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Baculovirus DNA Replication

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The *Baculoviridae* are a diverse family of viruses pathogenic for arthropods, particularly insects of the order Lepidoptera (Blissard and Rohrmann 1990). They are characterized by large, rod-shaped virions that contain double-stranded, supercoiled DNA genomes ranging in size from 88 kb to more than 160 kb, depending on the viral strain. The virions are enclosed in large proteinaceous structures known as occlusion bodies, which function to protect virions and allow them to remain viable for extended periods. Baculoviruses are divided into two genera on the basis of occlusion body morphology; the nuclear polyhedrosis viruses (NPVs) are characterized by many virions present in each polyhedron-shaped occlusion body, whereas the granulosis viruses (GVs) normally have a single virion in much smaller occlusion bodies. Baculoviruses have drawn widespread interest due to their remarkable ability to overexpress heterologous genes under the control of the strong polyhedrin gene promoter (Smith et al. 1983; Pennock et al. 1984). They are also being investigated for incorporation into insect pest management programs as alternatives to chemical insecticides (Leisy and van Beek 1992).

The best-characterized baculovirus, the *Autographa californica* multinucleocapsid NPV (AcMNPV), has a genome of 134 kb and is estimated to contain 154 genes (Fig. 1) (Ayres et al. 1994). A distinctive feature of the AcMNPV genome is the presence of eight homologous regions (*hr*s) composed of multiple repeated sequences that are dispersed throughout the genome (Cochran and Faulkner 1983; Guarino et al. 1986; Guarino and Summers 1986b; Ayres et al. 1994). *hr*s, which are prominently featured in this review, have been shown to act as enhancers of baculovirus early gene expression and are implicated as origins of baculovirus DNA replication.

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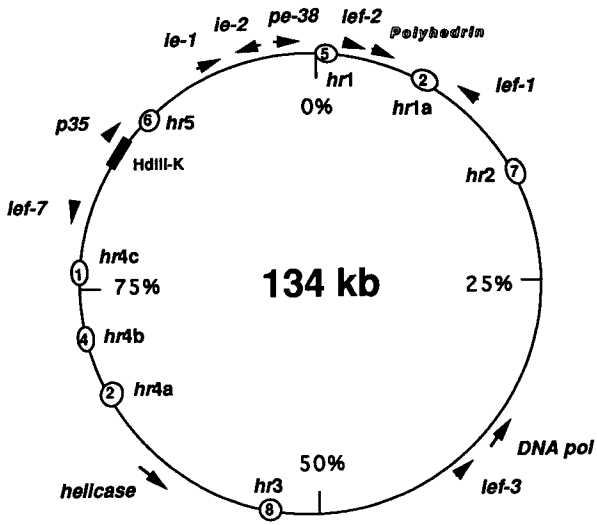


Figure 1 Location of homologous regions and genes involved in DNA replication on the AcMNPV genome. The polyhedrin gene is shown as a point of reference. The numbers within the circles indicate the number of repeated sequences within each *hr*. The arrows indicate the location and transcriptional direction of the genes involved in DNA replication. The location of the non-*hr* putative replication origin (HdIII-K) is indicated. Data for this figure are from Ayres et al. (1994).

Baculovirus genes are expressed in a transcriptional cascade in which each phase is dependent on the expression of genes during the previous phase. They can be divided into the general categories of early genes, which are transcribed by the host RNA polymerase II (Fuchs et al. 1983; Huh and Weaver 1990; Hoopes and Rohrmann 1991), and late genes, which are transcribed by a virus-specific RNA polymerase with a unique subunit composition (Yang et al. 1991). This polymerase is resistant to α-amanitin (an RNA pol II toxin) (Grula et al. 1981) and tagetitoxin (an RNA pol III toxin) (Glocker et al. 1993) and initiates transcription from within a 5-bp late promoter element with the sequence A/G/T TAAG (Blissard and Rohrmann 1990). Late gene expression is dependent on viral DNA replication and is not observed when DNA replication is inhibited (e.g., by aphidicolin) (Friesen and Miller 1986; Rice and Miller 1986–1987). Most late genes are transcribed after the onset of DNA replication with the levels of expression declining at later times after infection. However, expression of two genes termed "very late genes" reaches high levels very late in the infection. These genes are the polyhedrin gene, which encodes the major occlusion body protein, and

p10, which encodes a small poorly conserved protein that may be involved in occlusion body formation or cell lysis (van Oers et al. 1993; Gross et al. 1994).

Recently, a number of advances have been made in the characterization of the replication of baculovirus DNA, including the identification of putative replication origins and the development of assays that allowed the identification of the genes involved in DNA replication. In this review we summarize these data; a more extensive review is also available (Kool et al. 1995).

BACULOVIRUS REPLICATION ORIGINS

Two different strategies have been used to identify potential baculovirus replication origins. One employed the generation of defective interfering (DI) virus particles, which contain major genomic deletions but retain essential *cis*-acting sequences for DNA replication, and the other approach relied on testing the ability of cloned baculovirus DNA sequences to undergo DNA replication when transfected into infected insect cells.

Defective Genomes

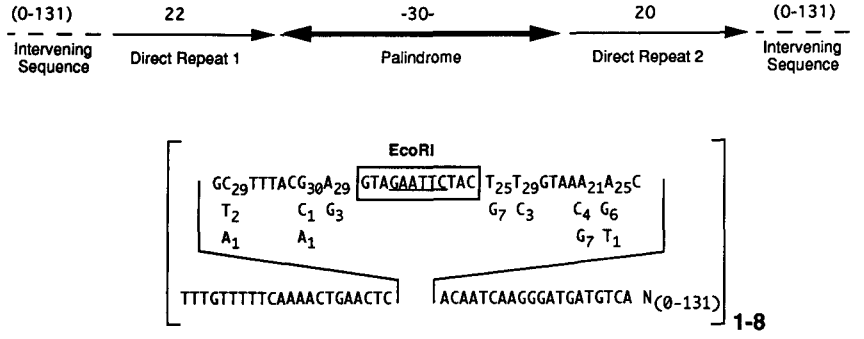
After undiluted serial passaging of AcMNPV, DI particles containing viral genomes with major deletions have been observed. Restriction enzyme analysis of viral DNA isolated after 40 serial passages showed the presence of seven supermolar *Eco*RI fragments (Kool et al. 1993a) that hybridized to DNA sequences flanking *hr*s. This suggested that the *hr*s were selectively retained in the defective genomes. In similar studies (Lee and Krell 1992, 1994), defective genomes analyzed after 81 passages were found to be heterogeneous in size with the majority migrating at about 50 kbp as determined by pulsed-field gel electrophoresis. In contrast to the results from Kool et al. (1993a), these defective genomes appeared to retain multiple repeats of a non-*hr*-containing sequence of less than 2.8 kb derived from the *Hin*dIII-K region of the parent AcMNPV genome (Fig. 1). The identification of different putative replication origins in these two studies suggests that both *hr* and non-*hr* sequences may act as replication origins in vivo.

hr Sequences as Baculovirus Replication Origins

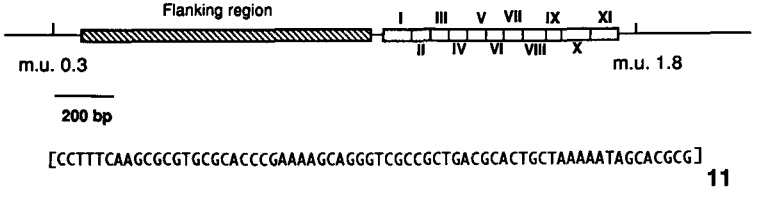
AcMNPV contains eight homologous regions (*hr*1, *hr*1a, *hr*2, *hr*3, *hr*4a, *hr*4b, *hr*4c, and *hr*5) (Fig. 1) that vary in size from about 0.2 kb to 1.0 kb. Each *hr* is composed of one to eight repeats of a highly conserved 72-bp

sequence element, which contains a 30-bp imperfect palindrome near its center (Fig. 2a). If converted to a cruciform structure, these palindromes would each have two mismatched regions outside a highly conserved 12-

a) AcMNPV



b) OpMNPV



c) LdMNPV

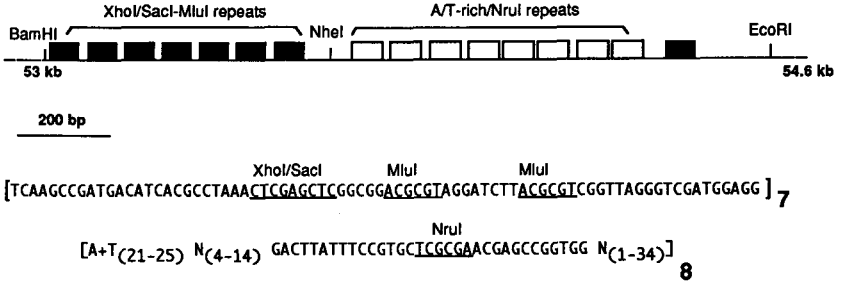


Figure 2 (See facing page for legend.)

bp core sequence containing an *Eco*RI site at its center. Individual repeats are separated by intervening sequences 0–131 nucleotides in length (Fig. 2a).

A transient assay employing *Dpn*I (Peden et al. 1980) has been used to examine infection-dependent replication of plasmids containing baculovirus DNA after their transfection into virus-infected insect cells (Pearson et al. 1992; Kool et al. 1993a). When transfected into AcMNPV-infected *Spodoptera frugiperda* cells, individual plasmids containing different *hr*s demonstrated the ability to replicate (Kool et al. 1993b; Leisy and Rohrmann 1993). (An *hr* with only one palindrome and flanking sequences, *hr*4c [Fig. 1], has also been reported [Ayres et al. 1994], but it lacks an *Eco*RI site and has not been functionally characterized.) Although the relative levels of replication for different *hr*s appear to be independent of the number of palindromes (e.g., *hr*4b, which contains four palindromes, replicated to a higher level than *hr*3, which contains eight palindromes) (Leisy and Rohrmann 1993), deletion mutagene-

Figure 2 Sequence organization of putative replication origins from three baculoviruses. (*a*) AcMNPV origin. A schematic representation of the general architecture of an *hr* is shown at the top. Below is shown the highly conserved 72-bp sequence element, which is present in 1–8 copies in the different homologous regions. The 30-bp palindrome is depicted above a consensus sequence of the conserved direct repeats found in *hr*s. The highly conserved 12-bp core sequence is boxed and the *Eco*RI site is underlined. The frequencies of the nucleotides in the variable positions of the palindrome (representative of 32 of the 35 palindromes) are shown as subscripts below each nucleotide. The letter N followed by subscripts indicates the length of the intervening sequences that are found between the repeats in different homologous regions. The consensus sequence for the repeated region flanking the palindrome is derived from 20 of the most conserved sequences. (*b*) OpMNPV *hr*-like origin. A schematic diagram showing the location of an origin containing a repeated element and essential flanking sequences. The efficiency of replication is dependent on the length of the flanking sequences (Ahrens et al. 1995b). The consensus sequence of the repeated region is shown below the diagram (Theilmann and Stewart 1992). (*c*) LdMNPV origin. A schematic diagram of an origin containing two repeated domains. This origin is composed of a domain containing seven repeats with overlapping *Xho*I/*Sac*I sites (*black boxes*), and *Mlu*I sites linked to a second domain containing eight repeats of an AT-rich sequence linked to a sequence containing a *Nru*I site (*open boxes*) (Pearson and Rohrmann 1995). A consensus sequence of these two domains is shown below the diagram. The letter N, followed by subscripts, indicates the nucleotides separating internal regions or separating the repeated consensus sequences.

sis of *hr*5, which contains six palindromes, indicated that the efficiency of replication was correlated with the number of palindromes present (Pearson et al. 1992).

Sequences similar to AcMNPV *hr*s have been found in a number of other baculoviruses, including *Bombyx mori* NPV, a close relative of AcMNPV (Maeda and Majima 1990; Majima et al. 1993), and *Choristoneura fumiferana* MNPV (CfMNPV) (Arif and Doerfler 1984; Kuzio and Faulkner 1984), which is less related to AcMNPV but has at least one set of repeated palindromes, some of which are more than 75% identical to AcMNPV palindromes (Xie et al. 1995). The genome of the *Orgyia pseudotsugata* MNPV (OpMNPV) was shown to contain five homologous regions by DNA-DNA hybridization. One OpMNPV *hr* has been characterized and found to act as an enhancer of early gene transcription (Theilmann and Stewart 1992) and to function as a replication origin in transient assays when linked to flanking sequences (Ahrens et al. 1995b). However, it lacks well-defined palindromes (Fig. 2b) and has only 50% sequence identity to AcMNPV *hr* sequences. The *Lymantria dispar* MNPV (LdMNPV), which is more distantly related to AcMNPV than BmNPV, CfMNPV, and OpMNPV, contains *hr*s located in eight regions of its genome (Pearson and Rohrmann 1995). Two of the *hr*s have been characterized and are composed of repeats of about 80 bp that include a series of palindromes unrelated to AcMNPV palindromes. Plasmids containing these sequences alone replicate poorly. However, LdMNPV *hr*4 is adjacent to a second domain composed of eight partially repeated sequences of 60–100 bp, each containing a 15- to 25-bp AT-rich sequence and a 6- to 10-bp palindrome containing an *Nru*I site (Fig. 2c). In combination, the two domains cause plasmids to replicate to high levels in LdMNPV-infected cells (Pearson and Rohrmann 1995).

Non-*hr*-containing Origins

In addition to *hr* sequences that may function as origins of DNA replication, two other non-*hr* putative origins have been characterized. The AcMNPV *Hin*dIII-K fragment, which lacks sequences closely related to AcMNPV *hr*s (Kool et al. 1994a), was initially implicated as a replication origin by the studies involving the generation of DI virus particles (Fig. 1) (see above). Subsequently, it was shown to replicate in an infection-dependent replication assay (Kool et al. 1993b; Lee and Krell 1994), although at less than 20% the level of *hr*-containing plasmids (Leisy and Rohrmann 1993). Similarily, the OpMNPV *Hin*dIII-N fragment (Pearson et al. 1993), which lacks *hr*-related sequences, was shown

to replicate at significant levels. However, *Hin*dIII-N replicates at only about 25% the efficiency of the OpMNPV *hr*-containing sequence (Ahrens et al. 1995b). Investigations of non-*hr*-containing replication origins from AcMNPV and other baculoviruses are not complete, and it is likely that a number of other such regions occur.

Form of Transiently Replicated Plasmid DNA

A circular topology is a prerequisite for transient replication of origin-containing plasmids (Kool et al. 1993b). Linearized origin-containing plasmids were not replicated. The *hr*-containing plasmid DNA replicated in AcMNPV-infected cells was shown to be of high molecular weight, suggesting that replication does not lead to the production of an exact replica of the input circular plasmid DNA. Partial digestion of the replicated DNA with a restriction enzyme that cut the input plasmid at a unique site led to the production of a "stepladder" pattern of fragments, consistent with the DNA being organized as a linear concatemer containing multiple copies of the plasmid (Leisy and Rohrmann 1993). Although the form of replicating genomic DNA in infected cells has not been described, a linear concatemeric structure of origin-containing plasmids may indicate that viral DNA replicates via a rolling circle. Defective genomes consisting of concatemers of sequences from the *Hin*dIII-K fragment (Lee and Krell 1992, 1994) also support a rolling-circle model for baculovirus DNA replication. With such a model, a single replication initiation event would lead to the production of multiple copies of the genome. The mechanism by which such a structure might be resolved into unit-length, circular genome segments is not known, but it could involve cleavage and religation to form monomeric circles before or during packaging.

Possible Role of Multiple Replication Origins

Eight putative origins have been identified in the AcMNPV genome. The role these origins play in viral DNA replication, and whether they are active simultaneously, is not clear. Deletion of *hr*5 from the AcMNPV or BmNPV genome had no apparent effect on the replication of these viruses (Rodems and Friesen 1993; Majima et al. 1993). Subsequently, Wu and Carstens (1995) showed that deletion of other individual *hr*s had no effect on replication of AcMNPV in cell culture. It is possible that the presence of multiple *hr* sequences in the AcMNPV genome may reflect their role as transcriptional enhancers of early genes rather than their role

in replication. The accelerated expression of early genes may be essential for the successful establishment of an infection. In contrast, only a single origin may be required per replication initiation event, and rolling-circle replication could lead to the production of multiple genomes. If factors required for the initiation of replication are limiting, the formation of an initiation complex may be rare and may occur only once on each DNA molecule. The selection of which origin initiates replication may occur at random. In such circumstances, functionally redundant origins may increase the probability of the formation of a functional preinitiation complex and thus increase the speed of the infection cycle. The limitation of replication factors would reduce the likelihood of multiple initiations on a single genome, which could lead to abortive replication via the production of complex branched structures that may not yield viable genomes.

GENES INVOLVED IN BACULOVIRUS DNA REPLICATION

A replication assay similar to that employed for the identification of the herpes simplex virus I replication genes (Challberg 1986) was developed for the identification of baculovirus replication genes. A set of overlapping cosmid clones encompassing the complete genome was transfected into uninfected insect cells along with a reporter plasmid containing a putative replication origin. A minimal set of cosmids was identified that replicated the plasmid DNA, and the specific genes were determined using subclones of the essential cosmids. This protocol was used to identify both essential and stimulatory genes for transient DNA replication of two baculoviruses, AcMNPV (Kool et al. 1994c; Lu and Miller 1995) and OpMNPV (Ahrens and Rohrmann 1995a,b; Ahrens et al. 1995a). The genes that have been implicated in DNA replication are described below and are summarized for AcMNPV in Table 1. Their location on the AcMNPV genome is shown in Figure 1.

DNA polymerase

In early studies (Kelly and Lescott 1981; Miller et al. 1981; Kelly 1982), it was noted that a new DNA polymerase activity was induced in baculovirus-infected cells. A $3' \rightarrow 5'$ exonuclease activity specific for single-stranded DNA was found to be tightly associated with *B. mori* NPV DNA polymerase (Mikhailov et al. 1986). Subsequently, the location and sequence of genes from six different baculoviruses that encode predicted proteins of about 115 kD and that contain motifs conserved among a number of DNA polymerases were determined (Tomalski et al.

Table 1 AcMNPV replication genes

Gene	MW[a]	Function(s)	Essential(E) Stimulatory (S)
DNA pol	114.3		E/S
helicase	143.2		E
ie-1	66.9	transcriptional activator binds *hr* sequences	E
lef-1	30.8		E
lef-2	23.9		E
lef-3	44.6	SSB[b]	E
p35[c]	34.8	inhibits apoptosis transcriptional activator	E/S
ie-2	47.0	transcriptional activator	S
pe-38	37.4	transcriptional activator	S
lef-7	26.6		S

For details see text.
[a]Molecular weights of predicted proteins are from Ayres et al. (1994).
[b]Hang et al. (1995).
[c]*p35* is not present in OpMNPV.

1988; Bjornson et al. 1992; Cowan et al. 1994; Chaeychomsri et al. 1995; Liu and Carstens 1995; Ahrens and Rohrmann 1996). Although baculovirus DNA polymerase genes were shown to be essential for DNA replication in two studies (Pearson et al. 1993; Kool et al. 1994c), in another study (Lu and Miller 1995), low levels of replication activity were observed in the absence of the baculovirus DNA polymerase gene. This suggests that under differing assay conditions a host DNA polymerase may act in combination with the other essential baculovirus replication genes to replicate plasmid DNA.

helicase

A baculovirus gene with limited homology to helicases was originally identified by sequencing an ORF containing a temperature-sensitive mutation that resulted in virus defective for DNA synthesis (Lu and Carstens 1991). This gene encodes a predicted protein of 143 kD that contains a variety of amino acid motifs common to a number of helicases, including NTP-binding and DNA/RNA-unwinding motifs. In addition, sequences in the helicase protein that are involved in specifying host range have been identified (Maeda et al. 1993; Croizier et al. 1994), which could indicate that this region might require association with a host protein in order to function.

ie-1

Immediate early gene 1 (*ie-1*) is the only baculovirus gene to date for which both spliced and unspliced transcripts have been identified (Chisholm and Henner 1988). The spliced forms produce a protein product with an additional 54 amino acids at the amino terminus. Plasmids expressing unspliced *ie-1* have been shown to be essential for baculovirus DNA replication (Kool et al. 1994c; Ahrens and Rohrmann 1995b; Lu and Miller 1995). Deletion analysis of AcMNPV *ie-1* indicates the presence of distinct transcriptional-activation and DNA-binding domains (Kovacs et al. 1992).

ie-1 has been shown to activate a variety of baculovirus early gene promoter-reporter constructs when they are co-transfected into uninfected insect cells (Guarino and Summers 1986a; Blissard and Rohrmann 1991; Lu and Carstens 1993; Rodems and Friesen 1993), and this activation is greatly enhanced when these constructs are linked to *hr* sequences. Extracts from *S. frugiperda* cells transfected with *ie-1* and IE-1 produced in in vitro transcription-translation reactions cause retardation of *hr*-containing DNA in gel shift assays (Choi and Guarino 1995).

In the baculovirus system, *ie-1* may be essential because it activates the expression of the other replication genes to levels sufficient to produce detectable amounts of replicated DNA. Alternatively, it may be directly involved in DNA replication by binding to an origin and catalyzing early steps that lead to the assembly of a replication complex.

lef-1, lef-2, lef-3

Whereas information on possible functions of *ie-1* and the DNA polymerase and helicase genes is available because of functional studies or their homologies with well-characterized genes from other organisms, little is known about three of the essential replication genes called late expression factors (*lef-1, 2,* and *3*). These genes were originally implicated as being essential for late gene expression (Todd et al. 1995). However, this may have been due to the dependence of the late gene expression assay on DNA replication. Several investigations indicate that these genes are directly involved in DNA replication of both AcMNPV and OpMNPV (Kool et al. 1994c; Ahrens and Rohrmann 1995a,b; Ahrens et al. 1995a; Lu and Miller 1995). Yeast two-hybrid analysis indicates that LEF-1 and LEF-2 form hetero-oligomers (Leisy and Rohrmann 1995). A motif found in single-stranded DNA-binding proteins is present in OpMNPV and AcMNPV LEF-3 (Ahrens et al. 1995a), and AcMNPV LEF-3 has been shown to bind to single-stranded DNA agarose columns

with high affinity and elute only at salt concentrations greater than 0.9 M (Hang et al. 1995).

p35

The AcMNPV *p35* gene was found to greatly stimulate replication in one study (Kool et al. 1994c), but using different assay conditions, other investigators suggest that it is essential for replication (Lu and Miller 1995). *p35* is an inhibitor of AcMNPV-induced apoptosis in *S. frugiperda* cells (Clem et al. 1991) and may also act as a transcriptional activator of early genes (Gong and Guarino 1994). It is possible that the direct effect of *p35* in transient replication assays through transcriptional activation is minor in comparison to the elevation of the replication signal due to inhibition of apoptosis, which would prevent the cells from dying. This theory is supported by the fact that OpMNPV, which shows a high degree of genome similarity to AcMNPV, lacks a homolog of the *p35* gene (Gombart et al. 1989). However, OpMNPV encodes a functional analog of *p35*, called Op-*iap* (*i*nhibitor of *ap*optosis), which bears no sequence identity with p35 but is able to inhibit AcMNPV-induced apoptosis in Sf-9 cells (Birnbaum et al. 1994). A related gene (Cp-*iap*) with similar properties is found in the genome of the *Cydia pomonella* GV (Crook et al. 1993). Both these genes can substitute for the *p35* gene in replication assays employing the other AcMNPV replication genes (Lu and Miller 1995). If apoptosis is induced by the replication of the transfected DNA, it would suggest that the cell has a mechanism that discriminates between normal chromosomal DNA replication and replication of foreign DNA or DNA replication not linked to the cell cycle. Alternatively, expression of one or more of the genes involved in DNA replication may directly induce apoptosis.

ie-2 and *pe-38*

Efficient DNA replication in the transient assay system requires additional genes that stimulate replication. Two of the stimulatory genes, *ie-2* (previously called *ie-n*) (Carson et al. 1988) and *pe-38* (Krappa and Knebel-Mörsdorf 1991), encode *trans*-activators of early gene transcription. In particular, *pe-38* has been shown to activate expression of the baculovirus *helicase* homolog. Furthermore, *ie-2* has been shown to stimulate *pe-38* expression (Lu and Carstens 1993) and *ie-1* expression (Yoo and Guarino 1994). *ie-2* and *pe-38* may selectively stimulate certain of the replication genes to higher levels than are necessary for minimal detection.

lef-7

lef-7 was first shown to stimulate the expression of a CAT construct under the control of either the p39-capsid or polyhedrin promoters (Morris et al. 1994) and was subsequently determined to stimulate DNA replication (Lu and Miller 1995).

pcna

The AcMNPV genome contains a gene with 42% amino acid sequence identity to rat proliferating cell nuclear antigen (PCNA) (Crawford and Miller 1988; O'Reilly et al. 1989), which is an essential component of several eukaryotic replication systems (Kornberg and Baker 1992). However, this gene is lacking in the closely related BmNPV genome (Gomi et al. 1994), and it has no effect on transient DNA replication (Kool et al. 1994c). Therefore, the role this gene plays in AcMNPV biology is not clear.

TRANSIENT REPLICATION OF DNA IN TRANSFECTED CELLS IS ORIGIN-INDEPENDENT

Although plasmid replication in virus-infected cells is sequence-dependent, replication becomes viral origin-independent when transfected naked DNA, instead of intact virions, is used to supply essential *trans*-acting factors. Plasmids containing the most efficient origins, as determined by the replication assay employing virus-infected cells, still show the strongest replication signals in transfected cells; however, other plasmids (including those lacking inserts) replicate to detectable levels (Kool et al. 1994b,c). In other eukaryotic systems, chromatin structure represses initiation of both transcription and replication (Wolffe 1991). Although the organization of DNA-binding proteins associated with baculovirus genomes is unclear, there is evidence for the association of a small, very basic protein with baculovirus DNA in virions (Wilson et al. 1987). If baculovirus DNA is packaged in a chromatin-like structure, transfection of DNA lacking these proteins may result in the deregulation and high-level expression of the genes involved in replication. High gene copy number may also play a role in origin-independent replication. Cells infected with virus at low moi on average contain only one to a few copies of each gene. In contrast, cells transfected with cloned viral replication genes contain much higher gene doses, potentially resulting in an overabundance of replication proteins, which may cause saturation of specific origin sequences by these proteins. The excess protein molecules

may bind to DNA sequences nonspecifically and, hence, cause initiation of replication from any plasmid. High levels of recombination in transfected cells could also influence these observations.

SPECIFICITY OF BACULOVIRUS DNA REPLICATION

As indicated above, some baculoviruses contain several putative replication origins that may differ in structure within the same virus and between viruses. The ability of two different baculoviruses to replicate heterologous origin-containing plasmids has been examined. It was found that transfection of OpMNPV-infected *L. dispar* cells with an AcMNPV *hr*5-containing plasmid resulted in only trace amounts of plasmid replication (Pearson et al. 1993). Conversely, only minimal levels of replication of plasmids containing the putative OpMNPV replication origins *Hin*dIII-N or an OpMNPV *hr*-containing plasmid were detected in AcMNPV-infected *S. frugiperda* cells (Ahrens et al. 1995b). These data indicate that although baculoviruses are often similar in genome structure and organization, the DNA sequences that constitute an origin of replication and the viral/host proteins that recognize and interact with these sequences may differ significantly. Recent investigations suggest that some replication genes are fully interchangeable between the two systems, whereas others show high degrees of specificity (C.H. Ahrens, unpubl.).

FUTURE INVESTIGATIONS

The identification of baculovirus replication origins along with the development of assays for monitoring transient DNA replication, and the identification of genes that are essential or that stimulate DNA replication, have opened a number of avenues of research. These include characterization of the role of specific DNA sequences in origin structure and of the biochemical function(s) of the replication proteins in the initiation of replication and during DNA synthesis. The functions of some of the essential replication gene products can be predicted by comparison with those from other organisms. For example, a DNA polymerase normally requires a processivity factor that keeps the polymerase core tightly associated with the template, thereby preventing it from dissociating. Another essential component may include an origin-binding protein that recognizes an origin and catalyzes the assembly of the replication complex, and a primase that would be involved in the synthesis of the RNA primers during replication. In addition, if, as the data with *hr*-containing

plasmids suggest, replication results in large genome concatemers, understanding the mechanism by which such structures are resolved into unit-length genomes is of particular interest. Contribution of host factors to the replication process also cannot be ruled out. Of high priority is the development of an in vitro replication system that can be used to define the role of each component of the replication complex in replicating DNA.

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