The *lef-3* Gene of *Autographa californica* Nuclear Polyhedrosis Virus Encodes a Single-Stranded DNA-Binding Protein

XIN HANG,[1] WEN DONG,[2] AND LINDA A. GUARINO[1,2,3]*

Departments of Biochemistry & Biophysics[1] and Entomology,[2] Texas A&M University, and The Center for Advanced Invertebrate Molecular Sciences, Institute of Biosciences and Technology,[3] College Station, Texas 77843-2475

The *Autographa californica* nuclear polyhedrosis virus (AcNPV) replicates in the nuclei of infected cells and encodes several proteins required for viral DNA replication. As a first step in the functional characterization of viral replication proteins, we purified a single-stranded DNA-binding protein (SSB) from AcNPV-infected insect cells. Nuclear extracts were chromatographed on single-stranded DNA agarose columns. An abundant protein with an apparent molecular weight of 43,000 was eluted from the columns at 0.9 to 1.0 M NaCl. This protein was not evident in extracts prepared from control cells, suggesting that the SSB was encoded by the virus. SSB bound to single-stranded DNA in solution, and binding was nonspecific with respect to base sequence, as single-stranded vector DNA competed as efficiently as single-stranded DNA containing the AcNPV origin of DNA replication. Competition binding experiments indicated that SSB showed a preference for single-stranded DNA over double-stranded DNA. To determine whether SSB was encoded by the *lef-3* gene of AcNPV, the *lef-3* open reading frame was cloned under the control of the bacteriophage T7 promoter. Immunochemical analyses indicated that LEF-3 produced in bacteria or in rabbit reticulocyte lysates specifically reacted with antiserum produced by immunization with purified SSB. Immunoblot analyses of infected cell extracts revealed that SSB/LEF-3 was detected by 4 h postinfection and accumulated through 48 h postinfection.

The genome of *Autographa californica* nuclear polyhedrosis virus (AcNPV) is a double-stranded, supercoiled DNA molecule of 134 kb and potentially encodes 150 proteins (1). The viral genome consists of unique sequences, with the exception of five regions of homologous sequences (*hr*s *1* to *5*) that are interspersed along the length of the genome (5). Each *hr* contains from two to eight 24-bp imperfect palindromes with naturally occurring *Eco*RI sites at their core. The *hr*s function as enhancers of early viral gene expression (11, 12). The baculovirus transactivator IE1 binds to DNA sequences within the enhancer regions (9, 10).

When the *hr*s were first described, Cochran and Faulkner (5) speculated that they might function as origins of replication. To test this hypothesis, Pearson et al. (26) transfected AcNPV-infected cells with plasmids containing *hr2* or *hr5*. Replication was monitored after recovery of plasmids and digestion with *Dpn*I. These experiments revealed that plasmids containing *hr*s replicated in infected cells but not in control cells. The conclusion that *hr*s function as origins of replication was confirmed by Kool et al. (16), who used a different strategy to identify potential origins of replication. Defective virus particles of AcNPV were generated by serial undiluted passage in insect cells. Analysis of the defective viral genomes revealed amplified sequences corresponding to the *hr3* and *hr5* regions of AcNPV DNA. However, a different result was obtained by Lee and Krell (19), who reported that defective genomes consisted of repeated sequences of a 3-kb DNA fragment corresponding to the *Hin*dIII-K region of the viral genome. This region of the AcNPV genome does not contain an *hr*, indicating that DNA replication may initiate at two structurally distinct type sequences in the baculovirus genome. It is currently not known

which origin is used during baculovirus replication. A recent report by Rodems and Friesen (27) indicates that *hr5* is not essential for virus growth. This suggests that the multiple origins are redundant in function.

To identify the viral proteins required for baculovirus DNA replication, a transient complementation assay was used (15, 17). These experiments revealed that proteins encoded by six viral genes (*dna pol*, *helicase*, *lef-1*, *lef-2*, *lef-3*, and *ie1*) are essential for replication of plasmids containing a viral origin of replication. In addition, products of the *ie2*, *pe38*, and *p35* genes stimulate the levels of DNA replication. The *dna pol* gene was first identified by using an oligonucleotide probe corresponding to an amino acid sequence that is conserved among other viral DNA polymerases (30); however, an enzymatic function has not been demonstrated for the protein encoded by this gene. The *helicase* gene was originally identified as the site of a mutation in the temperature-sensitive mutant ts8 (22). This mutant is unable to replicate viral DNA at the nonpermissive temperature. Computer analysis indicates that the protein contains a motif common to DNA helicases, although functional studies have not been conducted to confirm this hypothesis. The *lef* (lef = late expression factor) genes were mapped by using a reporter system for late gene expression (20, 24, 25). The transactivators *ie1*, *ie2*, and *pe38* are required for expression of early genes (2, 12, 23), and their role in DNA replication might be indirect. *p35* is a suppressor of apoptosis in infected cells and may also affect DNA replication indirectly by regulating timely expression of viral early genes (4, 8).

Most of the large DNA viruses encode single-stranded DNA-binding proteins (SSB) which are essential for replication of viral DNA (18). Therefore, we predicted that one of the genes shown to be essential for plasmid replication might encode a SSB. To test this possibility, we purified SSB from
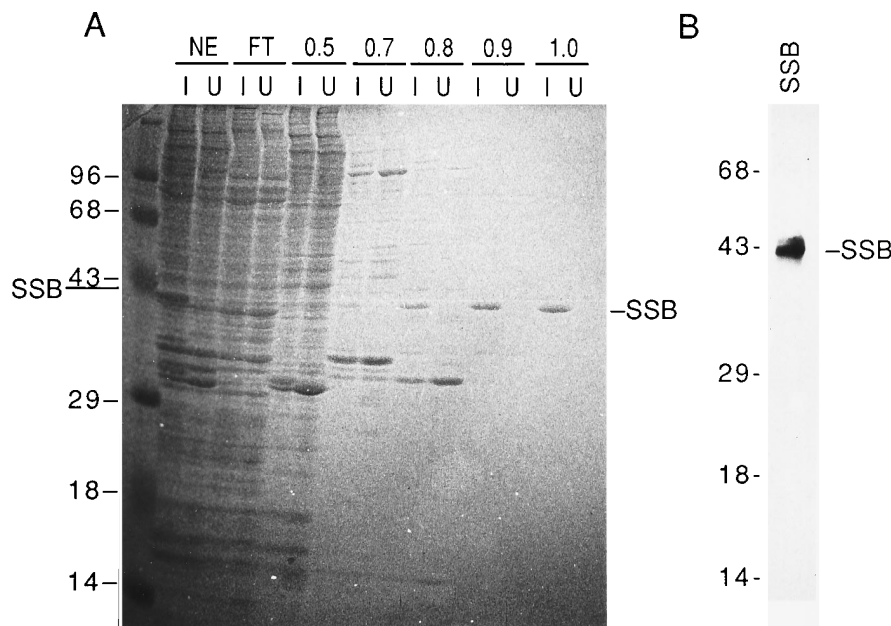

FIG. 1. ssDNA agarose chromatography of nuclear extracts. Nuclear extracts (NE) were prepared from AcNPV-infected (I) and uninfected (U) cells. The flowthrough (FT) and fractions eluted at the indicated concentrations of NaCl were analyzed by SDS-PAGE followed by staining with Coomassie blue. The migration of molecular size markers is shown on the left. The arrows indicate the position of the 43-kDa protein in the crude nuclear extract and the high-salt eluates. (B). SDS-PAGE analysis of the combined high-salt fractions. Proteins were identified by silver staining. The migration of molecular size markers is shown on the left, and the position of SSB is indicated by arrows.

AcNPV-infected cells. *Spodoptera frugiperda* cells (5×10^8 cells) were infected at a multiplicity of 10 (29) and harvested 16 h postinfection (p.i.). Infected cells were washed three times with phosphate-buffered saline and once with hypotonic buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 1 μg of leupeptin per ml). The cells were resuspended in 1 volume of hypotonic buffer, allowed to swell on ice for 10 min, and then lysed by 15 strokes in a Kontes Dounce homogenizer (B pestle). The homogenate was centrifuged at $2,000 \times g$ for 10 min to pellet nuclei. The nuclei were resuspended in an equal volume of hypotonic buffer containing 3.4 M NaCl. The extract was incubated for 1 h on ice and then clarified by centrifugation at $100,000 \times g$ for 60 min. The supernatant was dialyzed against buffer A (20 mM HEPES [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM dithiothreitol, 10% glycerol, 0.1 M NaCl) and then clarified by centrifugation. The soluble nuclear extracts were then adjusted to 10 mM EDTA and passed over a 1-ml column of single-stranded DNA (ssDNA) agarose (BRL). The column was washed with 1 ml of buffer A and then successively eluted with 2 ml of buffer A containing 0.2 M NaCl–1 M NaCl in 0.1 M increments.

Proteins from each fraction were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie blue (Fig. 1a). An abundant protein with an apparent molecular weight of 43,000 was eluted in the 0.8 to 1.0 M NaCl fractions. This protein was apparently a viral protein, as it was not observed in the uninfected cell extracts fractionated in parallel. Proteins eluting from ssDNA agarose at 0.9 and 1.0 M NaCl were pooled, treated with DNase I, and concentrated. The proteins were electrophoresed on a 12% gel and detected by silver staining (Fig. 1b). A single band was observed that migrated slightly faster than the ovalbumin marker (43 kDa), indicating that the protein was purified to homogeneity. The SSB was one of the
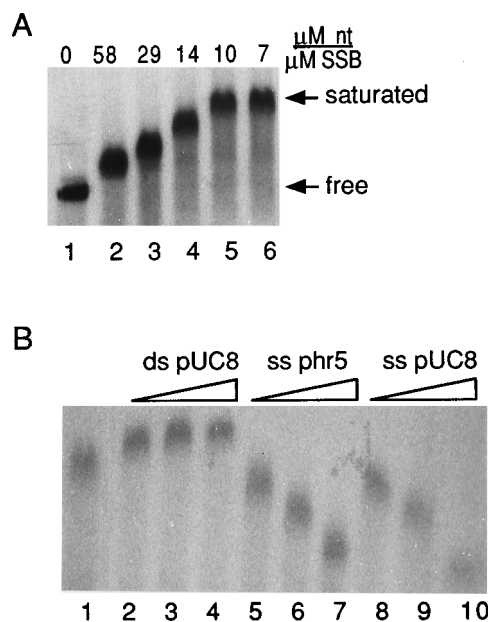

FIG. 2. ssDNA binding activity. (A) SSB binds ssDNA cooperatively. The plasmid p*hr5* contains a 484-bp fragment of *hr5* cloned into the *Mlu*I site of pIBI-24 (9). The plasmid was digested with *Hin*dIII, treated with calf intestinal phosphatase, and 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled DNA was denatured before use by heating to 100°C for 5 min. The standard assay mix contained 20 mM Tris (pH 7.8), 2 mM dithiothreitol, 1 mM EDTA, and 5′-end labeled pUC18 DNA and SSB in a total volume of 50 μl. The mixtures were incubated for 10 min at room temperature. After incubation, the samples were analyzed by 0.6% agarose gel electrophoresis. The gel electrophoresis running buffer was 20 mM Tris (pH 7.8), 0.4 mM sodium acetate, 0.2 mM EDTA (21). Electrophoresis was carried out at room temperature at constant voltage (7 V/cm) with buffer recirculation for 3 h. Gels were dried and exposed to X-ray film. (B) SSB binds ssDNA preferentially. End-labeled pUC18 DNA was incubated in the presence of 1.4 μg of SSB. Competitors were added in a 5-fold (lanes 2, 5, and 8), 10-fold (lanes 3, 6, and 9), or 20-fold (lanes 4, 7, and 10) molar excess: pUC18 DNA (lanes 2 to 4), thermally denatured p*hr5* (lanes 5 to 7), or thermally denatured pUC18 (lanes 8 to 10).
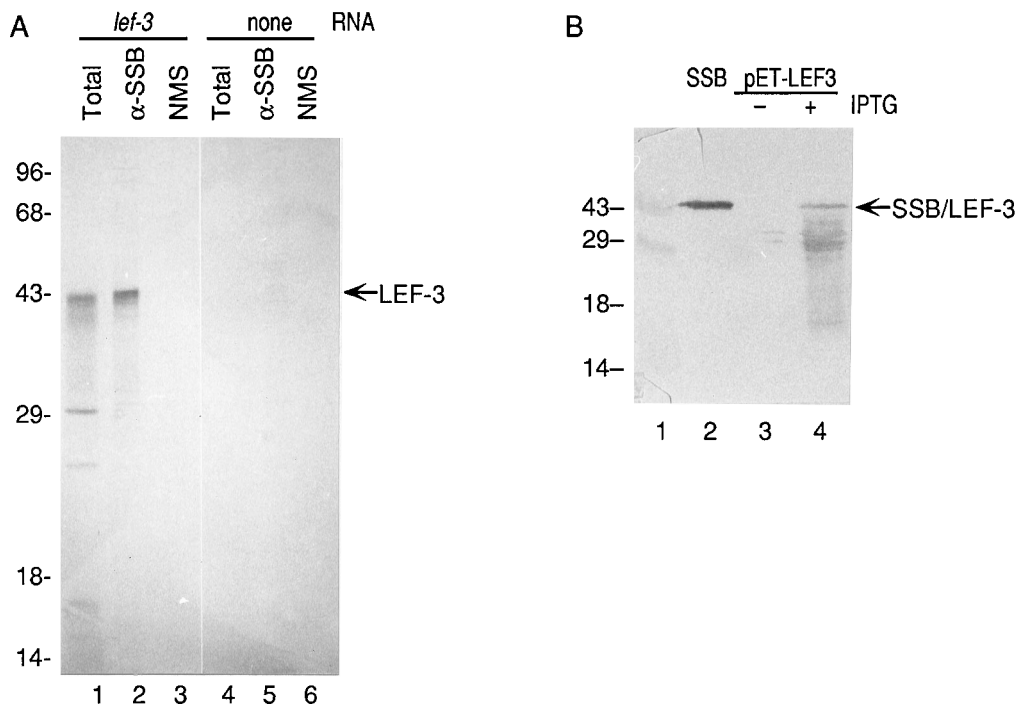
FIG. 3. SSB is encoded by the *lef-3* gene. (A) Immunoprecipitation of *lef-3* gene product. A plasmid containing the *Hin*dIII-B fragment of AcNPV DNA was digested with *Eco*RI and *Bgl*II. A 1,530-bp fragment containing the LEF-3 open reading frame (20) was cloned into the *Bgl*II and *Eco*RI sites of pET3a (28). The resulting plasmid (pET-LEF3) contains sequences coding for amino acid residues 6 to 385 of *lef-3* fused in frame with residues 1 to 13 of the T7 gene 10 protein. pET-LEF3 was used for in vitro transcription with T7 polymerase, followed by translation in rabbit reticulocyte extracts (lanes 1 to 3). As a control, translation reactions were performed in the absence of added RNA (lanes 4 to 6). [35S]methionine-labeled products were analyzed directly (lanes 1 and 4), after immunoprecipitation with mouse polyclonal serum prepared against purified SSB (lanes 2 and 5), or following immunoprecipitation with normal serum (lanes 3 and 6). The numbers at the left indicate the position and size in kilodaltons of molecular markers run on the same gel. The migration of the LEF-3 gene product is indicated by an arrow. (B) Immunoblot analysis of LEF-3 expressed in bacteria. Cultures of DE3(lysE) were transformed with pET-LEF-3. A recombinant was grown to an optical density at 600 nm of 0.6 and analyzed directly (lane 2) or induced with 0.5 mM IPTG (lane 3). After 2 h at 37°C, cells were collected by centrifugation and resuspended in SDS sample buffer. SSB purified from AcNPV-infected cells was added to lane 1. The proteins were electrophoretically transferred to nitrocellulose sheets by using a semidry apparatus (13). The nitrocellulose was reacted with monoclonal antibody 6G11, and immune complexes were detected by using alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G. Antibody against SSB was raised by injecting the purified protein mixed with an equal volume of complete Freund's adjuvant into BALB/C mice (20 μg in 200 μl per mouse). Mice were boosted three times at 3-week intervals with 30 μg of SSB mixed with an equal volume of incomplete Freund's adjuvant (13). Hybridoma cell lines were produced using standard procedures (13).

major proteins in infected nuclear extracts and can clearly be seen in the starting material (see arrow, Fig. 1a). The yield of purified protein was 6 μmol of SSB from 5×10^8 cells. Assuming a recovery of 100%, we calculate that there are approximately 7.2 million molecules of SSB per cell. The abundance of this protein is consistent with a structural role in viral DNA replication. SSB are needed in stoichiometric amounts, while the enzymes are less abundant, as they are only required in catalytic amounts.

To confirm that the retention of SSB on the column was due to binding of the protein to DNA, additional binding experiments were performed in solution (Fig. 2a). For these experiments, we used the plasmid p*hr5*, which contains a 484-bp *Mlu*I fragment of *hr5*, a potential origin of baculovirus DNA replication. In a series of parallel reactions, the amount of thermally denatured p*hr5* DNA was held constant at 110 μM and the concentration of SSB was varied from 1.9 to 15.2 μM. DNA-protein complexes were then separated by agarose gel electrophoresis. As shown in Fig. 2a, the amount of bound DNA increases with increasing protein concentration (lanes 1 to 6). A diffuse band was observed at a low protein-to-DNA ratio. This suggests that SSB binds with low to moderate cooperativity; however, this assay is not optimal for measurements of cooperativity. The binding data indicate that the DNA was saturated at an input ratio of 1 μmol of protein per 10 μmol of nucleotides.

To test whether binding of SSB to DNA was sequence specific, reactions were performed in the presence of an excess of thermally denatured pUC18 DNA or p*hr5* DNA (Fig. 2b). Both DNAs competed with equivalent efficiency (compare lanes 8 to 10 and 11 to 13), indicating that SSB does not bind preferentially to DNA containing origin of DNA replication. To test whether SSB bound double-stranded DNA (dsDNA), increasing molar amounts of pUC18 were added to the binding reactions. No competition was observed with a 20-fold molar excess of pUC18 DNA. We also tested the binding activity of SSB with ds p*hr5* or pUC8 DNA as a probe. These experiments also indicated that SSB did not bind dsDNA to a detectable extent (data not shown). Together, these data suggest that baculovirus SSB binds ssDNA preferentially and that it binds DNA in a non-sequence-specific fashion.

Plasmid replication in baculovirus-infected cells requires the products of the *dna polymerase*, *helicase*, *lef-1*, *lef-2*, *lef-3*, and *ie1* genes (15). The *lef-3* gene has a predicted molecular size of 44.5 kDa (20), which is close to the apparent size of SSB on SDS-PAGE (Fig. 1), indicating that SSB might be LEF3. To test this hypothesis, the open reading frame of *lef-3* was cloned under the control of the T7 promoter. This plasmid (pET-LEF3) was used for in vitro transcription, followed by in vitro translation in rabbit reticulocyte extracts. Translation products labeled with [35S]-methionine were directly analyzed by SDS-PAGE (Fig. 3, lane 1). LEF-3 produced in vitro migrated
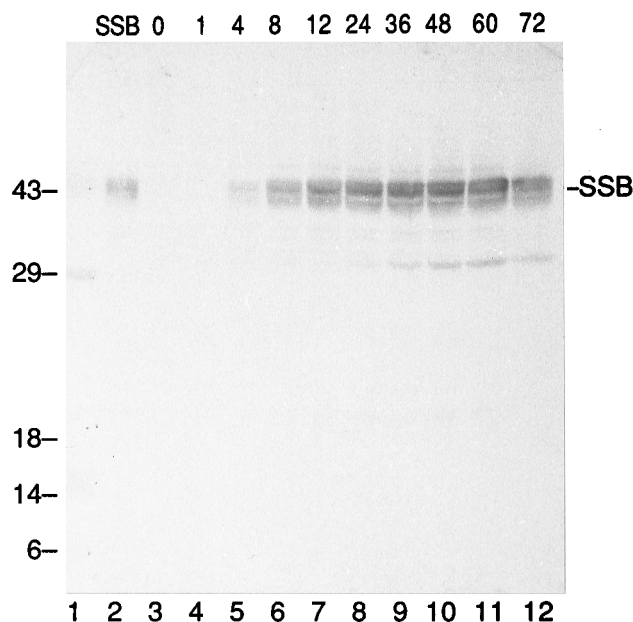
FIG. 4. Immunoblot analyses of SSB in AcNPV-infected cells. *S. frugiperda* cells in spinner culture were infected with AcNPV at a multiplicity of infection of 10. At the indicated times, 5 ml was removed from the culture, and the cells were pelleted by centrifugation. The pellet was resuspended in 1 ml of extraction buffer (50 mM Tris [pH 8.0], 100 mM NaCl, 1% Nonidet P-40), shaken for 20 min on ice, and clarified by centrifugation. Samples were boiled for 3 min and electrophoresed on 12% acrylamide gels. The numbers at the left indicate the positions of the protein standards, and the migration of SSB is indicated on the right.

slightly faster than the 43-kDa molecular marker, as did SSB purified from infected cells (Fig. 1). The *lef-3* translation product was specifically immunoprecipitated with a polyclonal antiserum raised in mice against purified SSB but not with control serum (compare lanes 2 and 3). As an added control, translation reactions were performed in the absence of added RNA. No labeled translation products were detected when analyzed directly or after immunoprecipitation (lanes 4 to 6).

To further confirm that SSB was encoded by the *lef-3* gene, LEF-3 was expressed in bacteria and immunoblots were probed with a monoclonal antibody raised against SSB. As shown in Fig. 4b, a monoclonal antibody specific for SSB, MAb 6G11, does not react with proteins in uninduced bacteria containing the *lef-3* gene under the control of bacteriophage T7 promoter. Addition of IPTG (isopropyl-β-D-thiogalactopyranoside) induced the synthesis of numerous proteins that reacted with MAb 6G11. The largest of the proteins comigrated with SSB purified from AcNPV-infected cells. The smaller bands probably represent proteolytic degradation products of LEF-3. We tested two other cloned hybridoma cell lines that were raised against SSB, and we found that these antibodies also recognized LEF-3 expressed in bacteria (data not shown). Together, these results are highly suggestive that *lef-3* encodes SSB.

We attempted to confirm that the *lef-3* gene product is a SSB. However, the LEF-3 expressed in bacteria was insoluble (data not shown), and, therefore, we were unable to attempt the DNA binding experiment. LEF-3 expressed in rabbit reticulocytes bound to ssDNA agarose. However, it eluted from the column at a lower salt concentration than SSB purified from infected cells (data not shown). This may reflect the fact that the protein is not folded correctly or that a posttranslational modification is needed for DNA binding activity.

To characterize the expression of SSB/LEF-3 in infected cells, a monoclonal antibody raised against purified SSB was used to probe immunoblots of infected cell extracts. SSB was first detected at 4 h p.i. (Fig. 4). Protein accumulated through 48 h and declined slightly after that. This pattern of protein synthesis is consistent with transcription mapping of the *lef-3* gene (20). *lef-3* RNA was synthesized by 3 h p.i., peaked at 6 h p.i., and then declined slightly by 12 h p.i. but remained detectable through 48 h p.i.

The *lef-3* gene was first identified as a factor required for expression of the baculovirus late genes capsid and polyhedrin (20). In baculoviruses, late gene expression is absolutely dependent upon viral DNA replication. Therefore, it is possible that the requirement for *lef-3* in late gene expression merely reflects the fact that transcription requires prior viral DNA replication. Alternatively, LEF-3 may have a direct effect on transcription of late genes. In at least three other viral systems, it has been demonstrated that SSB have distinct roles in DNA replication and stimulation of late gene expression. The adenovirus DNA-binding protein, the herpes simplex virus ICP8 protein, and the gene 32 protein of bacteriophage T4 have direct effects upon transcription of their cognate late genes (3, 6, 7). In addition, the DNA polymerase accessory proteins of T4 stimulate the opening of late promoters, and the replication fork acts as a mobile enhancer of T4 late gene expression (14).

REFERENCES

1. **Ayres, M. D., S. C. Howard, J. Kuzio, M. Lopez-Ferber, and R. D. Possee.** 1994. The complete DNA sequence of the *Autographa californica* nuclear polyhedrosis virus. Virology **202:**586–605.
2. **Carson, D. D., L. A. Guarino, and M. D. Summers.** 1988. Functional mapping of an AcNPV immediate early gene which augments expression of the IE-1 *trans*-activated 39K gene. Virology **162:**444–451.
3. **Chang, L. S., and T. Shenk.** 1990. The adenovirus DNA-binding protein stimulates the rate of transcription directed by adenovirus and adeno-associated virus promoters. J. Virol. **64:**2103–2109.
4. **Clem, R. J., and L. K. Miller.** 1993. Apoptosis reduces both the in vitro replication and the in vivo infectivity of a baculovirus. J. Virol. **67:**3730–3738.
5. **Cochran, M. A., and P. Faulkner.** 1983. Location of homologous DNA sequences interspersed at five regions in the baculovirus AcMNPV genome. J. Virol. **45:**961–970.
6. **Gao, M., and D. M. Knipe.** 1991. Potential role for herpes simplex virus ICP8 DNA replication protein in stimulation of late gene expression. J. Virol. **65:**2666–2675.
7. **Gauss, P., K. B. Krassa, D. S. McPheeters, M. A. Nelson, and L. Gold.** 1987. Zinc(II) and the single-stranded DNA binding protein of bacteriophage T4. Proc. Natl. Acad. Sci. USA **84:**8515–8519.
8. **Gong, M., and L. A. Guarino.** 1994. The apoptotic suppressor P35 increases expression of a delayed early gene of *Autographa californica* nuclear polyhedrosis virus. Virology **204:**38–44.
9. **Guarino, L. A., and W. Dong.** 1991. Expression of an enhancer-binding protein in insect cells transfected with the *Autographa californica* nuclear polyhedrosis virus IE1 gene. J. Virol. **65:**3676–3680.
10. **Guarino, L. A., and W. Dong.** 1994. Functional dissection of the *Autographa californica* nuclear polyhedrosis virus enhancer element *hr5*. Virology **200:**328–335.
11. **Guarino, L. A., M. A. Gonzalez, and M. D. Summers.** 1986. Complete sequence and enhancer function of the homologous DNA regions of *Autographa californica* nuclear polyhedrosis virus. J. Virol. **60:**224–229.
12. **Guarino, L. A., and M. D. Summers.** 1986. Interspersed homologous DNA of *Autographa californica* nuclear polyhedrosis virus enhances delayed-early gene expression. J. Virol. **60:**215–223.
13. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Herendeen, D. R., G. A. Kassavetis, and E. P. Geiduschek.** 1992. A transcriptional enhancer whose function imposes a requirement that proteins track along DNA. Science **256:**1298–1303.
15. **Kool, M., C. H. Ahrens, R. W. Goldbach, G. F. Rohrmann, and J. M. Vlak.** 1994. Identification of genes involved in DNA replication of the *Autographa*

californica baculovirus. Proc. Natl. Acad. Sci. USA **91:**11212–11216.

16. **Kool, M., P. M. M. M. Van den Berg, J. Tramper, R. W. Goldbach, and J. M. Vlak.** 1993. Location of two putative origins of DNA replication of *Autographa californica* nuclear polyhedrosis virus. Virology **192:**94–101.

17. **Kool, M., J. T. M. Voeten, R. W. Goldbach, and J. M. Vlak.** 1994. Functional mapping of regions of the Autographa californica nuclear polyhedrosis viral genome required for DNA replication. Virology **198:**680–689.

18. **Kornberg, A., and T. Baker.** 1992. DNA replication, 2nd ed. W. H. Freeman and Co., New York.

19. **Lee, H. Y., and P. J. Krell.** 1992. Generation and analysis of defective genomes of *Autographa californica* nuclear polyhedrosis virus. J. Virol. **66:**4339–4347.

20. **Li, Y., A. L. Passarelli, and L. K. Miller.** 1993. Identification, sequence, and transcriptional mapping of *lef-3*, a baculovirus gene involved in late and very late gene expression. J. Virol. **67:**5260–5268.

21. **Lohman, T. M., L. B. Overman, and S. Datta.** 1986. Salt-dependent changes in the DNA binding co-operativity of Escherichia coli single strand binding protein. J. Mol. Biol. **187:**603–615.

22. **Lu, A., and E. B. Carstens.** 1991. Nucleotide sequence of a gene essential for viral DNA replication in the baculovirus *Autographa californica* nuclear polyhedrosis virus. Virology **181:**336–347.

23. **Lu, A., and E. B. Carstens.** 1994. Immediate early baculovirus genes trans-activate the p143 gene promoter of *Autographa californica* nuclear polyhedrosis virus. Virology **195:**710–718.

24. **Passarelli, A. L., and L. K. Miller.** 1993. Three baculovirus genes involved in late and very late gene expression: *ie-1*, *ie-n*, and *lef-2*. J. Virol. **67:**2149–2158.

25. **Passarelli, A. L., and L. K. Miller.** 1993. Identification of *lef-1*, a baculovirus gene involved in late and very late gene expression. J. Virol. **67:**3481–3488.

26. **Pearson, M., R. Bjornson, G. Pearson, and G. Rohrmann.** 1993. The *Autographa californica* baculovirus genome: evidence for multiple replication origins. Science **257:**1382–1384.

27. **Rodems, S. M., and P. D. Friesen.** 1993. The *hr5* transcriptional enhancer stimulates early gene expression from the *Autographa californica* nuclear polyhedrosis virus genome but is not required for virus replication. J. Virol. **67:**5776–5785.

28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

29. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Tex. Agric. Exp. Stn. Bull. 1555.

30. **Tomalski, M. D., J. Wu, and L. K. Miller.** 1988. The location, sequence, transcription and regulation of a baculovirus DNA polymerase gene. Virology **167:**591–600.