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Identification of genes involved in DNA replication of the *Autographa californica* baculovirus

(nuclear polyhedrosis virus/*Spodoptera frugiperda*/apoptosis)

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ABSTRACT **By use of a transient replication assay, nine genes involved in DNA replication were identified in the genome of the *Autographa californica* baculovirus. Six genes encoding helicase, DNA polymerase, IE-1, LEF-1, LEF-2, and LEF-3 are essential for DNA replication while three genes encoding P35, IE-2, and PE38 stimulate DNA replication. No stimulation by the AcMNPV *pcna* gene, encoding a protein with sequence homology to proliferating-cell nuclear antigen, was observed. A pattern of amino acids found in a number of single-stranded-DNA-binding proteins was identified in the carboxyl-terminal region of IE-1.**

The *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) is the type species of the *Baculoviridae*, a large family of insect viruses, and has a circular, supercoiled DNA genome of ≈134 kb (1, 2). 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 of DNA replication (3–6). Seven of these origins (*ori*) are located within homologous regions (*hr*) (Fig. 1), which contain repeats of closely related imperfect palindromes (8). One origin is located within the *Hin*dIII-K fragment, which does not contain a *hr* (9).

To date only a putative helicase gene (10) and a putative DNA polymerase gene (11, 12) have been identified as essential for baculovirus DNA replication. In addition, a gene (*pcna*) encoding a protein resembling proliferating-cell nuclear antigen (PCNA), which is a DNA polymerase processivity factor in other systems, has been identified in AcMNPV (13), but its role in DNA replication has not been determined.

With a transient replication assay, six large regions of the AcMNPV genome were identified that contain one or more genes involved in DNA replication (7). In this report, this assay was used for the identification of six genes encoding proteins essential for AcMNPV DNA replication and three genes whose products stimulate DNA replication.

MATERIALS AND METHODS

Cells and Virus. *Spodoptera frugiperda* Sf9 cells (14) were cultured in TNM-FH medium (15), supplemented with 10% fetal bovine serum (FBS). The E2 strain of AcMNPV (16) was used as wild-type virus. Routine cell culture maintenance and virus infection procedures were carried out as described (17).

Plasmid Constructs. The nine replication genes were identified within six regions previously shown to be essential for DNA replication (ref. 7; see also Fig. 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 on the *Eco*RI-O fragment (18) and was cloned as an *Nru* I–*Eco*RI fragment (m.u. 7.5–8.7, ref. 2) into plasmid pUC19. *lef-2* is on *Eco*RI-I (19, 20) and was cloned as an *Mlu* I fragment (m.u. 1.9–2.6) with *Mlu* I–*Bgl* II linkers into the *Bam*HI site of pUC19. The DNA polymerase homolog (*dna pol*) and *lef-3* are on SstI-F (12, 21). *dna pol* was cloned as an *Sst*I–*Eco*RV fragment (m.u. 38.9–41.6) into pBKS(−) (Stratagene), and *lef-3* was cloned as an *Eco*RI–*Apa* I (m.u. 42.8–44.5) fragment into pJDH119 (22). *hel*, the gene encoding helicase (p143) in *Eco*RI-D (10) was cloned as an *Eco*RI–*Ssp* I fragment (m.u. 59.9–63.5) into pBKS(−). The *Eco*RI-S fragment (m.u. 86.8–87.7), containing the *p35* gene (23), was cloned into pUC19. *ie-1* (24) was cloned as a *Cla* I–*Hin*dIII fragment (m.u. 94.7–96.9) into pUC8. *ie-2* (25, 26), located on *Pst* I-N (m.u. 96.9–98.9), and *pe38* (27), located on a *Pst* I–*Eco*RI fragment (m.u. 98.9–100.0), were cloned into pUC19. The *pcna* gene (13, 28) was cloned as an *Eco*RI–*Hin*dIII fragment, containing *Eco*RI-T and part of *Eco*RI-M (m.u. 29.0–30.9), into pBKS(−). The reporter plasmid (pAcHL) contained homology region 2 (*hr2*) in the *Hin*dIII-L fragment (m.u. 18.4–20.5) cloned into pBKS(−). All the plasmids were transformed into *Escherichia coli* JM101 or DH5α by standard techniques (29). DNA isolation, purification with CsCl gradients or Qiagen columns, digestion with restriction enzymes, and agarose gel electrophoresis were carried out according to standard procedures (29) or the manufacturer's instructions.

Replication Assays. Transient replication assays were carried out as described (7), with minor modifications. *S. frugiperda* Sf9 cells were seeded onto six-well plates at a density of 2×10^6 cells per well in TNM-FH medium (15) supplemented with 10% FBS. After 24 hr, the medium was removed and 1 μg of the *ori*-containing plasmid pAcHL (*hr2*) was mixed with equimolar amounts of plasmids containing the replication genes, with 0.5 μg of DNA of a 5-kb plasmid as standard. The DNA was mixed with 200 μl of transfection buffer (25 mM Hepes/140 mM NaCl/125 mM $CaCl_2$, pH 7.1) and 200 μl of Grace's medium plus 10% FBS, penicillin G (50 units/ml), streptomycin (50 μg/ml; BioWhittaker), and Fungizone (amphotericin B, 375 ng/ml; Flow Laboratories) and was added onto the cells. After incubation for 4 hr at 27°C,

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Abbreviations: AcMNPV, *Autographa california* multinucleocapsid nuclear polyhedrosis virus; FBS, fetal bovine serum; HSV-1, herpes simplex virus 1; PCNA, proliferating-cell nuclear antigen; SSB, single-stranded-DNA-binding protein; m.u., map unit(s).

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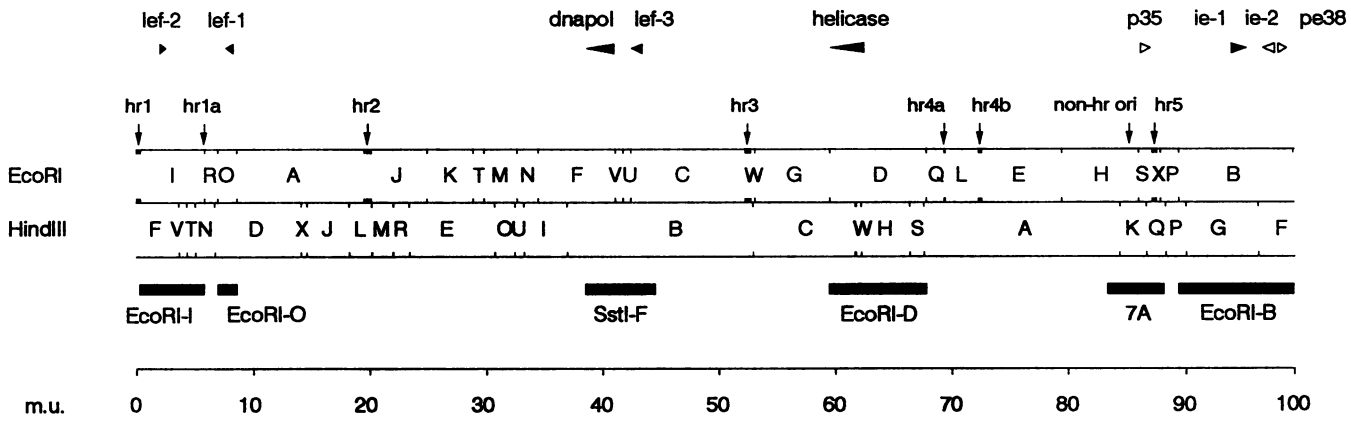

FIG. 1. *Eco*RI and *Hin*dIII restriction map of the AcMNPV genome. Six regions previously shown to be required for DNA replication (7) include *Eco*RI-I, *Eco*RI-O, *Sst* I-F, *Eco*RI-D, 7A, and *Eco*RI-B. Putative origins (*ori*) of DNA replication, including seven *hr* regions and one non-*hr* region are shown above the map together with the location of the essential (filled arrowheads) and stimulating (open arrowheads) replication genes. m.u., Map units.

the transfection medium was removed and 1 ml of fresh TNM-FH medium supplemented with 10% FBS was added. After 72 hr at 27°C, total DNA was isolated from the cells (17) and suspended in 64 μl of TE (10 mM Tris·HCl/1 mM EDTA, pH 8.0). An aliquot (16 μl) was digested with a combination of the restriction enzymes *Hin*dIII and *Dpn* I, the former to linearize the plasmids and *Dpn* I to determine whether the transfected plasmids had undergone replication in the insect cells and thus become resistant to cleavage by this methylation-sensitive restriction enzyme. After agarose gel electrophoresis, the DNA was transferred to membrane filters (GeneScreen*Plus*; New England Nuclear) by the method of Koetsier *et al.* (30). Radioactively labeled (31) pBSK(−) was used as probe for hybridization.

RESULTS

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. 1) (7). To locate the genes on these fragments that were required for DNA replication, subclones of each of these six regions were tested in a *Dpn* I-based replication assay for their ability to substitute for the original clone. From the minimal subclones, nine genes were identified that influenced DNA replication, including six that were essential for DNA replication and three which stimulated replication (Fig. 1, filled and open arrowheads, respectively). When nine plasmids containing each of these genes were transfected into uninfected *S. frugiperda* Sf9 cells, a high level of plasmid replication was observed (Fig. 2, lane 1). When either *lef-1*, *lef-2*, *lef-3*, *dna pol*, *hel*, or *ie-1* was omitted from the transfection mixture, no replication was detected (lanes 2–7, respectively) indicating that each of these genes is essential. When any one of the stimulating genes—*p35*, *ie-2*, or *pe38*—was omitted, lower levels of replication were observed (lanes 8–10, respectively). 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, with *p35* and *ie-2* stimulating replication to a much higher level than *pe38* (lanes 12–14). However, when each activator was omitted from the transfection mixture containing all the other replication genes, *p35* exhibited the most profound effect (lanes 8–10). IE-2 and PE38 are known transactivators of early gene transcription (25, 32), and P35 is known as an inhibitor of virus-induced apoptosis in *S. frugiperda* cells (33).

An AcMNPV gene (*pcna*) encoding a PCNA-like protein has been reported (13, 28). Homologs of this gene code for DNA polymerase processivity factors and are often found to be essential in other DNA replication systems (34). It was shown that deletion of *pcna* yielded viable virus that exhibited a delay in late gene expression (28). In addition, in a previous report it was shown that the *pcna* gene was not among the essential regions for DNA replication (7). However, these results did not rule out a stimulatory role for this gene, which may be most significant at the onset of DNA replication but be undetectable at the later times after transfection when total cellular DNA is routinely isolated with our protocol. Therefore to test whether *pcna* influenced DNA replication in the transient replication assay, *S. frugiperda* cells were transfected with the complete set of replication genes and pAcHL (containing *hr2*), with and without *pcna*, and total cellular DNA was isolated at various times after transfection. In both cases, newly replicated plasmid DNA was first detected at 18 hr after transfection and no significant difference was found in the levels or time course of plasmid replication (Fig. 3).

The results in Figs. 2 and 3 demonstrate that plasmid replication in the transient complementation assay is viral origin-independent. The reporter plasmid pAcHL (containing *hr2*) and all added plasmids replicate when all essential genes are present. This agrees with previous results which showed that plasmid replication became viral origin-independent when naked DNA instead of intact virions was used to supply essential trans-acting factors (7). The difference in gene copy number may play a role in this phenomenon, since cells infected with virus at a multiplicity of infection 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 overabundance of replication proteins. Replication origins may thus become saturated with origin-recognizing protein(s), and this excess of protein(s) may bind nonspecifically 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 for Fig. 2. This did not result in specific replication of the *ori*-containing plasmid but led to a precipitous decrease in replication signals for all plasmids (data not shown). When the amount of the replication genes was reduced to 10%, with a mixture of the *hr2*-containing plasmid and a pBS(−) control plasmid kept constant at 1 μg, pAcHL replicated to high levels but the control pBS(−) plasmid also replicated, although at a substantially lower level than pAcHL (data not shown). Therefore, under these

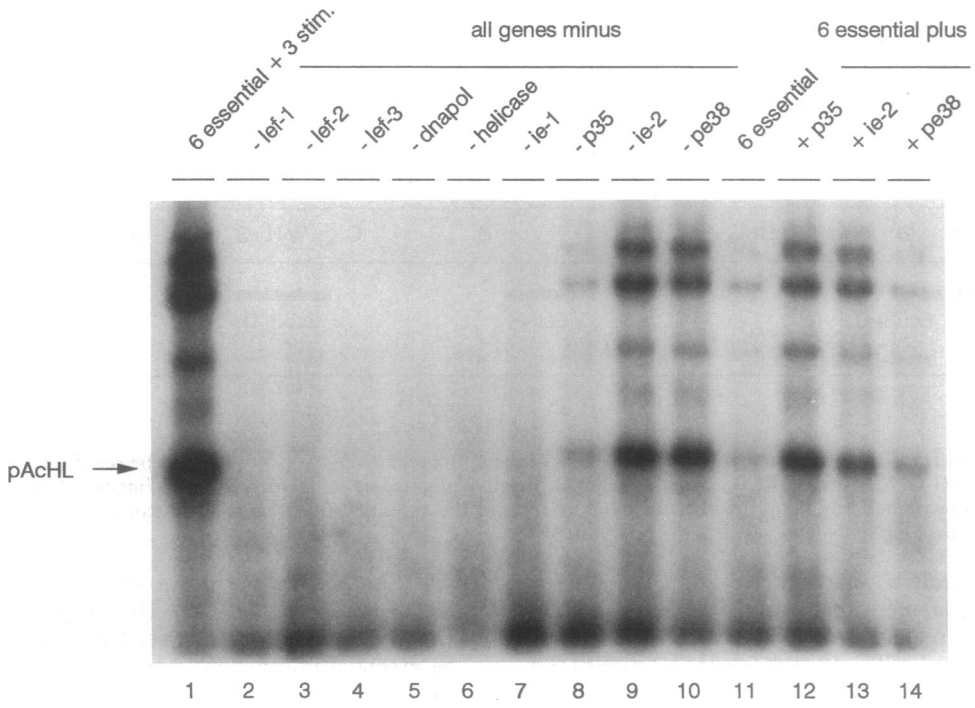

FIG. 2. Transient replication assay of nine AcMNPV replication genes. These were identified within six regions previously shown to be essential for DNA replication (ref. 7; see also Fig. 1). *S. frugiperda* Sf9 cells were cotransfected with equimolar amounts of plasmids containing the AcMNPV replication genes and 1 μg of pAcHL (*Hin*dIII-L) containing *hr2* as AcMNPV origin of DNA replication. Total cellular DNA was isolated 72 hr after transfection and digested with *Hin*dIII and *Dpn* I. Hybridization was carried out with ^{32}P-labeled pBKS(−) as probe. The contents of mixtures of transfected DNA are indicated above the lanes. The position of the *hr2*-containing reporter plasmid (pAcHL) is shown on the left. stim., Stimulatory.

altered conditions, this assay does not completely mimic the origin-specific replication observed in virally infected cells.

Two of the AcMNPV genes we identified as essential for replication have previously been shown to contain sequence motifs with similarity to DNA polymerase (12) and helicase (10). However, the other four essential genes have not been reported to be similar to other defined sequences in the data base. A component present in many DNA replication systems is a single-stranded-DNA-binding protein (SSB) (34). A common feature of SSBs is a set of aromatic and basic amino acids separated by nonconserved residues, which may be involved in DNA binding. Examination of the AcMNPV replication proteins revealed a SSB-like motif in the carboxyl-terminal region of the IE-1 sequence from amino acid 455 to 511. This motif is similar to the DNA-binding motif which was originally identified from alignments of herpes simplex virus 1 (HSV-1) UL29 with the gene 5 SSB from fd bacteriophage (35). 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. 4) (42).

DISCUSSION

Six genes, encoding helicase, DNA polymerase, IE-1, LEF-1, LEF-2, and LEF-3, were identified in the genome of AcMNPV as essential for transient DNA replication. The gene products of *p35*, *ie-2*, and *pe38* were found to stimulate this replication. No significant stimulation was detected from the AcMNPV PCNA, nor did the absence of this protein cause a delay in

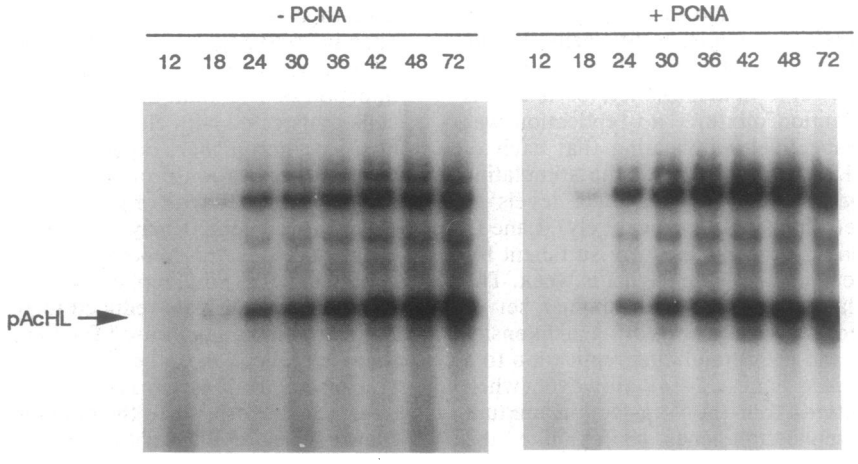

FIG. 3. Effect of *pcna* on the time course of transient plasmid DNA replication. *S. frugiperda* Sf9 cells were cotransfected with pAcHL (*Hin*dIII-L, *hr2*) as reporter plasmid and equimolar amounts of nine plasmids containing the AcMNPV replication genes with (*A*) or without (*B*) *pcna*. Total cellular DNA was isolated at the indicated times (in hours) after transfection and processed as described in the legend to Fig. 2.

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
HSV1	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

FIG. 4. A comparison of putative single-stranded-DNA-binding amino acid sequence motifs. The spacer regions between conserved aromatic and basic amino acids are designated by X with a number indicating the spacer residues present. Numbers at the margins of the sequence indicate the location of the motif in the amino acid sequence. The alignments from the gene *5* protein of bacteriophage fd (GP5); gene *32* protein of bacteriophage T4 (GP32), SSB of *E. coli*; and DNA-binding proteins from bacteriophage ike (PIKE), HSV-1, varicella–zoster virus (VZV), Epstein–Barr virus (EBV), and adenovirus type 5 (AD-5) are from Wang and Hall (35). Additional sequences are as follows: equine herpes virus (EHV) (36), human cytomegalovirus (HCMV) (37), mouse cytomegalovirus (MCMV) (38), and channel catfish virus (CCV) (39). The baculovirus IE-1 sequences are from AcMNPV (AcIE-1), (24), *Bombyx mori* NPV (BmIE-1) (40), *Orgyia pseudotsugata* MNPV (OpIE-1) (41), *Choristoneura fumiferana* MNPV (GenBank accession no. L04945), *Heliocoverpa zea* NPV (HzIE-1) (GenBank accession no. U03418), and *Spodoptera exigua* MNPV (SeIE-1) (E.A. van Strien, personal communication).

plasmid DNA replication in the transient replication assay. These results, together with the fact that a gene encoding a PCNA-like protein is absent from the genomes of the closely related *Bombyx mori* NPV (S. Maeda, personal communication) and *Orgyia pseudotsugata* MNPV (data not shown), suggest that *pcna* may not be directly involved in baculovirus DNA replication.

Two of the three identified stimulatory genes, *ie-2* and *pe38*, encode transactivators of transcription (25, 32). In particular, PE38 has been shown to activate expression of the baculovirus helicase homolog. Furthermore, IE-2 has been shown to stimulate *pe38* expression (32). In HSV-1 transient replication assays, the four identified transactivators are also not essential for replication of origin-containing plasmids (43). In contrast, in replication assays containing genes from the herpesviruses Epstein–Barr virus and human cytomegalovirus, the three transactivators identified for these viruses are essential (44–46).

The third stimulatory gene identified in AcMNPV, *p35*, has previously been shown to encode an inhibitor of virus-induced apoptosis in *S. frugiperda* cells (33). 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 (47). Therefore the apparent stimulatory effect observed for *p35* in the transient replication assays may not be caused by direct activation of the replication process but may be due to inhibition of apoptosis, thereby elevating the replication signal by preventing the cells from dying. Further evidence that *p35* does not play a direct role in replication comes from the observation that *Orgyla pseudotsugata* MNPV, which shows a high degree of genome similarity to AcMNPV, lacks a homolog of the *p35* gene (48). If P35 does not directly activate replication in *S. frugiperda* cells, that could suggest that apoptosis may be induced by the expression of one or more of the replication genes or by the replication of heterologous DNA. The former possibility 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 (49). If apoptosis is induced by the replication of the transfected DNA, this would suggest that the cell has a mechanism for discriminating between normal chromosomal DNA replication versus replication of foreign DNA, or DNA replication not linked to the cell cycle.

Four of the six genes (*lef-1*, *lef-2*, *lef-3*, and *ie-1*) we have implicated as being essential for DNA replication have also been shown to be necessary for the transcription of baculovirus late genes, in an assay in which late promoter–chloramphenicol acetyltransferase gene constructs are co-transfected into cells along with plasmids comprising specific regions of the viral genome (18, 20, 21). Transcription of late genes is dependent upon DNA replication (50), and the assay used for these investigations does not differentiate between genes required for replication or late transcription. Our results suggest that these genes may be essential for late transcription because of the role they play in DNA replication.

A review of well-characterized replication systems from a variety of organisms including *Escherichia coli*, bacteriophages T7 and T4, and the animal viruses simian virus 40 and herpesviruses indicates a common theme in the number and function of many of the components required for DNA replication in these systems (34). All these systems are composed of a helicase, DNA polymerase, DNA polymerase accessory protein, primase, and SSB components. In addition, there are often requirements for a topoisomerase and origin-binding proteins. A sequence related to a putative DNA-binding motif found in SSBs was found in IE-1. This motif is highly conserved in six baculovirus IE-1 proteins (42) suggesting that this pattern of amino acids is functionally important. SSBs are often required components of DNA replication systems and may function by stabilizing single-stranded regions at the replication fork (34). This could also explain the ability of IE-1 to strongly transactivate a number of baculovirus early genes (51); IE-1 may stabilize single-stranded DNA within promoter regions, thereby keeping these sequences accessible for transcriptional initiation. The ability of a SSB to activate transcription is not unique to IE-1, since HSV-1 UL29, the adenovirus SSB, the gene *32* product of bacteriophage T4, and SSB of *E. coli* exhibit similar roles in activation of transcription (52–55).

The *Baculoviridae* share a number of features in DNA structure and DNA replication with the *Herpesviridae*. 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 origin-containing plasmids into infected cells results in large concatemers of input plasmid DNA (6, 56), suggesting a rolling circle mode of replication. For three herpesviruses, HSV-1 (43, 57), Epstein–Barr virus (44), and human cytomeglovirus (45, 46), the genes involved in DNA replication have also been identified. Although the total number of replication genes differs among these three herpesviruses, a core set of six essential genes also are found in all three. It is likely that members of the essential set of baculovirus replication genes for which a function is not implied may have counterparts among the herpesvirus replication genes.

Although the identification in AcMNPV 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 origin-containing plasmid DNA, with the production of long concatemers of the genome, then other genes required for the resolution of these structures are most likely present. Furthermore, the involvement of host genes in the replication process cannot be ruled out.

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