin of replication (the replicator), and what protein or proteins are involved in recognizing the origin to initiate replication.

A characteristic of most baculoviruses that have been sequenced is the presence of homologous regions (*hrs*), first identified in AcMNPV, at various locations within the genome (25). The number and distribution of *hrs* is variable in different species of baculovirus (150). The eight AcMNPV *hrs* consist of repeated AT rich sequences of about 70 base pairs with an imperfect 30 base pair palindrome near their centre (Fig. 1). In other baculoviruses, *hrs* are generally AT-rich sequences within repeated palindromic motifs whose numbers vary between three (AnpeNPV, EcobNPV) and seventeen (SpltNPV) per genome. However, there are also some baculoviruses, notably some members of the *Betabacuolviruses*, which reportedly do not contain any *hrs* (TniSNPV, AdorGV, and CpltGV) (93, 159, 161). However, most of these viruses do contain one or more AT-rich direct repeats (*drs*) but with less conservation in sequence than *hrs*. The specific sequence, the repeated structure, and the presence of *hrs* or *drs* at distributed sites around the AcMNPV genome suggested that these may function as origins of viral DNA replication (25). Data to support this hypothesis was initially gathered from genetic experiments. Continuous passage of virus at high multiplicity of infection leads to sequential and cumulative deletions of viral DNA segments and the accumu- lation of defective interfering (DI) particles (74, 79, 81). Many of these DI genomes lack essential genes so they require co-infection with standard virus carrying full-length genomes (helper virus) for their replication. Because of the smaller genome size, replicating DI genomes can accumulate to high levels. Analysis showed that in many cases, *hr*-containing regions were consistently maintained in the DI genomes. These results suggested that the presence of *hrs* provided a selective advantage for the replication of these defective genomes by acting as origins of replication.

Although analyzing radiolabeled replicative intermediates and electron microscopy has been used to examine replicating molecules (36, 110), more detail information about potential viral origins of replication has come from transient replication assays where a library of cosmids or plasmids carrying different viral genomic regions were examined for their ability to replicate in transfected cells (55, 142, 156). In these assays, the transfected input plasmid DNA, produced in dam+ bacteria that methylate deoxyadenosine residues, is distinguished from newly

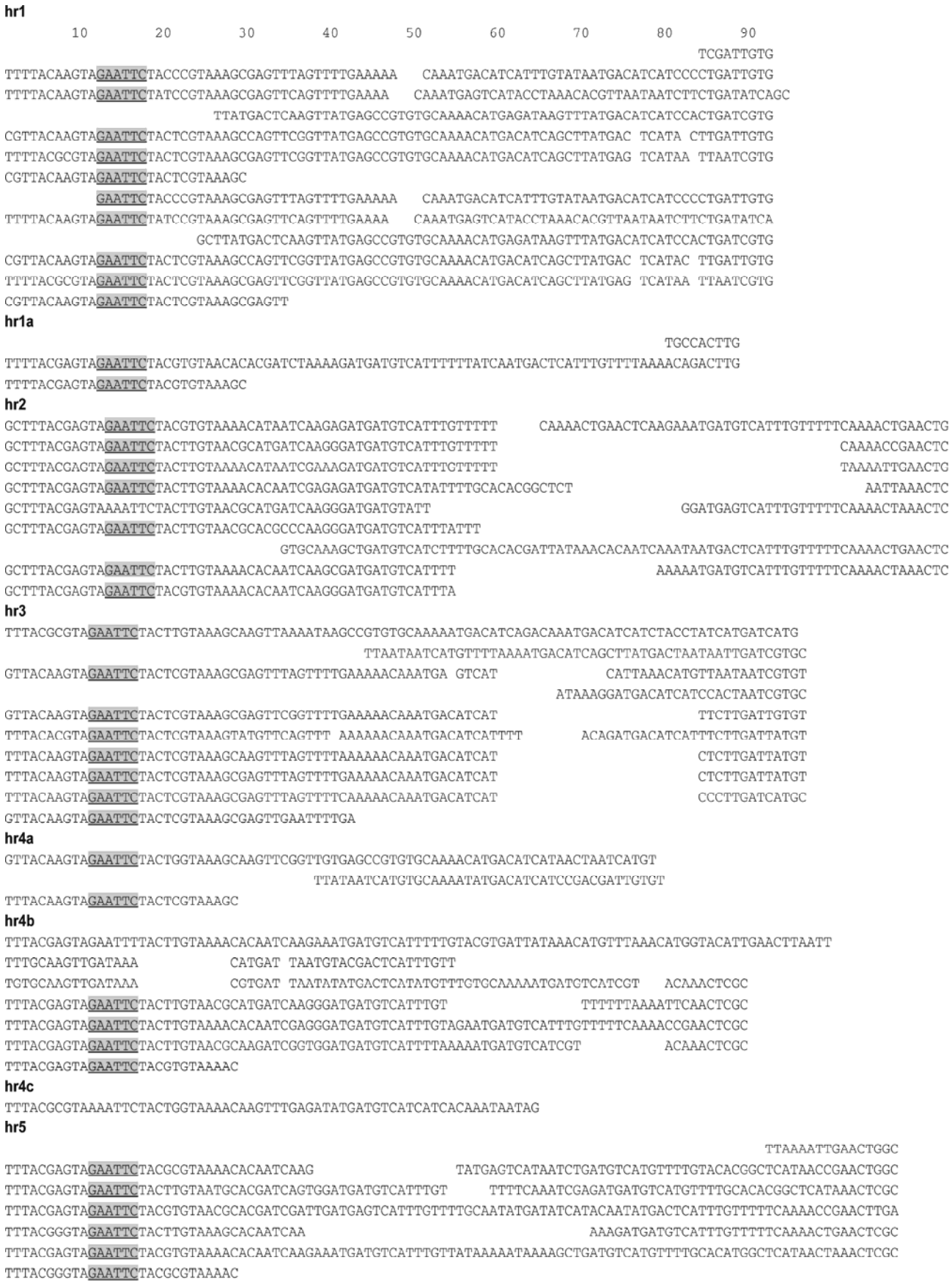

Fig. 1. Alignments of the AcMNPV homologous regions. The *hrs* are aligned to show the repeat sequences in each region. Spaces have been inserted to maintain maximum homologies. The central conserved *Eco*RI sites are underlined and shaded.

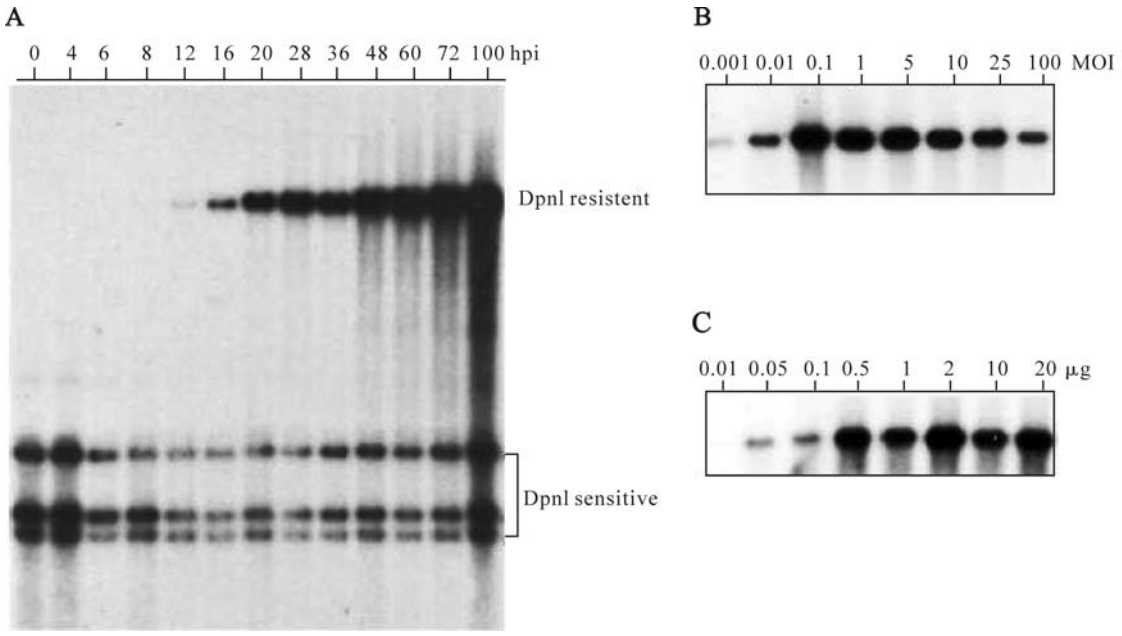

Fig. 2. Time course, effect of multiplicity of infection, and plasmid concentration on transient replication assays. (A) A plasmid carrying hr2 was transfected into Sf21 cells, and at 16 hours post transfection, the cells were infected with AcMNPV (moi of 1). Intracellular DNA was harvested at the indicated times after infection. Total intracellular DNA was digested with *Eco*RI and *Dpn*I, and assayed for plasmid replication by probing the Southern blots with ^{32}P-labelled pUC19 DNA. Replicated plasmid (*Dpn*I resistant) was easily detected by 12 hr post infection and continued to increase in amount until 100 hr post infection. (B) Cells were transfected with an hr2 carrying plasmid, then infected 6 hr later with AcMNPV at different moi (multiplicity of infection) and harvested at 48 hr post infection. Maximum replication was detected with a moi of 0.1, consistent with the transfection efficiency of about 20% (about one plasmid copy per infected cell). (C) Cells were transfected with the indicated amounts of an hr2-carrying plasmid, infected with AcMNPV (moi of 1) at 6 hr post transfection and harvested at 48 hr post infection. Maximum replication was detected with 0.5 μg of plasmid DNA.

replicated DNA that is not methylated, by the use of the restriction endonuclease *Dpn*I (30, 123). Since most *Dpn*I sites in insect and mammalian genomic DNA are not methylated *Dpn*I treatment of total intracellular DNA from plasmid-transfected cells can distinguish between input plasmid (methylated) and progeny plasmid (unmethylated) DNA that has replicated in the eukaryotic cells. The conclusion is that if a recombinant bacterial plasmid replicates in these cells, it must carry a viral origin of replication that is recognized and functional. Optimization of this assay for the baculovirus system indicates that specificity of replication of an origin-containing plasmid requires plasmid transfected cells to be infected with AcMNPV not earlier than 6 hours after transfection (163). The virus supplies the proteins required for recognition of a virus-specific origin in the plasmid. Maximum detection of replicating plasmid DNA occurs with relatively low multiplicities of virus infection (between 0.1 and 1) and small amounts of plasmid DNA (0.5 to 1.0 μg) (Fig. 2b,c). Under these conditions, replicating plasmid DNA is easily detected by 12 hours post infection and at high levels by 20 hr post infection, simulating authentic viral DNA replication. (Fig. 2a).

Application of this transient DNA replication assay to identify putative baculovirus origins of replication demonstrated that AcMNPV *hr1, hr1a, hr2, hr3, hr4a, hr4b,* (*hr4c* has not been tested), and *hr5*, when

present *in cis* on plasmids, are capable of supporting plasmid replication in transfected Sf cells (71, 72, 85, 122). In fact, it seems that a single *hr* palindrome with an essential *Eco*RI core site is sufficient to support plasmid replication (83, 122).

Non-*hr*-containing regions have also been characterized as potential origins of replication. A region within the AcMNPV *Hin*d III K fragment that is tandemly repeated in DI genomes and which contains repeated sequences, an AT-rich region and palindromes supports plasmid replication in transient assays (70, 79). Several early promoter regions of AcMNPV can also support plasmid replication in transient assays (163). The ability of non-*hr* origins to function as origins *in vivo* has also been demonstrated by a competitive PCR analysis of DNA extracted from infected cells (54).

It is clear that varying parameters of the transient assays can have a big impact on the conclusions of whether a region of the genome can support replication or not. If plasmid DNA is co-transfected with viral DNA, or virus infection occurs within 4 hr of transfection, any plasmid DNA will replicate, whether or not it carries a potential viral origin of replication (163). The non-specificity of the baculovirus replication machinery seems to be related to the state of the transfected DNA within the cell. The specificity of *hr*-directed plasmid replication requires a delay of at least five hours before introducing the helper functions of virus infection. Since DNA transfected into cells probably associates with host histones within the first 5 hours following transfection (160), the specificity of origin recognition is likely influenced by the chromatin structure of the DNA. This suggests that in order to direct the replication of plasmids because of the presence of *hr* sequences, plasmids without *hrs* must be sequestered with cellular proteins, likely histones, to prevent non-specific recognition by the viral proteins required for specificity of origin function. In addition, this suggests that the proteins required for baculovirus origin recognition can overcome this block, or that the viral DNA sequences that function as origins can adopt a structure that is specifically recognized by the initiator protein complex. Baculoviruses have evolved multiple regions including *hrs*, and other repeated sequences that are AT-rich and include palindromes that can function as origins of replication. An IE-1 binding motif (IBM) has been identified that is present in two copies within the *hr* palindrome, orientated in opposite directions and centered on the *Eco*RI site (84). Single copies of the IBM were found to correlate with negative regulation of other immediate early genes such as *ie-2* and *pe38*. Altering the sequence or spacing of IBMs seemed to also affect their replication capacity in transient assays suggesting that IBMs may be important in the specificity of initiation of replication (86). A map of the 111 IBM sites in AcMNPV is shown in Fig. 3. The significance of two large regions that lack any IBM (nt 44202-60155 and nt 102616-117071) is not known but the presence of so many potential IE-1 binding sequences is intriguing. It is possible that the formation of bent DNA or cruciform DNA as a result of the specific palindrome sequences within *hrs* or other non-*hr* origins and the binding of IE-1 may be distinguishing signals recognized by the baculovirus replication complex for replication initiation (80, 128).

GENES AND GENE PRODUCTS ESSENTIAL FOR BACULOVIRUS DNA REPLICATION

Genetic analysis of conditional lethal mutants provided the first clues as to which baculovirus genes were essential for DNA replication (48, 108). Physical mapping by marker rescue of two temperature sensitive mutations of AcMNPV (ts8 and tsB821), capable of normal replication at the permissive temperature but defective at the non-permissive temperature, and sequence analysis identified *ie-1* and *p143* and their respective protein products IE-1 and P143, as genes essential for viral DNA replication (90, 130). IE-1 is the major immediate early gene transcription activator (53), while P143 has DNA unwinding (helicase) activity (90, 99). Other potential replication genes including a viral DNA polymerase, and proliferating cell nuclear antigen were identified by amino acid sequence homology with known genes (111, 147).

With the development of the transient replication assay discussed above, it became possible to determine which regions of the genome were capable of supporting plasmid DNA replication in transfected cells. By starting with large regions of the AcMNPV genome cloned into cosmids or plasmids, and then by the process of elimination of unnecessary regions, a minimum set of AcMNPV genes required for plasmid replication was identified (69, 73, 91). The essential AcMNPV genes include *ie-1*, *lef-1*, *lef-2*, *lef-3*, *p143*, and *dnapol*, while genes that seem to stimulate replication depending upon certain assay conditions include *ie-2*, *pe38*, *p35*, and *lef-7* (Fig. 4) (69, 91). Many of these genes were also identified in screens for their ability to stimulate late gene expression and so are referred to as late expression factor (*lef*) genes (146). DNA replication genes are scattered throughout the genome but some clustering of certain genes on the genome can be seen across genera, including the *p143* region and the *dnapol-lef-3* region. Table 1 compiles a list of baculovirus core genes including those involved in AcMNPV replication. Since most of the research on baculovirus replication has been done with AcMNPV, this review will focus on the genes identified in this virus only.

IE-1 (Ac147)

As discussed above, *ie-1* is an immediate early gene, whose product IE-1 is a transcription activator regulating a variety of other viral promoters, including its own, to stimulate virus replication (8, 51, 89, 117, 133, 141). IE-1 also binds as a dimer to *hrs*, suggesting that IE-1 is an initiator, binding to origins of replication (86) and acting as the nucleation site for the formation of the baculovirus replisome (62). Since *hrs* also function as transcription enhancers (50, 52), separable domains within IE-1 have been identified that contribute to both its transcription transactivation and DNA binding functions (115-117). IE-1 expression also contributes to virus-induced apoptosis, while at the same time, initiating the synthesis of proteins required for DNA replication (136). The inclusion of P35 as an essential gene for viral DNA replication may therefore be related to its function as an anti- apoptotic protein to inhibit the induction of apoptosis by IE-1.

LEF-3 (Ac67)

Lef-3 is a delayed early gene, originally shown to have single-strand DNA binding (SSB) activity (56). This activity is required during DNA replication to ensure that double stranded DNA that has been converted to single strands by the DNA unwinding activity of initiators or helicase does not prematurely re-anneal or produce hairpins which would inhibit replication fork movement. This in turn allows the

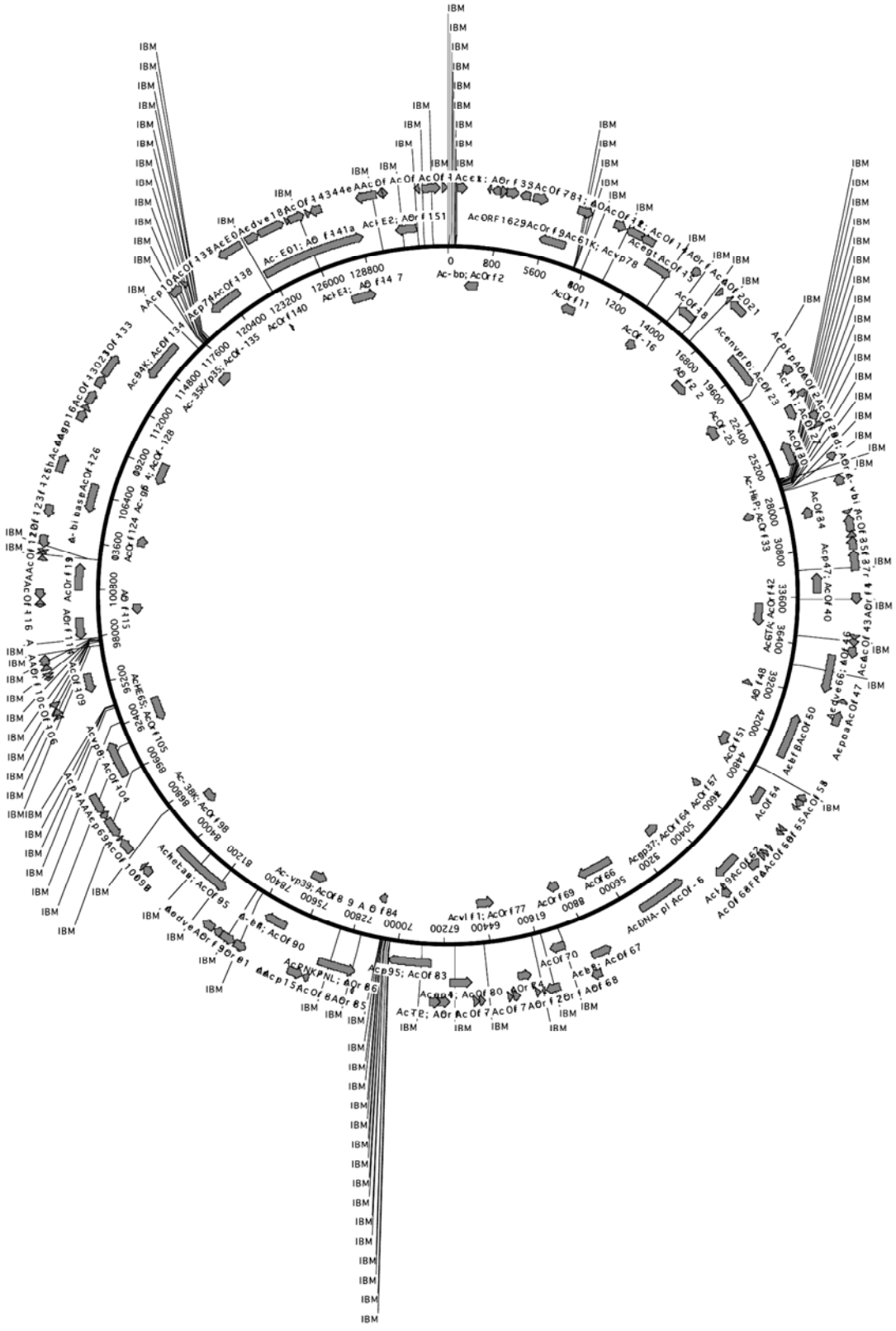

Fig. 3. Location of IE-1 binding motifs (IBM) in AcMNPV. A search of the AcMNPV genome for the consensus IE-1 binding motif (ACBYGTAA) identifies 111 sites. Many of these sites are concentrated within *hrs* but there are many other sites around the genome. Of note is the lack of these motifs in two areas, which are approximately located 180 degrees apart (from position 44205 to 60154 and position 102620 to 117070).

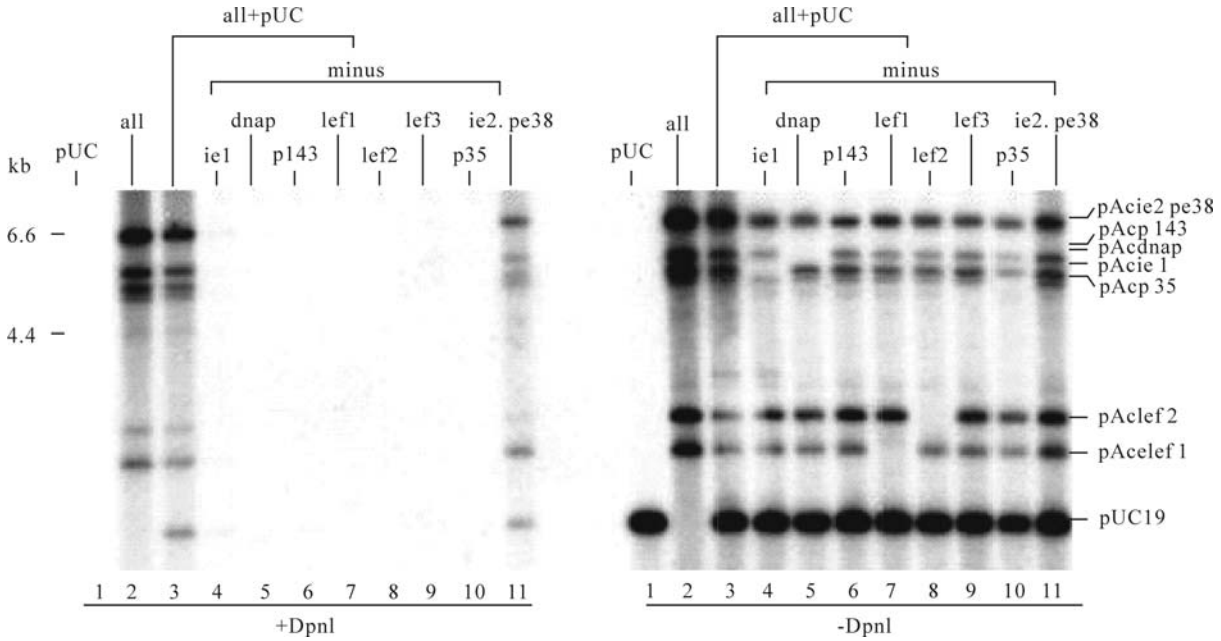

Fig. 4. Transient baculovirus DNA replication assay. Sf21 cells were co-transfected with pUC19 plus eight plasmids each expressing a different baculovirus gene: pAcie1 (ie-1), pAcdnapol (dnap), pAcp143 (p143), pAclef1 (lef1), pAclef2 (lef2), pAclef3 (lef3), pAcp35 (p35) and pAcie2pe38 (ie2,pe38) (lane 3). The assays were also carried out in the absence of one of these plasmids (lanes 4-11). As a control, Sf21 cells were also transfected with pUC19 (lane 1) or pUC19 plus all eight plasmids minus pUC19 (lane 2). Total cellular DNA was purified after 72 hr post transfection, and digested with *Ehe*I only (-DpnI) or with *Ehe*I and *Dpn*I (+DpnI). After agarose gel electrophoresis, the DNA was transferred to a Qiagen nylon membrane and probed with ^{32}P-labelled pUC19 DNA. All plasmids including pUC19 replicated in the presence of all the baculovirus genes (lane 2 and 3) while removing one of the essential genes resulted in no replication (lanes 10). The two genes ie-2 and pe38 appeared to be stimulatory since some replication occurred in their absence (lane 11).

DNA replication machinery to use the single-stranded DNA as template to produce progeny strands, to repair damaged DNA or to function in recombination pathways. SSBs are also likely to guide the formation of protein complexes which carry out these processes (34). LEF-3 has been isolated as a complex with IE-1 and P143 on viral DNA, indicating that it is an important component of the viral replisome (62). Several other functions have been attributed to LEF-3. It interacts with the viral helicase, P143, and is necessary for its nuclear localization (162) but LEF-3 is also required for other essential functions during DNA replication (22). LEF-3 interacts with a viral alkaline nuclease and is important in regulating its function (101, 102). The ability of LEF-3 to form high molecular weight oligomers and the ability of these complexes to facilitate both denaturing and reannealing activities depending on experimental conditions of temperature and redox state suggests that LEF-3 may also be required for homologous recombination during DNA replication (103).

P143 (helicase) (Ac95)

DNA helicases are motor proteins that use the binding and hydrolysis of nucleoside triphosphates, mainly ATP, to unwind double-stranded DNA in preparation for replication, recombination and repair (148). The physical mapping and sequence of the mutation site in a ts mutant that was defective for

DNA replication identified the *p143* gene, named for the predicted mass of its polypeptide product (143 kDa) (48, 90). P143 is a nuclear protein that binds both double-stranded and single-stranded DNA (77, 99). Helicase motifs (now suspected to generally identify nucleic-acid-stimulated NTPases) were identified in P143, leading to the prediction that it was a baculovirus helicase (90). Its ability to hydrolyze ATP and unwind double-stranded DNA has been confirmed (99). Site-directed mutagenesis of conserved amino acids in the ATPase- and DNA-binding domains, implied as conserved helicase motifs, confirmed that they were essential for P143 function in replication, and identifying these as important functions of this enzyme (88). As described above, the nuclear localization of P143, a requirement for replication, is mediated by LEF-3. However, it is not yet clear whether there is a direct interaction between P143 and LEF-3 as a prelude to nuclear transport or if other proteins are involved in this interaction (3, 62).

LEF-1 (Ac14) and LEF-2 (Ac6)

Primases are a class of RNA polymerases necessary for synthesizing short RNA primers on single-stranded DNA templates, providing the 3'-OH group necessary for recognition by DNA polymerase for elongation (40). As such, primase is an essential enzyme in all characterized DNA replication systems. LEF-1 and LEF-2, essential factors in baculovirus transient DNA replication assays, were shown to interact with each other by both yeast two hybrid assays and GST fusion affinity analysis (33). Mutational analysis of a predicted conserved primase motif in LEF-1 (WVVDAD to WVVQAD) resulted in the loss of function in replication assays, supporting the predicted function of this gene product (33). LEF-1 has primase activity, which surprisingly, produced products of hundreds of nucleotides in *in vitro* assays using M13 ssDNA as template (104). LEF-2 may provide an accessory role for more efficient priming by LEF-1 by enhancing its interaction with other proteins in the replisome, including DNAPOL and P143 (helicase). The LEF-1/LEF-2 complex has been recognized as a member of the archaeo-eukaryotic primase (AEP) super-family, included in the AEP proper clade (64).

DNAPOL (Ac65)

DNA polymerases are responsible for the elongation process of DNA replication by recognizing a free 3'-OH group of an RNA or DNA primer and extending it in a 5' to 3' direction, using ssDNA as a template (12). Synthesis on the leading strand can be extended continuously, but relies on the processivity of the enzyme to remain bound to the template. The processivity of many DNA polymerases is enhanced by the action of a protein complex called the sliding clamp which keeps DNA polymerase in close proximity to the DNA template (66). A baculovirus DNA polymerase activity was first detected in infected larvae and later, the gene was identified in the sequence of AcMNPV (107, 147). The baculovirus DNA polymerase has a 3' to 5' exonuclease function that is associated with enhancing the fidelity of polymerase activity (proof-reading) but no detectable 5' to 3' exonuclease activity, which is usually associated with RNA primer removal ahead of the polymerase during lagging strand synthesis (57, 98). DNA replication fidelity is a key determinant of genome stability so it is not unexpected that the baculovirus DNA polymerase maintains 3' to 5' exonuclease activity. The suggestion that it does not carry 5' to 3' exonuclease activity suggests that either

there is no requirement for this function because it is not needed during the process of baculovirus replication, or perhaps another host cell enzyme is used during replication. As mentioned above, DNA polymerases usually require processivity factors but there is no evidence that these are required by the baculovirus polymerase. Even with small amounts of purified enzyme, very long products were synthesized both on singly primed M13 DNA and a poly (dA)-oligo (dT) template (98). This suggests that the baculovirus DNA polymerase is robust and capable of replicating long stretches of leading strand template.

Other AcMNPV genes have been shown to provide a stimulatory role in transient replication assays in some reports (69, 91). However, these genes are not present in many of the sequenced baculovirus genomes, so they may be more specific to AcMNPV replication than to baculoviruses in general. They include *ie-2* (ac151) (present in 13 out of 53 sequenced baculoviruses), *pe38* (ac153) (present in 17 out of 53 sequenced baculoviruses), *p35* (ac135) (present in 8 out of 53 sequenced baculoviruses), *lef-7* (ac125) (present in 22 out of 53 sequenced baculoviruses). *ie-2* and *pe38* are immediate early AcMNPV genes that transactivate delayed early promoters in transient expression assays (14, 75, 76). Mutations in IE-2 or deletion of LEF-7 resulted in delayed DNA replication in a cell-line specific manner, supporting the stimulatory role they play in transient replication assays (21, 127). *pe38* and *ie-2* stimulated expression from the early *p143* gene promoter, suggesting that their enhancing role in replication assays is a result of enhancing the expression of genes required for viral DNA replication (89). The role of P35 in stimulating DNA replication by inhibiting apoptosis has been discussed above. The fact that mutations in the *p35* gene reduce BV titres supports this proposition (24).

BACMIDS AND BACULOVIRUS REPLICATION

Bacmids (a baculovirus genome carrying independent origins for replication in either bacteria or insect cells) were originally developed as a shuttle vector system to prepare recombinant baculoviruses in *E. coli* prior to transfection into insect cells (92). The system takes advantage of the site-specific transposition properties of the Tn7 transposon to simplify and enhance the process of generating recombinant bacmid DNA. This system can be modified to investigate any baculovirus gene by replacing authentic baculovirus genes with a selectable marker, suitable for screening in *E. coli*. The phenotype of the knockout virus can then be investigated by transfecting knockout bacmid DNA, purified from bacteria, into insect cells and studying the resulting infectious process. Knockout bacmids are generated in *E. coli* by replacing the target viral gene with an antibiotic resistance gene, using a lambda phage Red recombinase system expressed from a plasmid (29). A number of baculovirus genes have now be studied using this technology, including several genes involved in viral DNA replication (87, 113, 114, 151-153, 165) These studies support the essential role of DNAPOL in baculovirus replication as shown by transient replication assays, but also identified other genes that appear to play a role, *in vivo*, in normal DNA replication (including processing or packaging). These include DNA binding protein (DBP), alkaline nuclease (AN), ME53, and LEF-11 (87, 113, 151, 165).

DBP (Ac25)

A second baculovirus single-strand DNA binding protein was first identified in BmNPV-infected BmN cells (100). DBP binds to both ss- and ds-DNA, and when bound, protects DNA against exonuclease activity. When DBP was deleted in a recombinant bacmid, viral DNA replication was reduced but not eliminated and produced less than full genomic length products (151). This defect in producing normal genomic DNA correlated with a defect in virion assembly. DBP shares some properties with LEF-3, but has a greater affinity for subnuclear structures that may be important for late processing of replicative intermediate DNA within the replication factories of the virogenic stroma (105). The data indicate that DBP is an essential gene for production of infectious virus and the virogenic stroma but not essential for viral DNA replication (151).

AN (Ac133)

A knockout bacmid of the baculovirus alkaline nuclease (*an*) gene replicated normal amounts of viral DNA but was not capable of producing normal genomic length DNA (113). This phenotype is similar to that of the DBP-knockout. Since members of the alkaline nuclease family are involved in repair of dsDNA breaks and homologous recombination (2), the baculovirus AN may be required at a late stage of DNA processing, after the majority of the genome has been replicated and before the viral DNA is packaged into nucleocapsids. AN forms a complex with LEF-3, so the combined activities of this complex including binding ssDNA and recombination may facilitate the processing of replicative intermediates produced by initiation at multiple origins of replication or of branched intermediates produced by strand invasion and replication (101, 102). The interaction of AN with the ssDNA-binding protein LEF-3 may be required for the late processing of replicated DNA during DNA packaging into preformed nucleocapsids.

LEF-11 (Ac37)

LEF-11 is required in transient transcription assays for the stimulation of late and very late genes but is not required for DNA replication in these assays (91, 146). Thus, it was surprising when a LEF-11 knockout bacmid was demonstrated to be defective in DNA replication (87). Since no amino acid homology to any known protein has been identified, the role of LEF-11 during baculovirus DNA replication remains to be elucidated.

ME53 (Ac139)

Unlike the other immediate early transcribed genes *ie-1*, *ie-0*, *ie-2* and *pe38*, *me53* is not essential in transient replication or transcription assays but is essential *in vivo* for DNA replication (67, 165). ME53 has a potential zinc finger motif that might be important in sequence-specific recognition of DNA but since no amino acid homology to any known protein has been identified, the role that ME53 plays during baculovirus DNA replication remains to be elucidated.

MECHANISM OF BACULOVIRUS DNA REPLICATION

As discussed above, much progress has been made in identifying the viral genes required for AcMNPV DNA replication, and investigating their function during this process. However, it is still not clear how these genes interact with the viral genome to complete the replication process. Little is known about the mechanism of baculovirus replication or the structure of the replication intermediates. In most models of DNA replication, origins of replication are recognized

by the initiator complex that partially melts the ds-DNA. This acts as a nucleation site for formation of a pre-replicative complex (pre-RC) that includes a helicase which further unwinds the doubled stranded DNA. A DNA polymerase-primase complex is recruited to the initiation site to allow the formation of the replisome, which then extends replication forks either bidirectionally or unidirectionally until advancing replication forks from adjacent replicons merge (166).

Because baculoviruses have circular genomes, replication may initially proceed by a theta structure, similar to other circular genomes such as *E. coli,* papillomaviruses or as in the circularized herpes virus genome (9, 35, 129, 138, 158). Replication would initiate at an origin, and then two replication forks would act bidirectionally to either replicate the entire genome, or, because there are multiple predicted baculovirus origins (*hrs*, early promoters, non-*hr* origins), to merge with on-coming replication forks from adjacent replicons. This model would predict circular replicative intermediates (RIs) or perhaps 'onion-skin' RIs with multiple copies of regions including the origin of replication, depending on the efficiency of the origin recognition and firing (95). The selective activation (rereplication) or repression of origins of replication appears to be common in insects and may lead to the polyploidy seen in many insect tissues and cells in culture (43, 44). Duplication of baculovirus sequences is common, and in many cases, has involved reiteration of sequences adjoining origins of replication including the variable number of repeat sequences in *hrs*, direct repeats, multiple copies of genome segments in defective genomes, and other duplications adjacent to *hrs* or non-*hr* origin regions including baculovirus repeated open reading frame (*bro*) genes (7, 16, 71, 79, 124). These types of variation have likely been generated as a result of the baculovirus replication mechanism.

However, most of the experimental evidence generated to date suggests that baculovirus DNA replication includes a rolling circle mechanism. This has been suggested by the structural characteristics of replicated plasmids in AcMNPV infected cells (85, 164). Partial restriction enzyme digestion of intracellular replicated plasmid DNA with a single cutter enzyme reveals ladders of multiples of unit-length plasmid DNA, indicating the presence of head to tail concatemer plasmid DNA synthesized by the baculovirus replication machinery. These data are consistent with a rolling circle mechanism of replication resulting in long linear segments of DNA consisting of multiple copies of the template DNA. A similar conclusion was reached when replicative intermediates of AcMNPV were analyzed by field inversion gel electrophoresis (113, 118, 151, 154). Of the viral DNA that was not trapped in the well, partial digestion with an enzyme with a single restriction in the AcMNPV genome revealed a banding pattern of genome length multimers, suggesting that viral DNA replication occurred by a rolling circle mechanism.

Replicating plasmid DNA in co-transfected cells also results in plasmid DNA becoming integrated into the viral genome, at multiple locations with no sequence specificity (164). This plasmid DNA persists in this form in budded virions over several passages. The presence of integrated plasmid DNA suggests that recombination may also be involved in baculovirus DNA replication. High frequency recombination is a hallmark of baculovirus infections, as clearly demonstrated by the ease with which recombinant viruses can

be generated by marker rescue of ts mutants (15, 48, 106), or in the generation of recombinant baculoviruses for foreign gene expression (140). These recombination events are dependent upon active virus DNA replication, suggesting recombination may be a component of the replication mechanism (28, 97, 164). Evidence to support recombination-dependent DNA replication mechanism also comes from the characterization of the baculovirus alkaline nuclease gene, a member of a family of enzymes involved in DNA repair and recombination that includes the phage lambda exonuclease (Red alpha) and the herpes virus alkaline exonuclease (2, 10, 125). Deletion of the AN gene does not reduce the level of viral DNA replication, indicating that AN is not essential for the process of replication (113). However, there is evidence that the products of AN-knockout bacmid replication are subgenomic in size, and may consist of unresolved branched molecules which also can not be properly packaged into new virions. Recombination might also be a mechanism for the acquisition of genes from other co-infecting viruses or their insect hosts during baculovirus evolution and may account for the diversity of gene content between baculovirus species (58, 60, 63, 78).

There is evidence that baculovirus replication and transcription are coupled. IE-1 is essential for both transactivating transcription of other viral genes, and for DNA replication (121, 136, 141). Early promoters can function as origins of DNA replication, at least in transient assays (163) and induction of late gene transcription is dependent upon viral DNA replication (48, 61, 131, 144). Immediate early gene expression is critical for the induction of the viral genes required for viral DNA replication, the delayed early genes, but the specific role that transcription plays in the initiation of viral DNA replication has not been identified. Genes that comprise the viral-specific RNA polymerase (*p47*, *lef-4*, *lef-8* and *lef-9*) are essential for late gene expression, but are not required for viral DNA synthesis (15, 17, 119). Therefore, the induction of expression of these RNA polymerase genes is concurrent with the initiation of viral DNA replication. Whether there is a direct action during the process of DNA replication that stimulates late gene expression or it is simply an increase in template copy number coupled to the presence of other late expression factors that leads to the switch from early to late gene expression remains to be determined.

Nothing is known of how specific origins from the multiple possible origins might be selected. None of the AcMNPV *hrs* are essential for DNA replication (20, 132). Deleting two hrs which depletes a large stretch of the genome of any *hr* (about 84 kbp) has a minimal effective on DNA replication (20). It is possible that only one origin is recognized and used once per round of replication, perhaps because at the time of origin initiation, there are limited supplies of the necessary viral gene products. For example, P143 helicase is present at very low amounts in infected cells (77). The initiation at only one origin, whether it is an *hr*, the non-*hr* origin, or an early promoter could still lead to bidirectional replication so if the major process is rolling circle replication leading to head to tail concatameric progeny DNA, how the switch from theta bidirectional to unidirectional rolling circle replication might be regulated is not known. There is also no information on how the initial replication primer is synthesized. Since a ts mutation in IE-1 leads to a delay in DNA replication, it would be

interesting to know what the constraints are in this situation. Once replication has started, it is possible that intermediates of recombination could be used as DNA primers, leading to branched replicative intermediates. Baculovirus replication has been compared with that of herpes virus, another large DNA virus where after the linear genome is circularized soon after infection, it is postulated that there is an initial phase of origin-dependent replication, followed by a phase of origin-independent rolling circle replication, which would produce the concatemeric intermediates (82, 134). However, it has also been proposed that the branched structure of the replication intermediates in HSV-1 could be due to a combination of rolling circle replication and recombination, or to continuous reiniti- ation at the origins producing multiple replication forks (Y branches), or to a mechanism of replication similar to that of the T4 phage, where strand invasion during recombination generates a new origin of replication (96, 109, 135, 139). Definitive proof of any of the proposed replication mechanisms has yet to be established, but continued research into baculovirus replication will certainly provide insights into these concepts and hypotheses.

CONCLUSIONS

The availability of many baculovirus genomic sequences, coupled with rapid progress in identifying genes essential for viral DNA replication has lead to the description of core baculovirus genes including those essential for viral DNA replication. DNA replication requires a set of functionally conserved proteins and pathways. However, because of the distinctive properties of baculoviruses, including their unique and specific host ranges, it is probable that they have evolved ways of exploiting the host backgrounds to efficiently duplicate their genomes in ways different than other systems. There is still much to learn about baculovirus replication. Many enzymatic activities and structures that are thought essential for DNA replication have not yet been identified for baculoviruses. These include ligases to join free ends of DNA, topoisomerases to unknot DNA unwound by helicase or insert DNA during recombination, RNAses for primer removal, sliding clamps and alternative DNA polymerases acting on the leading and lagging strands, enzymes for recognition and cleaving concatemers to regenerate genome length circular molecules, DNA packaging signals, as well as those activities involved in recom- bination. The baculovirus model system can provide a view of processes modified for the purpose of virus survival. This may also provide an insight into the possibilities of mechanisms that occur within their host insect species leading to new concepts in the continuing development of baculoviruses as expres- sion systems, biological pesticides, and vectors for gene therapy in mammalian hosts.

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