

NOTES

Expression of Baculovirus Late and Very Late Genes Depends on LEF-4, a Component of the Viral RNA Polymerase Whose Guanyltransferase Function Is Essential

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Baculovirus *lef-4* encodes one subunit of the viral RNA polymerase. Here, we demonstrate the essential nature of LEF-4 by RNA interference and bacmid knockout technology. Silencing of LEF-4 in wild-type virus-infected cells suppressed expression of structural genes, while early expression was unaffected, demonstrating its essential role in late gene expression. After transfection of insect cells with *lef-4* mutant bacmid, no viral progeny was produced, further defining its central role in infection. Cotransfection with wild-type *lef-4* plasmid restored normal replication, but plasmid encoding a guanyltransferase-deficient version failed to rescue. These results emphasize the importance of the mRNA capping function of LEF-4.

Baculoviruses are unique among eukaryotic DNA viruses because early genes are transcribed by host RNA polymerase II (5, 13), while late genes are transcribed by a virus-encoded RNA polymerase (6, 8, 10). In *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), the core viral polymerase is composed of four viral proteins, LEF-4, LEF-8, LEF-9, and p47 (10). LEF-8 and LEF-9, which contain motifs common to β and β' RNA polymerases, are believed to form the catalytic site (19, 26). LEF-4 is an mRNA capping enzyme (7, 9, 15), and the function of p47 is unknown.

The LEF proteins were originally mapped as late expression factors by transient assays that used a late reporter construct and fragments of viral DNA (25). The *lef-4* gene was also identified as the site of a temperature-sensitive (ts) mutation with a late expression phenotype (2, 24). To address the essential function of LEF-4, we performed RNA silencing experiments and gene knockout experiments using bacmid technology. Previous studies have demonstrated the value of these techniques in the study of essential viral genes in *Spodoptera frugiperda* cells (4, 14, 17, 18, 20, 28).

First, a high quality LEF-4 antiserum was produced in rabbits using LEF-4 protein expressed in bacteria (9). *S. frugiperda* cells were infected with AcMNPV at 10 PFU/cell, and detergent-based nuclear extracts were prepared from infected cells 4, 8, 16, 24, 48, and 72 h postinfection (p.i.). As previously described, the cell pellet was lysed in 0.5% NP-40 and centrifuged and the supernatant (cytoplasmic fraction) was adjusted to 0.1 N NaOH (21). The pelleted nuclei were resuspended in 1% NP-40 and adjusted to 0.1 N NaOH. Immunoblot experi-

ments showed that LEF-4 antiserum recognized a polypeptide of about 50 kDa from 16 through 72 h p.i., without cross-reactivity (Fig. 1).

To conduct the RNA silencing experiments, specific double-stranded RNA (dsRNA) was prepared using the T7 RiboMax Express RNAi system (Promega). The cloned HindIII-C fragment of the AcMNPV genome served as DNA template to generate dsRNA corresponding to 543 bp at the 5' terminus of *lef-4* (primer 1a, 5'-GGGTAATACGACTCACTATAGGGGCGATTTTGTGATTGAG-3'; primer 1b, 5'-GGCAAATTCA TATTCGAGACG-3'; primer 2a, 5'-GGGTAATACGACTC

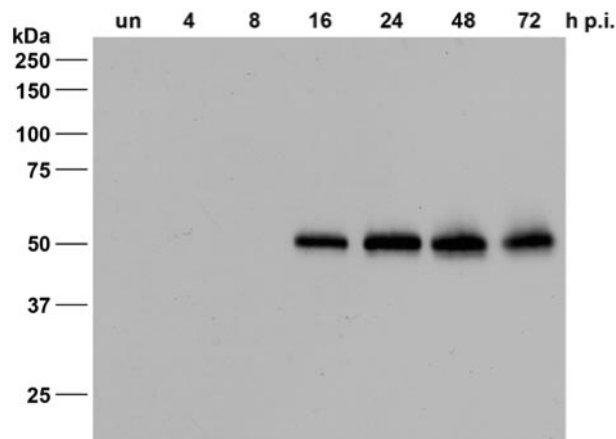


FIG. 1. Expression of LEF-4 in AcMNPV-infected *S. frugiperda* cells. Detergent-based nuclear extracts were prepared from uninfected *S. frugiperda* cells ("un") or from infected cells at 4, 8, 16, 24, 48, and 72 h p.i. Proteins were resolved on sodium dodecyl sulfate-10% polyacrylamide gels and stained with the rabbit anti-LEF-4 antiserum. Protein size markers are given on the left.

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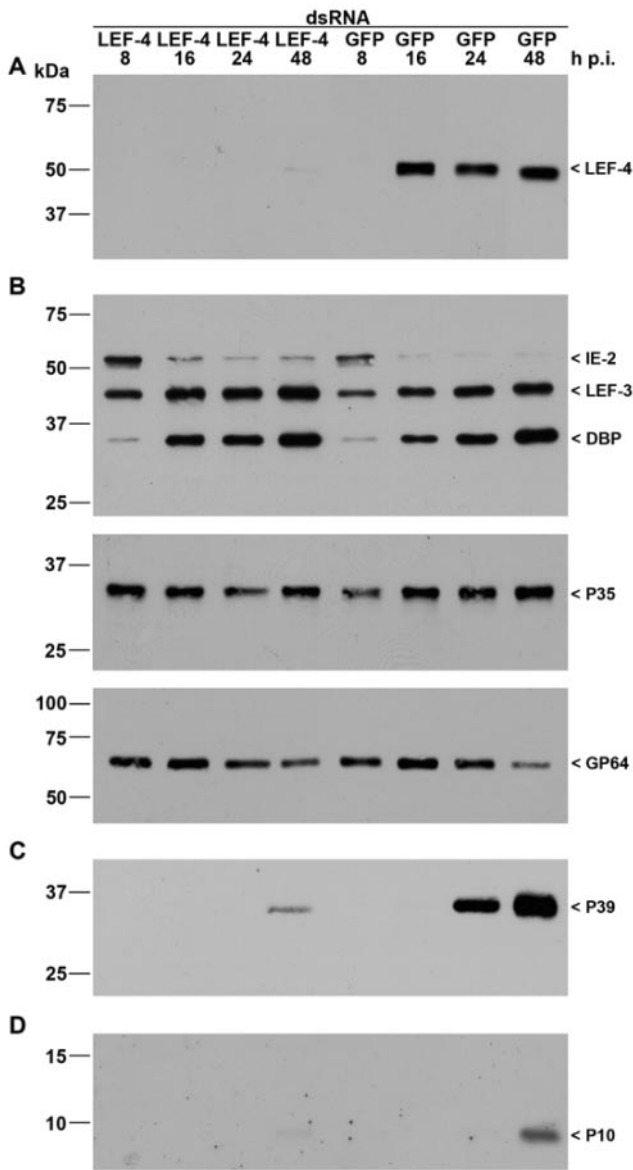


FIG. 2. Inhibition of viral gene expression by *lef-4* silencing. *S. frugiperda* cells were transfected with either LEF-4 or GFP dsRNA. Cells were subsequently infected with AcMNPV (10 PFU/cell) at 20 h posttransfection, and detergent-based nuclear and cytoplasmic extracts were prepared from infected cells 8, 16, 24, and 48 h p.i. Proteins were resolved on sodium dodecyl sulfate-10% polyacrylamide gels and transferred to nitrocellulose membranes (A, B, and C) or resolved on sodium dodecyl sulfate-15% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (D). (A) LEF-4 was stained with rabbit anti-Lef-4 antiserum. (B) Early gene expression was analyzed with rabbit antisera raised against IE2 (16), LEF-3 (3), DBP (22), or P35 (11) or with mouse monoclonal anti-GP64 (AcV5) (12). (C) Late gene expression was analyzed with mouse monoclonal anti-P39 (P10C6) (30). (D) Expression of the very late protein P10 was detectable with rabbit anti-P10 serum (29). Expression of LEF-4, IE2, LEF-3, DBP, P39, and P10 was analyzed on samples of nuclear protein fractions, and P35 and GP64 expression was detected in cytoplasmic fractions as described previously (21). Protein size markers are shown on the left, and the identities of the viral proteins are indicated on the right.

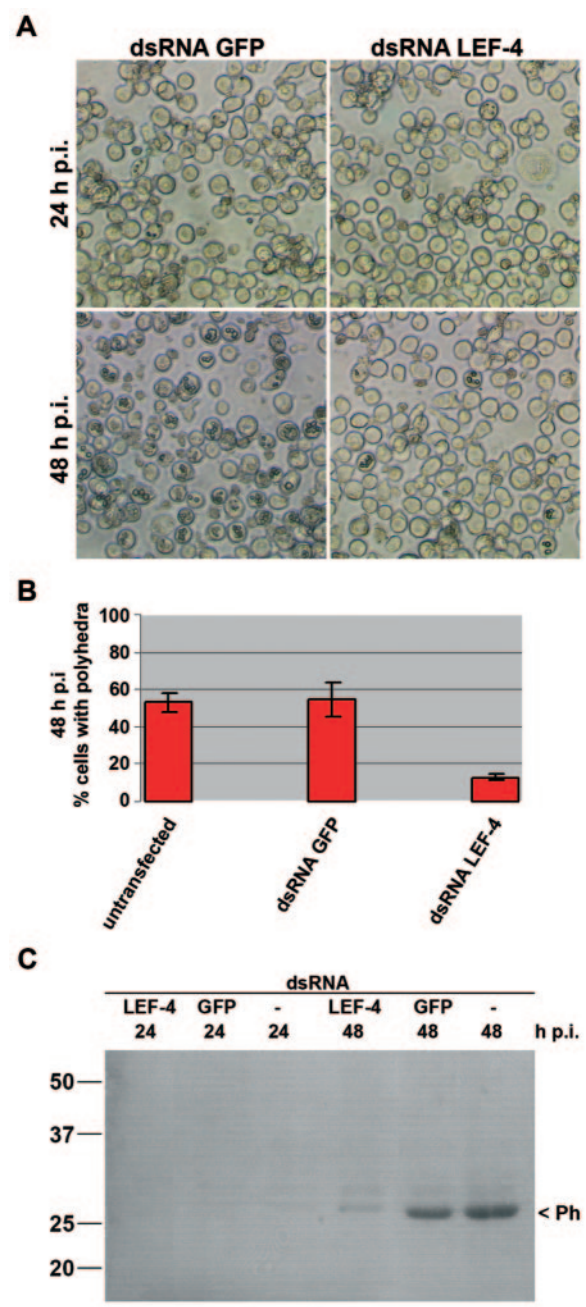


FIG. 3. Polyhedron formation and polyhedrin expression upon *lef-4* inhibition in AcMNPV-infected *S. frugiperda* cells. Cells were transfected with either LEF-4 dsRNA or GFP dsRNA, infected with AcMNPV (10 PFU/cells) at 20 h posttransfection, and analyzed at 24 and 48 h p.i. (A) Phase-contrast images are shown, and (B) polyhedron-containing cells were quantitated at 48 h p.i. (C) Cells transfected with LEF-4 dsRNA, GFP dsRNA, or untransfected cells were infected, and detergent-based nuclear extracts were prepared at 24 and 48 h p.i. Proteins were resolved on sodium dodecyl sulfate-10% polyacrylamide gels and transferred to nitrocellulose, and polyhedrin was viewed by Ponceau staining. Numbers at left are molecular masses in kilodaltons.

ACTATAGGCAAATTCATATTCGAGACG-3'; primer 2b, 5'-GGGGCGATTTTGTGATTGAG-3'). As a control we used dsRNA corresponding to a 600-bp region of the green fluorescent protein (GFP) gene. *S. frugiperda* cells were transfected

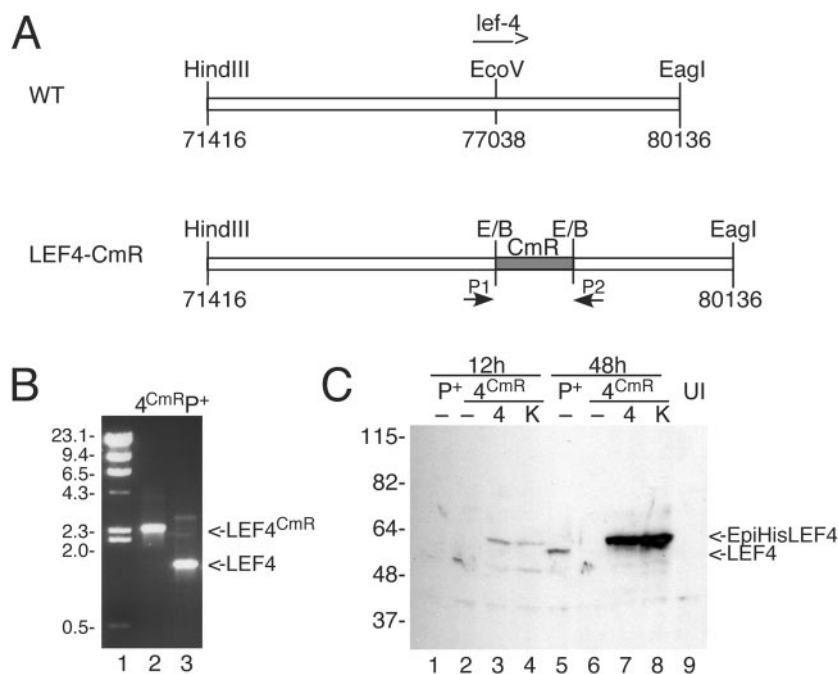


FIG. 4. Construction of BacP⁺/LEF-4^{CmR}. (A) Cloning strategy. A plasmid containing the left 7 kb of the HindIII-C fragment of AcMNPV was digested with EcoRV, and a 1-kb fragment of pBR325 containing the CAT resistance marker was inserted. The resultant plasmid was transformed into BJ5183 cells containing a modified version of the AcMNPV genome, followed by selection on chloramphenicol, as previously described (1). (B) PCR screening. Correct insertion of CAT into *lef-4* was verified by PCR using primers that flank the *lef-4* open reading frame. Lane 2, BacP⁺/LEF-4^{CmR}; lane 3, BacP⁺. The positions of molecular mass markers (lane 1) are indicated on the left in kilodaltons, and the migration of *lef-4* and *lef-4* with the CAT insertion is shown on the right. (C) Immunoblot analysis of LEF-4 expression in transfected cells. Cells were transfected with BacP⁺ (P⁺, lanes 1 and 5) or BacP⁺/LEF-4^{CmR} (4^{CmR}, lanes 2 to 4 and 6 to 8) and cotransfected where indicated with pHSEpiHisLEF-4 (4, lanes 3 and 7) or pHSEpiHisLEF-4(K255A) (K, lanes 4 and 8). Cells were harvested at 12 h (lanes 1 to 4) or 48 h (lanes 5 to 8) posttransfection. The blot was probed with rabbit LEF-4 antiserum. Untransfected cells were analyzed as a negative control (lane 9). The positions of molecular mass markers are indicated on the left in kilodaltons, and the migration of LEF-4 and EpiHisLEF-4 is indicated on the right.

with 5 μ g of LEF-4 dsRNA or GFP dsRNA as calcium phosphate precipitates (BD BaculoGold). Cells were subsequently infected with AcMNPV (10 PFU/cell) at 20 h posttransfection, followed by detergent-based nuclear extraction (21). Immunoblot analysis showed that transfection of LEF-4 dsRNA silenced expression of LEF-4. Transfection of the control GFP dsRNA, however, did not affect LEF-4 expression, thus demonstrating specificity (Fig. 2A). The efficiency of *lef-4* silencing indicates that a high percentage of cells must have taken up the dsRNA.

To study the effect of LEF-4 suppression on viral gene expression, we analyzed proteins expressed early, late, and very late during infection. IE2, an immediate-early protein, is detectable from 2 until 12 to 24 h p.i. in *S. frugiperda* cells (16). The loss of LEF-4 had no effect on IE2 levels, consistent with the assumption that the *ie2* promoter is transcribed by host RNA polymerase II (Fig. 2B). We also found no effect on expression patterns of LEF-3 (3), DBP (22), or P35 (11) in infected cells, consistent with the idea that LEF-4 is not involved in early gene expression (Fig. 2B). Surprisingly, we observed comparable levels of the membrane protein GP64 in cells treated with LEF-4 dsRNA or GFP dsRNA (Fig. 2B). Since GP64 is known to be regulated by both early and late promoters, we expected that protein levels would be lower in the *lef-4*-silenced cells. The similar amounts of protein might be coincidental, because accumulation of GP64 in normal in-

fection is a function of synthesis combined with the loss of protein due to budding of virus particles. In the case of *lef-4*-silenced cells, less budding presumably occurs, due to lower levels of synthesis of viral structural proteins (see below).

To examine the effect of LEF-4 on late genes, protein extracts were probed with antibody against the capsid protein P39 (30). Strong expression of P39 was observed at 24 and 48 h p.i. in cells treated with GFP dsRNA, while only a weak P39 signal was detected in cells with suppressed LEF-4 (Fig. 2C). The very late protein P10 was also shown to be dependent upon LEF-4 synthesis. P10 levels were undetectable in LEF-4 suppressed cells, while P10 was evident in the control cells at 48 h p.i. (Fig. 2D). Furthermore, polyhedrin levels were reduced. Immunoblot analysis showed a significant reduction in accumulation of polyhedrin protein in cells treated with LEF-4 dsRNA, compared to cells treated with GFP dsRNA or untreated cells (Fig. 3C). Microscopic analysis revealed the presence of polyhedron-positive cells at 24 and 48 h p.i. in the LEF-4-silenced cells, although the number of cells with polyhedra was about fivefold lower than that of the control GFP-treated cells (Fig. 3A and B). The few cells that were polyhedron positive had equivalent numbers of polyhedra per cell, and those polyhedra formed with the same kinetics as in the GFP dsRNA-treated and untreated controls (Fig. 3A and data not shown). This suggests that cells with polyhedra likely rep-

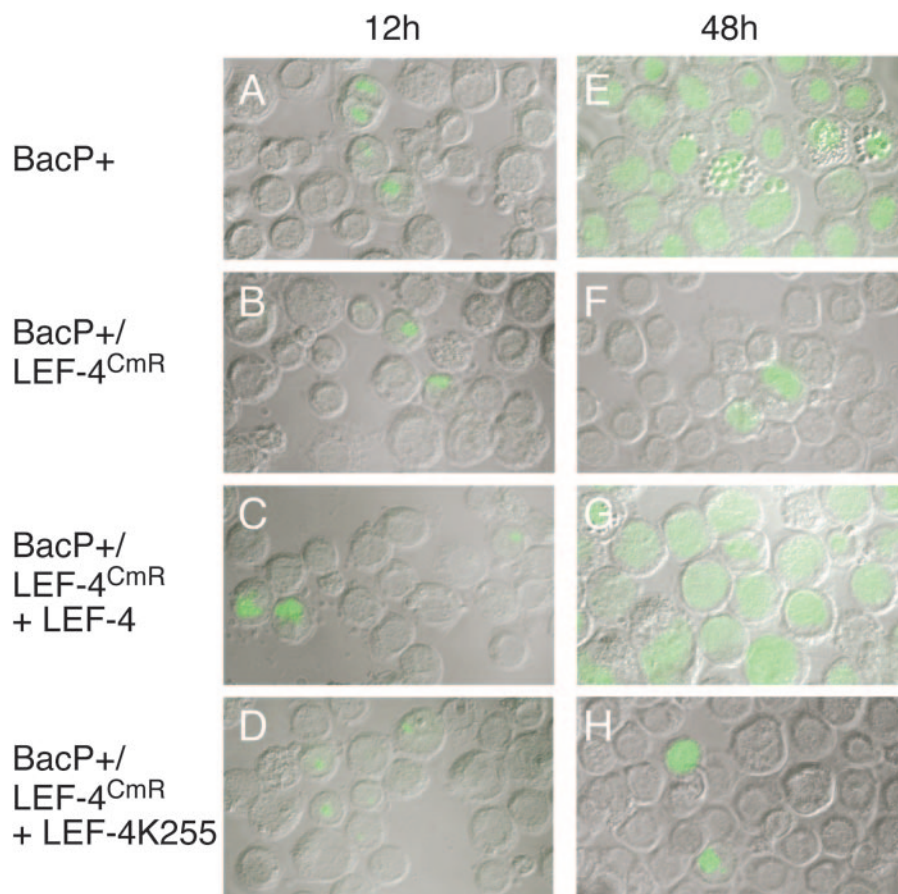


FIG. 5. Infectivity of *lef-4* mutant viruses. *S. frugiperda* cells were transfected with BacP⁺ (A and E), BacP⁺/LEF-4^{CmR} (B and F), or BacP⁺/LEF-4^{CmR} in the presence of plasmids encoding wild-type LEF-4 (C and G) or a mutant version of LEF-4 that lacks guanylttransferase activity (D and H). Cells were harvested at 12 or 48 h p.i. and processed for immunofluorescence using a mouse monoclonal antibody raised against IE1. Images were collected using a Zeiss ApoTome/Axioplan 2 microscope. Images shown represent merged images of phase contrast and green fluorescence to visualize percentages of cells expressing IE1.

resent the population of cells that did not take up LEF-4 dsRNA.

Our results strengthen the identification of LEF-4 as an essential component of the viral RNA polymerase that is responsible for late and very late transcription. The role of LEF-4, however, remains open. Previous results showed that the *lef-4* ts mutant L104F failed to produce infectious virus at the nonpermissive temperature (2). Interestingly, a mutant version of LEF-4 with this substitution was not impaired for guanylttransferase activity (15). To address whether LEF-4 is essential for its capping activity, we constructed a virus with an interrupted *lef-4* gene.

The bacmid BacP⁺ is derived from the Bac-to-Bac cloning vector (Invitrogen), which has the AcMNPV genome cloned into a single-copy plasmid (1). BacP⁺ has a reconstructed polyhedrin gene and lacks transposition sites for cloning into the polyhedrin locus. The *lef-4* gene was inactivated by inserting a 1-kb BstBI fragment of pBR325, containing the chloramphenicol acetyltransferase (CAT) resistance marker under the control of its own promoter, into the EcoRV site of an 8.7-kb genome fragment containing *lef-4* (Fig. 4A). The resulting plasmid was digested to excise the viral DNA that was used to transform BacP⁺-containing BJ5183 cells by electropora-

tion. Recombinants were selected by plating on chloramphenicol, and interruption of the *lef-4* open reading frame with CAT was confirmed by PCR using plasmids that flank the *lef-4* gene (Fig. 4B).

Purified bacmid DNA was transfected into Sf9 cells, which were analyzed at 12 h p.i. by immunofluorescence using antibody against the immediate-early IE1. Approximately 500 total cells were scored for IE1 expression. We found 4 to 12% positive cells, indicating similar transfection efficiencies for the two DNAs (Fig. 5A and B). At 48 h posttransfection, virtually all cells transfected with BacP⁺ were positive for IE1 (Fig. 5E), indicating that viral infection had spread to adjacent cells, but

TABLE 1. Infectious virus produced from transfection of bacmids

Bacmid	Plasmid	Titer at 48 h posttransfection
BacP ⁺	None	2×10^7
BacP ⁺ /LEF-4 ^{CmR}	None	0
	pHSEpiHisLEF-4	5.3×10^6
	pHSEpiHisLEF-4(K255A)	2×10^{2a}

^a All clones recovered were reversions.

the percentage of IE1-positive cells in BacP⁺/LEF-4^{CmR} had not significantly increased (Fig. 5F). The lack of replication was verified by plaque assay of the culture medium at 48 h posttransfection (Table 1).

To determine whether capping enzyme activity was necessary for viral infection, we cotransfected BacP⁺/LEF-4^{CmR} and pHSEpiHisLEF-4 (27), encoding an epitope-tagged version of *lef-4* or a mutant version of the same plasmid encoding an alanine substitution at lysine 255 (K255A). We have previously shown that this mutant is not active in guanylyltransferase assays (15). Immunoblot analysis showed that the two proteins were expressed at equivalent levels by 12 h posttransfection (Fig. 4C). The wild-type *lef-4* plasmid rescued the mutant bacmid DNA. The pattern of IE1 immunofluorescence was similar to that seen with the parental bacmid (Fig. 5C and G), and the yield of infectious virus was approximately 25% of that obtained with the parental clone (Table 1), indicating that the level of recombination between the bacmid and plasmid was very high. Cotransfection with the K255A mutant, however, did not significantly change the pattern of IE1 immunofluorescence (Fig. 5D and H). Some progeny virus was obtained (Table 1), but direct sequencing of four clones revealed that their genomes contained wild-type *lef-4*. This was possible because the BacP⁺/LEF-4^{CmR} construct was made by inserting the CAT gene into the LEF-4 open reading frame without deleting any *lef-4* sequences, including the K255 locus. The fact that only wild-type mutants were obtained with the K255A plasmid indicates that guanylyltransferase activity is required for late gene expression and production of progeny virions.

The strategy used in these studies differs somewhat from previous protocols relying on bacmid systems (18, 23, 28). First, we did not construct a "repaired" virus control by inserting the *lef-4* gene into the polyhedrin locus. Instead, we relied on the efficient recombination ability of baculoviruses to produce viable virus. The fact that the yield of infectious virus obtained from cotransfection of a wild-type *lef-4* plasmid and BacP⁺/LEF-4^{CmR} was 25% of that obtained with the parental bacmid indicates that in vivo recombination is very efficient. This was also evident in the high spread of IE1 fluorescence at 48 h posttransfection. Second, we did not construct bacmids that expressed reporter genes in order to follow infection. Instead we visualized expression of a viral gene.

Taken together, our results further characterize the essential nature of LEF-4 and demonstrate that the guanylyltransferase function of LEF-4 is essential for productive infection. In addition, LEF-4 may also be required for its RNA triphosphatase activity and another function that was disrupted by the L105F substitution (2). Biochemical assays of a protein with an L105F substitution revealed that it was normal with respect to guanylyltransferase and had only a modest decrease in RNA triphosphatase activity, which is probably insignificant (15). Since these are the only two enzymatic activities that the protein is known to possess, it is possible that L105 is important for structural integrity of the polymerase complex, and high temperature inhibits replication because the polymerase dissociated although the enzymatic functions of LEF-4 are unaffected. Analysis of additional LEF-4 mutants should help to define roles of LEF-4 in viral infection.

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