JOURNAL OF VIROLOGY, May 1997, p. 3574–3579 Vol. 71, No. 5
0022-538X/97/$04.00+0

The Baculovirus Single-Stranded DNA Binding Protein, LEF-3, Forms a Homotrimer in Solution†

J. T. EVANS AND G. F. ROHRMANN*

Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331-7301

Received 11 December 1996/Accepted 5 February 1997

LEF-3 is one of six proteins from *Autographa californica* multinucleocapsid polyhedrosis virus required for transient DNA replication and has the properties of a single-stranded DNA binding protein. In this report we demonstrate that LEF-3 interacts with itself in both yeast two-hybrid assays and glutathione *S*-transferase fusion affinity assays. LEF-3 deletion clones which were unable to interact with full-length LEF-3 also failed to support transient DNA replication, suggesting that this interaction is required for the proper function of LEF-3. LEF-3 was purified to homogeneity and characterized by analytical ultracentrifugation and native polyacrylamide gel electrophoresis. These studies revealed that LEF-3 was present as a 132-kDa complex, indicating that its native conformation is that of a homotrimer. This result was confirmed by cross-linking with glutaraldehyde followed by matrix-assisted laser desorption/ionization mass spectrometry.

Autographa californica multinucleocapsid nuclear polyhedrosis virus (Ac*M*NPV) has a genome of 134 kb that encodes approximately 150 genes (1). Transient replication assays have been used to identify six essential and three stimulatory genes involved in baculovirus DNA replication (23, 32). The six genes required for DNA replication encode the following proteins: DNA polymerase and helicase, whose functions are implied by DNA sequence homology (31, 42); LEF-3, a single-stranded DNA binding protein (16); IE-1, a transcriptional activator (15) which also binds to putative replication origins (4, 14, 27, 30, 38); and two proteins, LEF-1 and LEF-2, neither of which have been assigned specific functions but which have been shown to interact with one another (10). The three gene products which are stimulatory for DNA replication include two additional transcriptional activators, IE-2 (2) and PE-38 (28), and P35, which blocks apoptosis (5, 18) and therefore may not function directly in DNA replication.

Characterization of the interactions between the products of these nine genes by using the yeast two-hybrid system revealed several interactions. We recently described the interaction of LEF-1 and LEF-2 and demonstrated that mutants which failed to interact also failed to support transient DNA replication (10). In this report, we describe the interaction between LEF-3 and itself and provide evidence indicating that LEF-3 forms a homotrimer in solution.

MATERIALS AND METHODS

Insect cells. *Spodoptera frugiperda* (Sf-9) cell (44) monolayers were cultured in TNM-FH medium (20) supplemented with 10% fetal bovine serum. Suspension cultures were maintained in serum-free SF900 II SFM medium (GIBCO-BRL) supplemented with penicillin G (50 U/ml), streptomycin (950 μg/ml) (both from BioWhittaker Inc.), and fungisone (375 ng/ml) (GIBCO-BRL). Cell culture maintenance was carried out according to published procedures (41).

Bacterial and yeast cells. All bacterial plasmids were maintained in *Escherichia coli* DH5α. *Saccharomyces cerevisiae* Y166 (*MAT***a** *ura3-52 leu2-3,112 his3*Δ*200 ade2-101 trp1-901 gal4*Δ *gal80*Δ RNR::GAL-URA3 LYS2::GAL-HIS3 GAL-LacZ) was used for the yeast two-hybrid assays.

Plasmid constructs. All baculovirus constructs were originally derived from the Ac*M*NPV E2 strain (40). *lef-1* was originally cloned as an *Nru*I-*Eco*RI fragment (map units [m.u.] 7.5 to 8.7 [23, 24]) into pUC19. *lef-2* was cloned as an *Mlu*I fragment (m.u. 1.9 to 2.6), and *lef-3* was cloned as an *Eco*RI-*Apa*I fragment (m.u. 42.8 to 44.5) into pUC19. *lef-1*, *lef-2*, and *lef-3* were subcloned into pBluescript (pKS−) (Stratagene, Inc.). *Nco*I sites were generated at the ATG start codon of each gene by site-directed mutagenesis (39) to form pKSLEF1(NcoI), pKSLEF2(NcoI), and pKSLEF3(NcoI), respectively. The primers used for mutagenesis are shown in Table 1. Mutagenesis changed the second amino acid of LEF-1 from leucine to valine and left LEF-2 and LEF-3 unchanged. pKSLEF1(NcoI), pKSLEF2(NcoI), and pKSLEF3(NcoI) functioned in transient replication assays as well as the parent clone, indicating that the sequence change had no effect on their ability to support transient DNA replication (data not shown).

Yeast two-hybrid clones. Full-length *lef-1* and *lef-3* were cloned into pASI and pACTII (gifts from Steve Elledge) (8) by digesting pKSLEF1(NcoI) and pKSLEF3(NcoI) with NcoI-*Bam*HI and ligating the fragment into pASI and pACTII cut with the same enzymes. C-terminal deletion constructs of LEF-3 (amino acids [aa] 1 to 370 and 1 to 314) were made by exonuclease III digestion (17), followed by subcloning into pASI. LEF-3 N-terminal deletion constructs pASLEF3 (aa 311 to 385, 244 to 385, 165 to 385, and 77 to 385) were created by introducing NcoI sites into pKSLEF3(NcoI) at codon positions 244, 311, 165, and 77 by site-directed mutagenesis followed by subcloning of *Nco*I-*Bam*HI fragments into pASI. The primers used are shown in Table 1.

GST fusions and in vitro transcription and translation constructs. Glutathione *S*-transferase (GST) fusion proteins were constructed with pGEX CS-1 vector (a gift from Bill Dougherty) modified from pGEX (Pharmacia) such that it contained an *Nco*I site downstream of the GST gene that allowed direct cloning of our constructs. C-terminal deletion constructs of LEF-3 (aa 1 to 370 and 1 to 314) were made as described above. *lef-1*, *lef-2*, and *lef-3* (full length, aa 1 to 370, and aa 1 to 314) were subcloned into pGEX CS-1 with *Nco*I-*Bam*HI (for pGEXLEF1, pGEXLEF2, and pGEXLEF3) or *Nco*I-*Sma*I (for pGEXLEF3aa1-370 and pGEXLEF3aa1-314). The in vitro transcription and translation plasmid pKSLEF3(TnT) was constructed by digesting pKSLEF3(NcoI) with *Nco*I-*Apa*I, treating the digestion products with T4 DNA polymerase, and religating the vector, thus eliminating the *lef-3* promoter region and bringing the bacterial T3 promoter (from pKS−) within 40 bp of the translational start site for *lef-3*.

Seven-His fusion clone of *lef-3*. A seven-histidine N-terminal fusion construct of *lef-3* was made using pTrc-7Hpro vector (a gift from Bill Dougherty) modified from pTrc 99A (Pharmacia Biotech) such that it contained an *Nco*I site downstream of seven histidine codons; this allowed direct cloning of our constructs by using *Nco*I and *Bam*HI.

Miscellaneous procedures. (i) Site-directed mutagenesis. Site-directed mutagenesis was accomplished by following the procedure of T. A. Kunkel et al. (29, 39). The primers used to generate the clones used in this study are shown in Table 1.

(ii) Yeast two-hybrid transfections and liquid assays, GST fusion affinity assays, and transient DNA replication assays. These procedures were carried out as previously described (10).

LEF-3 purification. (i) Nuclear extracts. Nuclear extracts were prepared at 16 h postinfection as previously reported by Glocker et al. (12).

(ii) Purification. All procedures were carried out at 4°C. Fifteen milliliters of nuclear extract (from 1.5 liters of infected Sf-9 cells) was dialyzed against buffer E (20 mM HEPES [pH 7.5]), 5 mM KCl, 1.5 mM $MgCl_2$, 10 mM EDTA, 10%

* Corresponding author. Mailing address: ALS 1007, Department of Agricultural Chemistry, Oregon State University, Corvallis, OR 97331-7301. Phone: (541) 737-1793. Fax: (541) 737-0497. E-mail: rohrmann@bcc.orst.edu.

† Technical report 11,134 from Oregon State University Agricultural Experiment Station, Corvallis.

TABLE 1. Primers used for site-directed mutagenesis

Clone[a]	Location(s) of *Nco*I sites (codon no.)	Sequence of primer
pKSLEF-1(NcoI)	1	GTTCAAAGGGCACCATGGTAGTGTGCAATTAT
pKSLEF-2(NcoI)	1	AGAAGCCGCGAACCATGGCGAATGCA
pKSLEF-3(NcoI)	1	TCGACAACAGCACCATGGCGACCAAA
pKSLEF-3(NcoI)	1 and 244	CGAGGCTAAAGAAACCATGGCATATTCAAATTTG
pKSLEF-3(NcoI)	1 and 311	TCTACCTCTTCCGCCATGGGCAAATGGAA
pKSLEF-3(NcoI)	1 and 165	CAACGACGATCCCATGGACGTGGTTCAAG
pKSLEF-3(NcoI)	1 and 77	GCAATCTTTCAAAGCCATGGAAGAAGGC

[a] The last four clones were created from pKSLEF-3(NcoI), which has an *Nco*I site at codon 1.

glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) plus 100 mM NaCl and then clarified by centrifugation. The extract was passed over a 20-ml single-stranded DNA agarose (GIBCO-BRL) column previously equilibrated in buffer E–100 mM NaCl. The column was washed with 50 ml of buffer E–100 mM NaCl and 100 ml of buffer E–500 mM NaCl. LEF-3 was eluted from the column with a 20-ml gradient from 500 mM to 1 M NaCl in buffer E. Fractions containing LEF-3 (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) were dialyzed against buffer E and concentrated in a Centriplus 10-kDa concentrator (Amicon). Protein concentration was determined by protein assay (Bio-Rad).

The concentrated protein was diluted to an appropriate concentration with buffer E, and 100-μl aliquots were applied to a prepacked Superose 12 HR 10/30 column (Pharmacia Biotech) preequilibrated in buffer E. The column was resolved at a flow rate of 0.05 ml/min, and 100-μl fractions were collected. Peak fractions were pooled, concentrated in a Centricon 10-kDa microconcentrator (Amicon), and frozen at −80°C.

PAGE, Western blotting, and polyclonal antiserum production. SDS–12% PAGE was performed as described by Sambrook et al. (39). Gels were either fixed and stained with Coomassie brilliant blue (Bio-Rad) or used for Western blots as previously described (36, 37). Western blots were probed with a 1:1,000 dilution of rabbit polyclonal antiserum to LEF-3, washed, incubated with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad), and developed with a chemiluminescence blotting substrate in accordance with the manufacturer's instructions (Boehringer Mannheim).

Rabbit polyclonal antiserum for LEF-3 was prepared by expressing an N-terminal seven-His-tagged fusion construct of LEF-3 (see Plasmid constructs) in *E. coli* DH5α followed by purification on Ni-nitrilotriacetic acid resin (Qiagen) according to the manufacturer's instructions. Purified His-tagged LEF-3 was subcutaneously injected into a New Zealand White rabbit with complete Freund's adjuvant for the initial injection and incomplete Freund's adjuvant for subsequent injections (2 to 3 weeks between injections). Rabbit antiserum was collected 1 week after the third injection and tested by Western blot analysis.

Native PAGE was performed on a Hoefer SE 600 gel electrophoresis unit with a 4 to 12% gradient polyacrylamide gel. Standards used in native PAGE were from Sigma (gel filtration molecular weight marker kit). Gels were fixed and stained with Coomassie brilliant blue.

MALDI. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) was performed on a custom-built time-of-flight mass spectrometer (7, 11, 19). A voltage of 24 kV was used to accelerate ablated ions from the sample stage into the instrument's flight tube. Every mass spectrum was recorded as the sum of 30 consecutive spectra, each produced by a single pulse of 355-nm photons from a Nd:YAG laser (Spectra Physics). Matrix ions or ions from an added standard were used for mass calibration.

All samples were analyzed with a standard saturated solution of HCCA (α-cyano-4-hydroxy cinnamic acid; Aldrich) in a matrix of 33% acetonitrile–10% formic acid. For each mass analysis, 0.5 μl of the solution to be analyzed was mixed in a 1:3 ratio with the matrix solution and 0.5 μl of this mixture was deposited on the sample stage. At the first sign of crystal formation (generally 10 to 15 s after deposition when viewed under a stereomicroscope) the droplet was gently wiped off with a lab tissue, leaving a seed layer of crystallites on the surface of the sample stage. Another 0.5-μl portion of the mixture of matrix and the solution to be analyzed was then deposited on top of the seed layer and allowed to dry. The crystals were washed with water and used for MALDI.

Cross-linked samples were prepared by adding glutaraldehyde directly to purified LEF-3 (2.0 mg/ml) to give final concentrations of 0.1 to 6% glutaraldehyde. Samples were left at 23°C for 1 h and then immediately analyzed by MALDI.

Analytical ultracentrifugation. Purified LEF-3 was dialyzed overnight against buffer F (20 mM HEPES, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and adjusted to an optical density at 280 nm of 0.25 with the same buffer. Sedimentation equilibrium experiments were carried out with the Beckman Optima XL-A analytical ultracentrifuge. The rotor temperature was 20°C and the speed was fixed at 6,000 rpm. An equilibrium analysis was carried out after a 36-h run (equilibrium had been reached since no change in distribution occurred over a 6-h period). The analysis of the data used a non-linear, least-squares fitting routine of the program XL-A version 2.41 (34). The solution density used for the equilibrium analysis was 1.00538 g/cm^3, and the partial specific volume for LEF-3 was calculated as 0.728 cm^3/g (6, 35).

RESULTS

LEF-3 interaction experiments. A yeast two-hybrid system based on the modular nature of the GAL4 DNA binding and activation domains (3, 9, 13, 21, 33) was used to examine the interactions of the nine baculovirus gene products previously identified as being involved in DNA replication (23, 32). All nine of the replication genes were cloned into pASI, containing the GAL4 DNA binding domain, and pACTII, containing the GAL4 activation domain (8). An initial screening of these clones revealed several interactions, including an interaction involving LEF-3 with itself in both plate and liquid assays (Fig. 1, row 1). No β-galactosidase activity was detected when double recombinants containing the LEF-3 DNA binding domain or activation domain fusions and the control reciprocal vectors were made (data not shown). This indicated that the full-length fusion proteins did not have any intrinsic capacity to transcriptionally activate the GAL4 promoter. The original pASI plasmid (without fusion protein) has some capability for transactivating GAL4 promoters when transfected alone. However, when an insert is introduced into the multiple-cloning site, this capability is eliminated. Fusions of LEF-1 were used as negative controls for these experiments because they did not interact with LEF-3 in the yeast two-hybrid system or the GST fusion affinity assays (data not shown; Fig. 2, lane 2). Yeast transformed with LEF-3 (in pASI or pACTII) grew slowly on dropout plates and in liquid cultures. Plates were incubated at 30°C for 5 to 6 days (3 to 4 days is normal) before plate assays, and liquid cultures were grown for 24 to 36 h (12 to 16 h is normal) before liquid assays were performed. The slow growth of yeast transformed with LEF-3 did not affect the reproducibility of the plate or liquid assays.

Several N- and C-terminal deletion clones of LEF-3 were constructed (aa 1 to 370, 1 to 314, 311 to 385, 244 to 385, 165 to 385 and 77 to 385; Fig. 1, rows 2 to 7), all of which failed to interact with full-length LEF-3 in yeast two-hybrid assays. These data suggested that either the interaction domain in LEF-3 contains components of both the N- and C-terminal regions or the deletions we examined disrupt secondary structures required for interaction.

To confirm the LEF-3 interaction observed in the yeast two-hybrid system, we examined the interaction between in vitro-transcribed and -translated LEF-3 and GST fusion proteins (Fig. 2). GST fusion constructs were made with *lef-1*, *lef-2*, *lef-3*, *lef-3* aa1-370, and *lef-3* aa1-314 (called pGEXLEF1, pGEXLEF2, pGEXLEF3, pGEXLEF3aa1-370, and pGEXLEF3aa1-314, respectively), expressed in *E. coli*, and whole-cell extracts were prepared. The expression of a fusion protein of the expected size was confirmed by incubating the whole-cell extract

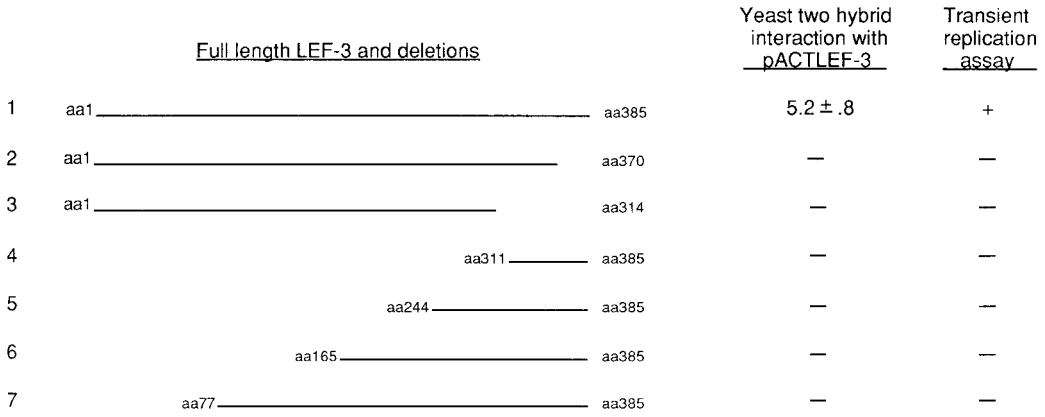

FIG. 1. Diagram of full-length LEF-3 and deletion clones. On the left are diagrams showing the portions of LEF-3 present in the mutants analyzed. Clone 1 is the full-length LEF-3. In the middle column, the levels of *lacZ* expression in yeast that contained both the LEF-3 clones diagrammed (in pAS1) and the full-length LEF-3 cloned into pACTII are shown. *lacZ* activity was determined for double recombinants which gave a blue color on plate assays; those which gave no blue color are indicated by a dash. All deletion clones (clones 2 to 7) were negative on plate assays. Full-length LEF-3 was positive on plate assays, and the number represents *lacZ* expression calculated from four liquid assays after two independent transformations (± standard deviation). Specific activity is expressed as nanomoles per minute per milligram of protein. The right column shows the ability of each construct to function in transient replication assays.

with glutathione Sepharose beads and washing with phosphate-buffered saline to remove unbound protein. The retained proteins were eluted and sized by SDS-PAGE. All five constructs showed protein fusion products of the expected size (data not shown). Each GST fusion was bound to glutathione Sepharose beads and incubated with ^{35}S-radiolabeled LEF-3 generated by in vitro transcription and translation reactions. After extensive washing, the bound proteins were eluted, separated by SDS-PAGE, and analyzed by autoradiography (Fig. 2). The input in vitro-transcribed and -translated LEF-3 is shown in lane 1. A major band of 44 kDa is present, consistent with initiation at the first ATG. Several minor bands of lower molecular weights are also present and correspond to translation from several in-frame downstream ATGs. When the radiolabeled LEF-3 was incubated with the GST fusions bound to glutathione Sepharose beads, the following was observed (Fig. 2): LEF-3 did not interact with GST-LEF1 (lane 2) or GST-LEF2 (lane 3); LEF-3 did interact with GST-LEF3 (lane 6) but did not interact with GST-LEF3aa1-370 (lane 4) or GST-LEF3aa1-314 (lane 5).

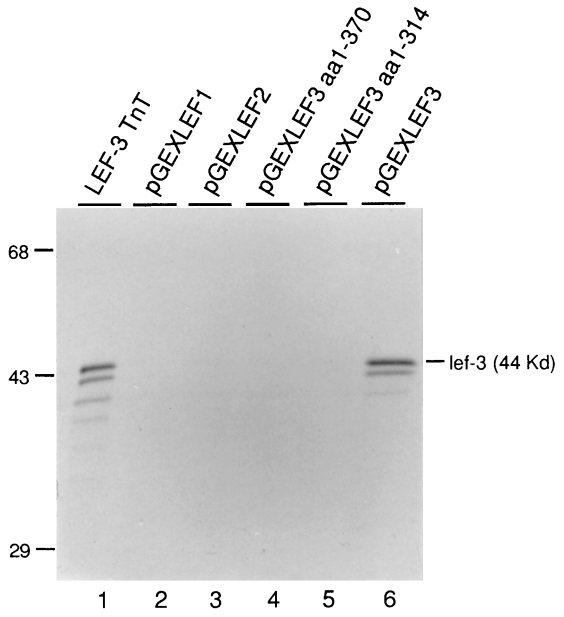

FIG. 2. Analysis of the interaction of GST fusions with in vitro-transcribed and -translated LEF-3. Lane 1 shows the input in vitro-transcribed and -translated ([^{35}S]methionine-labeled) LEF-3. The subsequent lanes show the labeled LEF-3 which was retained by glutathione Sepharose beads previously incubated with GST fusions of LEF-1 (lane 2); LEF-2 (lane 3); LEF-3, aa 1 to 370 (lane 4); LEF-3, aa 1 to 314 (lane 5); or full-length LEF-3 (lane 6).

Replication assay with LEF-3 and LEF-3 deletions. LEF-3 is required for transient replication of an origin-containing plasmid (23). To determine if the interaction between LEF-3 and itself was a prerequisite for its ability to function in baculovirus DNA replication, selected clones (aa 1 to 370, 1 to 314, 311 to 385, 244 to 385, 165 to 385, and 77 to 385 [Fig. 1, rows 2 to 7]) were placed behind their native promoters and used in transient DNA replication assays (23, 25). All of the deletion clones used were negative for interaction with full-length LEF-3 and failed to support DNA replication (data not shown and Fig. 1), suggesting either that LEF-3 oligomerization is required for the proper function of LEF-3 or that the deletions disrupted secondary structures required for both interaction and replication.

LEF-3 purification and oligomeric structure determination. Determination of the oligomeric structure of LEF-3 required isolation of the protein in a purified form. To accomplish this, 16-h-postinfection nuclear extracts were prepared (12) and subjected to single-stranded DNA agarose chromatography by using a modification of the method of Hang et al. (16). LEF-3 eluted from the single-stranded DNA agarose column in 800 mM to 1 M NaCl, resulting in 80 to 90% purification of LEF-3 (Fig. 3a). A second protein of 140 to 150 kDa coeluted with LEF-3 from the single-stranded DNA agarose column (Fig. 3a), suggesting that it interacted with LEF-3. Western blot analysis with antihelicase (P143) polyclonal antiserum identified this protein as helicase (P143) (unpublished data). These two proteins were separated by Superose 12 gel filtration, resulting in a highly purified LEF-3 that migrated with an apparent molecular mass of 44 kDa by SDS-PAGE (Fig. 3b). While Superose 12 gel filtration was an adequate technique for purification, the LEF-3 complex that passed through the column eluted at variable apparent sizes ranging from 205 to 255 kDa depending upon the amount of LEF-3 loaded onto the

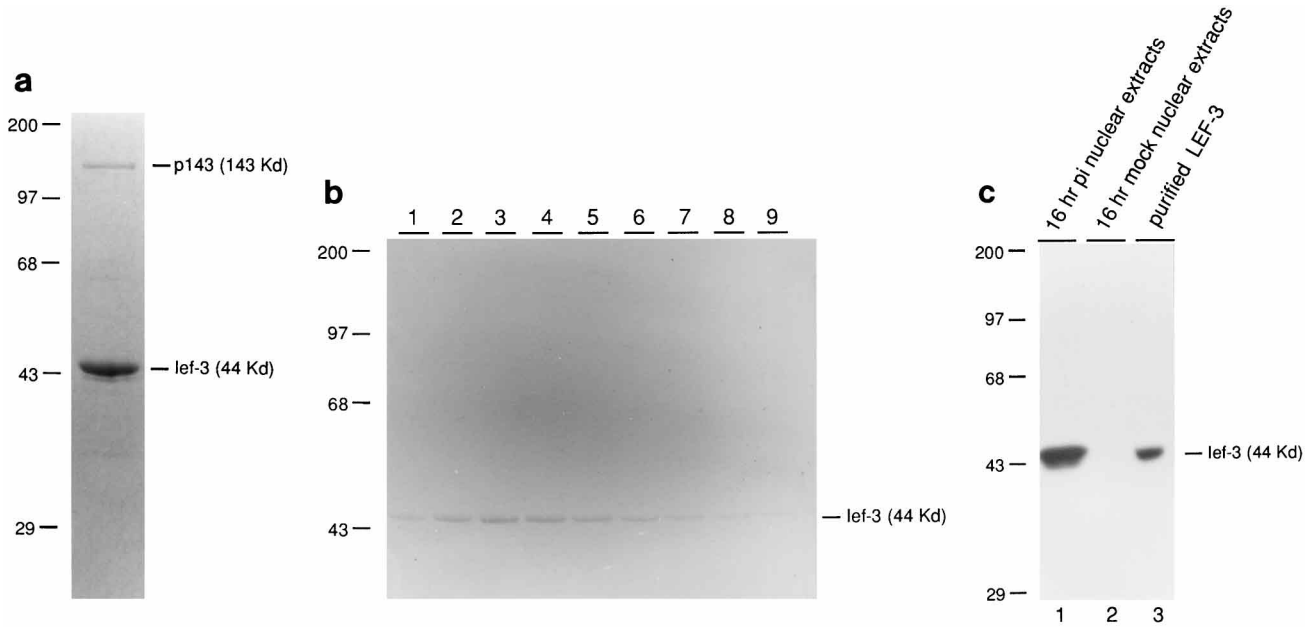

FIG. 3. Purification of LEF-3. (a) SDS-PAGE of peak fractions from single-stranded DNA agarose chromatography after concentration in a Centriplus 10-kDa concentrator. The positions of the molecular mass markers (in kilodaltons) are on the left, and the positions of helicase (P143) and LEF-3 are shown on the right. (b) SDS-PAGE of 100-μl fractions containing the LEF-3 protein peak from Superose 12 gel filtration. The numbers shown at the top represent fractions collected after 9 ml of buffer E passed through the column from the time of loading. (c) Western blot analysis of purified LEF-3 with LEF-3 polyclonal antiserum. Lane 1, 16-h-postinfection (pi) nuclear extracts; lane 2, 16-h mock-infected nuclear extracts; lane 3, purified LEF-3.

column. This variability made it difficult to assign an accurate molecular weight to the purified complex.

To confirm that the purified protein corresponded to the Ac*M*NPV *lef-3* gene, polyclonal antiserum was produced against an N-terminal histidine-tagged LEF-3 which was expressed in *E. coli* and purified with Ni-nitrilotriacetic acid resin (Qiagen). The antiserum specifically recognized LEF-3 in 16-h-postinfection nuclear extracts and in purified LEF-3 (Fig. 3c, lanes 1 and 3) but did not recognize any proteins from mock-infected 16-h nuclear extracts (lane 2). Purified LEF-3 was tested for single-stranded DNA binding protein (SSB) activity in gel shift assays with ^{32}P-labeled single-stranded DNA, resulting in observations similar to those previously reported (16) (data not shown).

Nondenaturing PAGE and analytical ultracentrifugation. Native PAGE (4 to 12% gradient PAGE) analysis of purified LEF-3 revealed a single band, indicating that the majority of protein present was complexed as a single oligomeric structure and not as a mixture of different oligomeric forms (Fig. 4). The molecular mass of the complex was calculated to be about 131 kDa from a curve derived from standards run on the same gel. This suggested that LEF-3 may form a homotrimer in solution.

To further investigate the oligomerization of LEF-3, sedimentation equilibrium of purified LEF-3 was carried out with a Beckman Optima XL-A analytical ultracentrifuge. The technique of sedimentation equilibrium depends upon the distribution of the solute in the sample well at equilibrium, where the distribution due to diffusion is balanced by the distribution due to sedimentation. This allows calculation of the average molecular weight of the solute (43). Figure 5 shows the distribution of LEF-3 in the sample well after 36 h at 6,000 rpm (20°C). Equilibrium analysis at 36 h showed that purified LEF-3 formed a complex of 132 kDa, indicating that LEF-3 is a homotrimer.

MALDI of cross-linked LEF-3. The oligomeric structure of LEF-3 was also examined by cross-linking the protein using 0.1 to 6% glutaraldehyde followed by MALDI analysis. MALDI can cause a slight oligomerization of proteins, so it is necessary to compare cross-linked with non-cross-linked samples when analyzing subunit structures (11). Figure 6a shows non-cross-

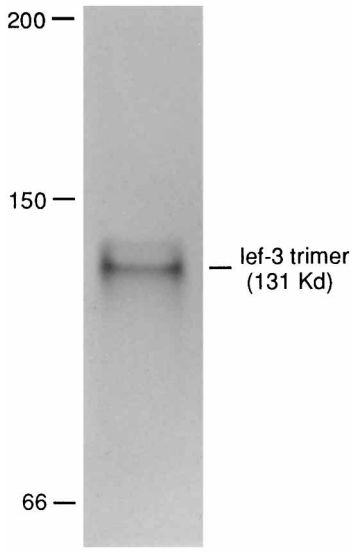

FIG. 4. Native PAGE (4 to 12%) analysis of purified LEF-3. Purified LEF-3 was subject to native PAGE for 6 h at 150 V. The standards used were from Sigma (gel filtration molecular weight marker kit). The gel was stained with Coomassie brilliant blue (Bio-Rad). Molecular mass markers in kilodaltons are on the left.

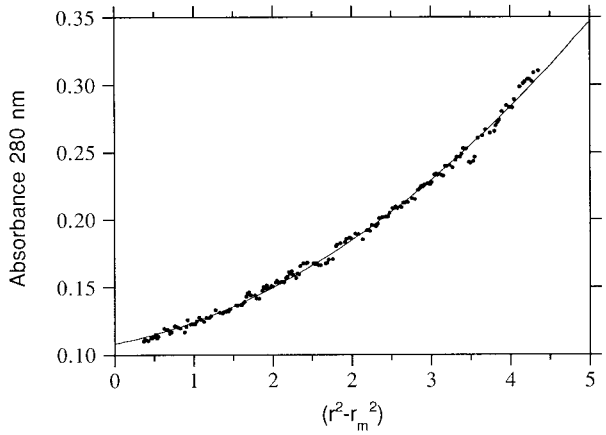

FIG. 5. Sedimentation equilibrium of purified LEF-3 after 36 h at 6,000 rpm (20°C). The absorbance was monitored at 280 nm and is plotted as a function of the radial position, where r^2 is the square of the distance from the axis of rotation and r_m^2 is the square of the distance of the meniscus from the axis (both expressed in centimeters squared). The equilibrium analysis was carried out as described in Materials and Methods.

linked LEF-3 analyzed by MALDI. The 44.6-kDa single-charged monomer is the major species; however, the oligomerization caused by MALDI can be seen in the single- and double-charged species in the form of dimers, trimers, and tetramers. The double-charged dimer and tetramer peaks are masked by the single-charged monomer and dimer peaks.

MALDI analysis of LEF-3 cross-linked with 0.1% glutaraldehyde is shown in Fig. 6b. This sample clearly shows an increase in the size of the single- and double-charged trimer peaks (141,300 and 70,700 Da, respectively) relative to the monomer peaks. Peak area analyses indicated that the cross-linked single- and double-charged trimer peaks were five and seven times larger, respectively, than the corresponding non-cross-linked peaks relative to the monomer peak in each MALDI run. The sizes of the dimer and tetramer peaks increased slightly in the cross-linked sample but did not change significantly (less than onefold change) relative to the monomer peak in each of the MALDI runs. The increase in size of the trimer peaks in the cross-linked samples clearly indicates that LEF-3 is a homotrimer.

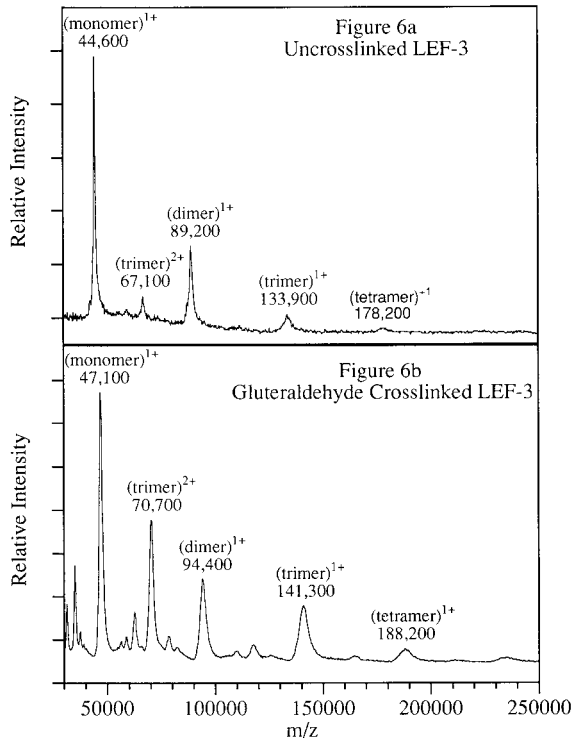

FIG. 6. MALDI of purified LEF-3. (a) Non-cross-linked LEF-3; (b) LEF-3 cross-linked with 0.1% gluteraldehyde.

The increased molecular weights of the cross-linked complexes are due to the addition of the glutaraldehyde cross-linker. At 0.1% glutaraldehyde (100.1 molecular weight), about 25 molecules of glutaraldehyde were added per subunit of LEF-3. Although at higher concentrations of glutaraldehyde (0.2 to 6%), the sizes of the trimer peaks did not increase significantly, the molecular weight increased proportionally with the increasing concentration of glutaraldehyde (data not shown).

DISCUSSION

In this report, we have demonstrated by both yeast two-hybrid analysis and GST fusion affinity experiments that LEF-3 interacts with itself. LEF-3 deletion constructs which disrupted this interaction also eliminated the ability of the protein to function in transient replication assays, suggesting that this interaction is essential for the proper function of LEF-3. It is also possible that these deletions disrupted secondary structures required for both interaction and replication.

We purified LEF-3 to homogeneity and used native PAGE, analytical ultracentrifugation, and chemical cross-linking to determine the oligomeric structure of LEF-3. The purified LEF-3 monomer was determined to have an actual molecular mass of 44.6 kDa by MALDI, which is close to what would be predicted from the amino acid sequence (44.5 kDa). Native PAGE, analytical ultracentrifugation, and chemical cross-linking studies demonstrated that LEF-3 forms a homotrimer in solution.

SSBs have been reported to form monomers, homodimers, and homotetramers (26), and the human SSB RP-A forms a heterotrimer consisting of 70- (Rpa1), 34- (Rpa2), and 13- (Rpa3) kDa subunits (22, 45). LEF-3 is the first SSB reported to form a homotrimer.

During the purification of LEF-3 by single-stranded DNA agarose chromatography, a larger protein of approximately 140 to 150 kDa coeluted with LEF-3 in 800 mM to 1 M NaCl (Fig. 3a). This protein followed the same elution profile as LEF-3, indicating that the two proteins may have interacted. Western analysis using antihelicase (P143) polyclonal antiserum indicated that the 140- to 150-kDa protein was helicase (P143) (unpublished data). Helicase (P143) has a calculated molecular mass of 143 kDa, and its gene has been identified as one of the six genes required for replication of an origin-containing plasmid (23, 32). The interaction evidence presented here and previously reported for LEF-1 and LEF-2 (10) suggests that Ac*M*NPV DNA replication involves a complex set of interactions between a viral DNA replication complex and both double- and single-stranded DNA. Further analysis of the interactions between baculovirus replication proteins should lead to an understanding of the roles each of these proteins plays in baculovirus DNA replication.

ACKNOWLEDGMENTS

The assistance of Lilo Barofsky of the Mass Spectrometry Facility and Services Core Unit of the Environmental Health Science Center at Oregon State University and Dean Malencik and Sonia Anderson with

analytical ultracentrifugation is gratefully acknowledged. We also thank Bill Dougherty and Steve Elledge for providing the plasmid vectors used in this study. We thank Douglas Leisy for suggestions and criticisms of the manuscript. The hospitality of M. Chamberlin and C. Kane and members of their laboratory to G.R. during his sabbatical leave is gratefully acknowledged.

This project was supported by grants from the NSF (MCB-9630769), ACS (SG-208), and NIEHS (ES 000210).

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