JOURNAL OF VIROLOGY, Apr. 1997, p. 3114–3119 Vol. 71, No. 4
0022-538X/97/$04.00+0

Characterization of the Interaction between the Baculovirus Replication Factors LEF-1 and LEF-2†

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Received 23 September 1996/Accepted 16 January 1997

The *Autographa californica* multinucleocapsid nuclear polyhedrosis virus has six genes required and three genes stimulatory for transient DNA replication. We demonstrate that the products of two of these genes, LEF-1 and LEF-2, interact in both two-hybrid assays using *Saccharomyces cerevisiae* and glutathione *S*-transferase fusion affinity assays. Using yeast-two-hybrid assays, we mapped the interaction domain of LEF-2 to amino acids between positions 20 and 60. Extensive deletion analyses of LEF-1 failed to reveal a delimited interaction domain, suggesting that there may be essential secondary structural elements that are inactivated by these deletions. All clones expressing LEF-1 and LEF-2 that were unable to interact also failed to support significant levels of transient DNA replication, suggesting that this interaction is required for DNA replication. Sequence analysis of LEF-1 revealed a primase-like motif, WVVDAD. When this motif was mutated to WVVQAD, LEF-1 no longer supported transient DNA replication.

Baculoviruses are a diverse family of invertebrate viruses with large covalently closed, circular, double-stranded DNA genomes (3). The most well-characterized baculovirus, *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (Ac*M*NPV), has a genome of 134 kb which encodes approximately 150 genes (1). Although baculoviruses have received widespread attention in recent years due to their ability to hyperexpress heterologous genes under the control of very late promoter elements (2, 35), little is known about their replication cycle. Recently, methods for the analysis of defective genomes and the development of transient replication assays have been used to identify putative origins of DNA replication (22, 24, 30, 31, 36). Subsequently, a modification of the transient assay system was used to identify a subset of six essential and three stimulatory genes involved in baculovirus DNA replication (21, 33). The six genes required for DNA replication encode the following proteins: DNA polymerase and helicase, whose functions are implied by DNA sequence homology (32, 41); LEF-3, a single-stranded DNA binding (SSB) protein (14); IE-1, a transcriptional activator (13) which also binds putative replication origins (6, 12, 27, 37); and LEF-1 and LEF-2, to which functions have not yet been assigned. The three stimulatory gene products include two additional transcriptional activators, IE-2 (4) and PE-38 (28), and a gene, P35, which blocks apoptosis (7, 16) and therefore may not function directly in DNA replication.

In order to examine the functional interrelationships of baculovirus replication factors, we have characterized interactions occurring among the nine proteins involved in replication and investigated the functional significance of these interactions. In this report, we describe the interaction between Ac*M*NPV LEF-1 and LEF-2 by two-hybrid analyses using *Saccharomyces cerevisiae* and glutathione *S*-transferase (GST) fusion affinity experiments.

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† Technical report 11120 from the Oregon State University Agricultural Experiment Station.

MATERIALS AND METHODS

Insect cells. *Spodoptera frugiperda* (Sf-9) cells (42) were cultured in TNM-FH medium (18) supplemented with 10% fetal bovine serum (FBS). Cell culture maintenance was carried out according to published procedures (40).

Bacterial and yeast cells. All bacterial plasmids were maintained in *Escherichia coli* DH5α. *S. 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 and was the gift of Steve Elledge.

Plasmid constructs. All baculovirus constructs were originally derived from the Ac*M*NPV E2 strain (39).

Yeast-two-hybrid clones. *lef-1* was originally cloned as an *Nru*I-*Eco*RI fragment (map units [m.u.] 7.5 to 8.7 [21, 23]) into pUC19. *lef-2* was cloned as an *Mlu*I fragment (m.u. 1.9 to 2.6) into pUC19. *lef-1* and *lef-2* were both subcloned into pBluescript (pKS$^-$) (Stratagene, Inc.), and *Nco*I sites were generated at the ATG start codon of each gene by site-directed mutagenesis (38) to form pKSLEF1(NcoI) and pKSLEF2(NcoI), respectively. The primers used are shown in Table 1. Mutagenesis changed the second amino acid of LEF-1 from leucine to valine and left the LEF-2 amino acid sequence unchanged. *Nco*I-*Bam*HI inserts from these constructs were subcloned into the yeast vectors pASI and pACTII (gifts from Steve Elledge) (8), creating fusions with the GAL4 DNA binding and GAL4 activation domains, respectively.

C-terminal deletion constructs of LEF-1 (aa1-241 [containing amino acids {aa} 1 to 241], aa1-222, aa1-215, and aa1-197) were made by exonuclease III digestion (15). pASLEF1aa1-129 was created by digesting pASLEF1 with *Eco*RI and *Dra*I (*Dra*I cuts at codon 129) followed by subcloning the fragment into *Eco*RI-*Sma*I-digested pASI. C-terminal deletion aa1-175 and N-terminal deletion aa175-266 were created by digesting pKSLEF1(NcoI) with *Nde*I, which cuts at codon 175 of *lef-1*, filling in with T4 DNA polymerase, and then digesting with *Nco*I and *Bam*HI. The resulting fragments were subcloned into pASI cut with *Nco*I-*Sma*I (for N-terminal deletion clone pASLEF1aa1-175) or *Sma*I-*Bam*HI (for C-terminal deletion clone pASLEF1aa175-266). The remaining N-terminal deletions were constructed by introducing *Nco*I sites at codon positions 243, 223, 192, 134, 98, and 46 (Table 1) followed by subcloning of *Nco*I-*Bam*HI fragments into pASI. Site-directed mutagenesis was used to change the putative primase domain WVVDAD located at aa 73 to 78 to WVVQAD, creating pKSLEF1 (NcoI)(WVVQAD) (Table 1). The mutant was subcloned into pASI with *Nco*I-*Bam*HI. The mutations were confirmed by DNA sequence analysis.

LEF-2 deletions were constructed as follows: aa1-96, aa1-60, and aa60-210 were removed from pKSLEF2(NcoI) with *Nco*I-*Sal*I, *Nco*I-*Hpa*I, and *Hpa*I-*Bam*HI, respectively, and cloned into pASI. The two remaining LEF-2 C-terminal deletion clones were created by using site-directed mutagenesis to insert *Nco*I sites at the indicated positions (codon 20 and codon 40) followed by subcloning into pASI with *Nco*I-*Bam*HI (Table 1).

GST fusions and TnT constructs. 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. *lef-1*, *lef-2*, and *lef-3* were subcloned by ligating *Nco*I-*Bam*HI inserts into pGEX CS-1 cut with the same enzymes, resulting in pGEXLEF1, pGEXLEF2, and pGEXLEF3, respectively. The in vitro transcription-translation (TnT) plasmid, pKSLEF1(TnT), was constructed by digesting pKSLEF1 with *Nco*I-*Hin*dIII and religating the vector, eliminating the viral

TABLE 1. Primers used for site-directed mutagenesis

Clone no.[a]	Designation	*Nco*I site (codon no.)	Sequence of primer
1	pKSLEF1(NcoI)	1	GTTCAAAGGGCACCATGGTAGTGTGCAATTAT
2	pKSLEF1(NcoI)(WVVDAD)		GAATGGGTCGTACAGGCCGATTACAAAA
3	pKSLEF1(NcoI)	46	ATTTTGATACCTCCATGGCACAATTGTATA
4	pKSLEF1(NcoI)	98	TTTACATTGGCGCCATGGCGTTTCTGTTG
5	pKSLEF1(NcoI)	134	GTTTAAAATCACGTCCATGGCTCAAAATGTTCG
6	pKSLEF1(NcoI)	192	AGTATTGGCCGGCCATGGACAGGGATATT
7	pKSLEF1(NcoI)	223	TTCTCGTTGTATAACCATGGAATTGCTAGACA
8	pKSLEF1(NcoI)	243	ACTGGTGGATGTGCCATGGTGACAACGACA
9	pKSLEF2(NcoI)	1	AGAAGCCGCGAACCATGGCGAATGCA
10	pKSLEF2(NcoI)	20	AGCGTCATGTTTAGCCATGGAAGCTACATATTTAATT
11	pKSLEF2(NcoI)	40	ACCCTAACTCCATCCATGGTATTCTACAATG
12	pKSLEF3(NcoI)	1	TCGACAACAGCACCATGGCGACCAAA

[a] Clones 2 to 8 were created by using clone 1 as the template. Clones 10 and 11 were created by using clone 9 as the template.

promoter region, and bringing the bacterial T3 promoter (from pKS$^-$) within 40 bp of the translational start site for *lef-1*.

Replication assay clones. *lef-1*, *lef-2*, and *lef-3* were cloned into pKS$^-$ as described above. DNA polymerase, *p143* (helicase), *ie-1*, *p35*, *pe-38*, and *ie-2* were subcloned from pUC19 into pKS$^-$ (21). The *lef-1* construct with the mutated putative primase domain (WVVQAD) was cloned as described above. The reporter plasmid (pAcHdL) contained the putative replication origin, *hr*2, in the *Hin*dIII-L fragment (m.u. 18.4 to 20.5) cloned into pKS$^-$. pKSLEF1 aa1-241, and aa1-222 were cloned downstream of the *lef-1* promoter by digesting pASLEF1 (aa1-241 or aa1-222) with *Sal*I-*Bam*HI (*Sal*I is at codon 11 of *lef-1* and *Bam*HI is in the pASI multiple cloning site) and ligating the fragment into pKSLEF1 cut with the same enzymes. pKSLEF1 aa134-266, aa98-266, and aa46-266 were created by cutting pKSLEF1(NcoI) (*Nco*I site at ATG and codon 134, 98, or 46) with *Nco*I, religating, and screening for clones that lacked the *Nco*I fragment. pKSLEF2 aa1-96 was created by cutting pKSLEF2 with *Sal*I-*Bam*HI, blunt ending with T4 DNA polymerase, and religating with T4 DNA ligase. pKSLEF2 aa1-60 was constructed similarily with *Hpa*I-*Xba*I. pKSLEF2 aa40-210 and aa60-210 were created by digesting pASLEF2 aa20-210 or aa40-210 with *Nco*I-*Bam*HI and ligating the fragment behind the *lef-1* promoter by digesting pKSLEF1(NcoI) with *Nco*I-*Bam*HI.

Site-directed mutagenesis. Site-directed mutagenesis (29, 38) employed the primers shown in Table 1 to generate the clones used in this study.

Yeast-two-hybrid assay. (i) Transfections. All DNA used for transfections was prepared by alkaline lysis plasmid purification followed by phenol extraction (38). Yeast transformations were performed by spinning down 1 ml of overnight yeast culture (Y166 or single recombinant clones) and resuspending the cell pellet in 500 μl of transformation buffer (40% polyethylene glycol 3350, 100 mM LiCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 11 μg of sonicated calf thymus DNA). Plasmid DNA (1 to 2 μg) was added to cells plus transfection buffer and left at 23°C for 6 to 8 h. Cells were then washed three times with H_2O and plated on appropriate media.

(ii) Liquid yeast-two-hybrid assays. β-Galactosidase assays (17) were performed as follows. Single colonies were inoculated into 2 ml of appropriate media and incubated overnight at 30°C with shaking. A 1.7-ml aliquot of overnight culture was spun down and resuspended in 100 μl of Z buffer (0.06 M Na_2HPO_4, 0.04 M $NaH_2PO_4 \cdot H_2O$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H_2O$, 0.04 M β-mercaptoethanol). An equal volume of 0.5-mm-diameter glass beads (Biospec Products) was added followed by vortexing three to four times for 1 min. An additional 100 μl of Z buffer was added, and the extract was spun down in a microcentrifuge for 5 min at 10,000 × *g*. Supernatant (50 μl) was added to 950 μl of Z buffer and 200 μl of ONPG (*o*-nitrophenyl-β-D-galactopyranoside) (4 mg of ONPG per ml, 0.1 M $NaPO_4$ [pH 7.0]) and incubated at 30°C for 1 h; 400 μl of 1 M $NaCO_3$ was added to stop the reaction. The protein concentration was determined by a protein assay (Bio-Rad, Inc.). The specific activity of the extract was calculated by using the following formula: $OD_{420} \times 1.6/0.0045 \times$ protein (mg/ml) × extract volume (ml) × time (min). OD_{420} is the optical density at 420 nm of the product, *o*-nitrophenol. The factor 1.6 corrects for the reaction volume. Extract volume is the volume assayed (in milliliters). The factor 0.0045 is the optical density of a 1-nmol/ml solution of *o*-nitrophenol. Specific activity is expressed as nmoles per minute per milligram of protein.

The expression of fusion proteins was confirmed with an antibody to the hemagglutinin (HA) epitope (Babco) by Western blot analysis.

GST fusion TnT interaction experiments. GST fusion constructs were grown in 100 ml of 2× YT (38) to an OD_{595} of 0.5 to 0.6 and induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h at 37°C. Cells were spun down, resuspended in 5 ml of PBS (120 mM NaCl, 2.7 mM KPO_4 [pH 7.4]), and sonicated three times for 1 min. Triton X-100 was added to a final concentration of 1%, and the mixture was incubated at 23°C for 30 min. The bacterial cell extract was spun in a Sorvall centrifuge at 12,000 × *g* for 10 min at 4°C, and the supernatant was frozen in 1-ml aliquots. Expression of GST fusion proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

In vitro TnT reactions were performed with a rabbit reticulocyte lysate TnT system (Promega) according to the manufacturer's instructions. TnT reactions were labeled with [^{35}S]methionine (New England Nuclear). For interactions, 25 μl of glutathione Sepharose beads (Pharmacia) was incubated with 500 μl of bacterial extract containing the appropriate fusion protein for 30 min at 23°C. The beads were washed three times with 1 ml of phosphate-buffered saline (PBS). TnT reaction products (5 μl) plus 50 μl of PBS was added to the beads and incubated for 1 h at 23°C and washed five times with 1 ml of PBS. A 45-μl aliquot of 1× SDS-PAGE sample buffer (38) was added to the beads, boiled for 5 min, resolved on 12% SDS-PAGE, and analyzed by autoradiography.

Replication assay. Transient-replication assays were performed as described previously (21, 25). Briefly, Sf-9 cells were plated into six-well plates at a density of 1.25×10^6 cells/well in TNM-FH plus 10% FBS and allowed to attach overnight at 30°C. A 0.5-μg aliquot of each DNA construct to be transfected (including reporter construct pAcHdL) was mixed with 400 μl of transfection buffer (25 mM HEPES, 140 mM NaCl, 125 mM $CaCl_2 \cdot 2H_2O$ [pH 7.1]). All DNAs were prepared on midi columns (Qiagen, Inc.). The medium was replaced with 400 μl of Grace's medium–10% FBS, and the 400 μl of transfection buffer plus DNA was added. Transfection was allowed to proceed for 4 h at 27°C, and the transfection buffer was then replaced with 1 ml of TNM-FH (plus 10% FBS). After 72 h at 27°C, total DNA was isolated from the cells (40) and resuspended in 100 μl of TE (10 mM Tris, 1 mM EDTA [pH 8.0]). DNAs (10 μg) from each sample (concentration determined by OD_{260}) were digested overnight with *Hin*dIII-*Dpn*I, electrophoresed on a 0.8% agarose gel, transferred to membrane filters (GeneScreen Plus; New England Nuclear), and probed with ^{32}P-labeled pKS$^-$.

RESULTS

Interaction of full-length LEF-1 and LEF-2. A yeast-two-hybrid system based on the modular nature of the GAL4 DNA binding and activation domains (5, 10, 11, 19, 34) was used to examine the interactions of the nine baculovirus gene products implicated in previous studies (21, 33) as being involved in DNA replication. In this system, the GAL4 DNA binding and activation domains have been separated onto two different plasmids (pASI and pACTII, respectively) which have different auxotrophic markers for selection of single or double recombinants in yeast (8). These plasmids can be used to make fusions with proteins of interest and the GAL4 activation domain or GAL4 DNA binding domain. When cotransfected, if the fusion partners with the DNA binding domain and activation domain interact, the GAL4 activation domain is brought into proximity with the *lacZ* and *ura* promoters, causing transactivation of these genes. Interaction can be tested by both β-galactosidase assays (colormetric colony lift assay or liquid assay) and growth on URA$^-$ plates.

All nine of the replication genes were cloned into pASI and pACTII (8). An initial screen of these clones revealed several interactions, including one involving LEF-1 and LEF-2 (Fig. 1,

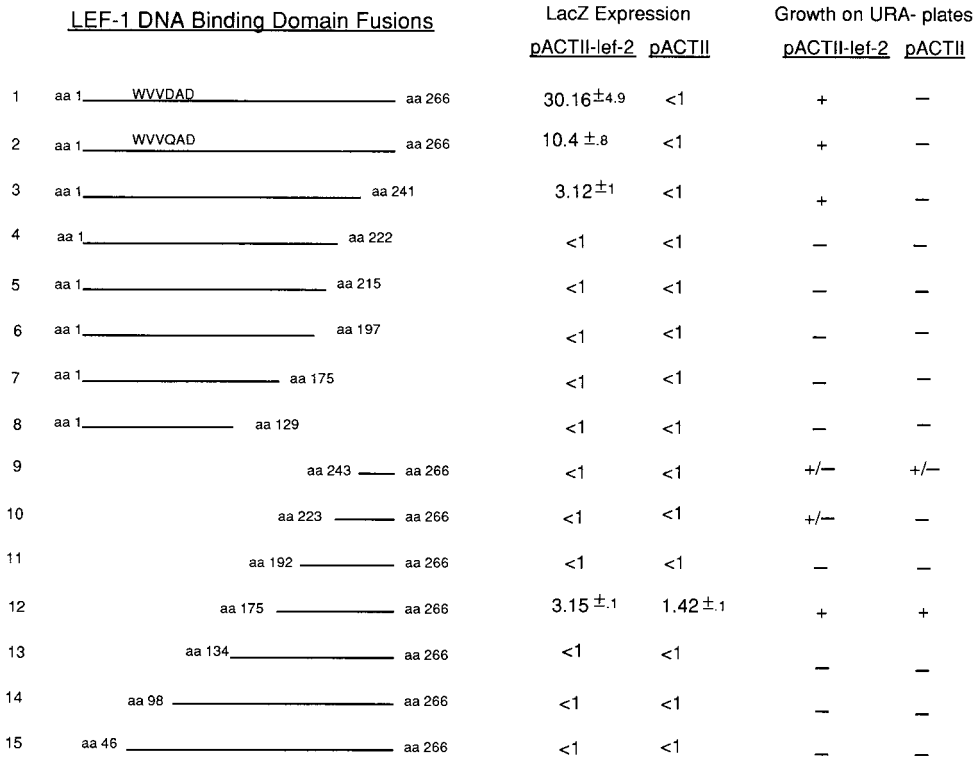

FIG. 1. Characterization of the interaction domain of LEF-1. On the left are diagrams showing the portions of LEF-1 present in the mutants analyzed. Clone 1 is the full-length LEF-1, and clone 2 is a full-length construct with a mutation in a putative primase domain. In the middle columns are shown the levels of *lacZ* expression from yeast that contained both the LEF-1 deletion clones diagrammed (in pAS1) and the full-length *lef-2* gene cloned into pACTII or pACTII with no insert (negative control). *lacZ* expression was calculated from four liquid assays from at least two independent transformations (± values are standard deviations). Specific activity is expressed as nmoles per minute per milligram of protein. Growth on plates lacking uracil is shown in the two right-hand columns (+, –, and ± indicate growth, no growth, and slow growth, respectively, on Ura^- plates after 4 days at 30°C). Protein expression was confirmed by Western blot analysis using a monoclonal antibody (Babco) to the HA epitope.

row 1, and Fig. 2, row 1). No β-galactosidase activity was detected when double recombinants, containing the DNA binding domain or activation domain fusions and the control reciprocal vectors encoding no fusion protein, were made (Fig. 1, row 1, and Fig. 2, row 1). This indicated that the full-length fusion proteins had no intrinsic capacity to transcriptionally activate the β-galactosidase promoter. In addition, no oligomerization between LEF-1 and itself or LEF-2 and itself was detected when pACTII and pASI fusions of LEF-1 or LEF-2 were cotransfected into yeast (data not shown).

To confirm the interaction observed in the yeast-two-hybrid system, we used GST-TnT affinity experiments (Fig. 3). GST fusion constructs were made with *lef-1*, *lef-2*, and *lef-3* (called pGEXLEF1, pGEXLEF2, and pGEXLEF3, 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 with glutathione Sepharose beads, washing with PBS to remove unbound proteins, and sizing the retained proteins by SDS-PAGE. All three constructs showed protein fusion products of the correct size

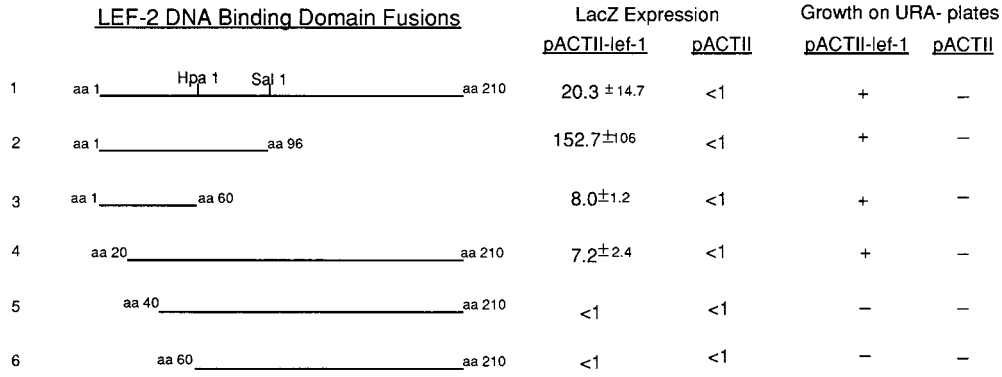

FIG. 2. Characterization of the interaction domain of LEF-2. On the left are diagrams showing the portions of LEF-2 present in the mutants analyzed. Clone 1 contains the full-length sequence. In the middle columns are shown the levels of *lacZ* expression when yeast contained both the LEF-2 deletion clones diagrammed (in pAS1) plus the full-length *lef-1* gene cloned into pACTII or pACTII alone with no fusion protein (negative control). For details, see the legend to Fig. 1.

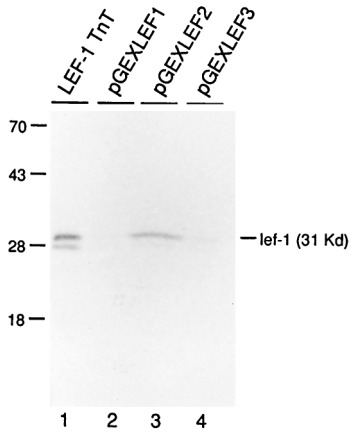

FIG. 3. Analysis of the interaction of GST fusions with in vitro-transcribed and -translated LEF-1. Lane 1 shows the input in vitro-transcribed and -translated ([^{35}S]methionine-labeled) LEF-1. The subsequent lanes show the labeled LEF-1 which was retained by glutathione Sepharose beads previously incubated with GST fusions of LEF-1 (lane 2), LEF-2 (lane 3), or LEF-3 (lane 4). Molecular masses (in kilodaltons) are indicated to the left of the gel.

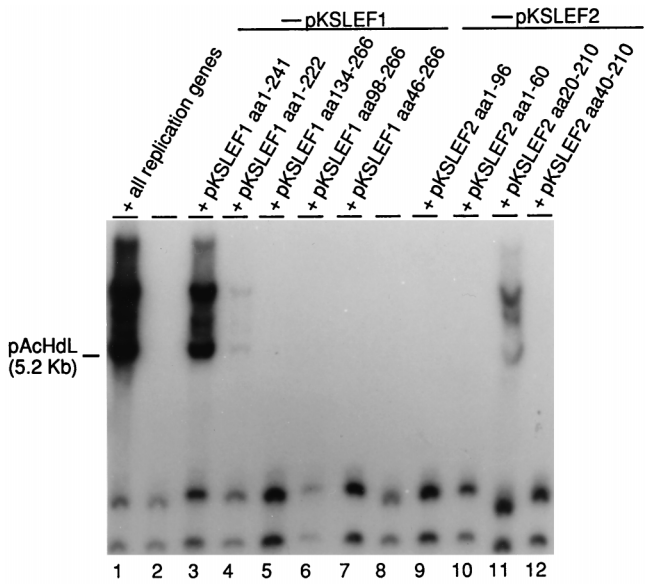

FIG. 4. Characterization of the ability of LEF-1 and LEF-2 mutants to support transient DNA replication. Lane 1 shows transfection with the nine previously characterized replication genes (*lef-1*, *lef-2*, *lef-3* [SSB], DNA polymerase, *p143* (helicase), *ie-1*, *ie-2*, *pe-38*, and *p35*) (21, 33). Lanes 2 and 8 show replication when LEF-1 or LEF-2, respectively, is not present. Lanes 3 to 7 and 9 to 12 show the replacement of LEF-1 or LEF-2 with the indicated deletion clones.

(data not shown). Each GST fusion was bound to glutathione sepharose beads and then incubated with ^{35}S-radiolabeled LEF-1 generated by the in vitro TnT reactions. After extensive washing, the bound proteins were eluted and analyzed by SDS-PAGE and autoradiography (Fig. 3). The input in vitro-transcribed and -translated LEF-1 is shown in lane 1. A major band of 31 kDa, which is consistent with initiation at the first ATG, is present. A second, minor band of lower molecular weight is also evident and corresponds to a pair of in-frame downstream ATGs at codon positions 13 and 14. When the radiolabeled LEF-1 was incubated with GST fusions of LEF-1, LEF-2, and LEF-3 bound to glutathione Sepharose beads, the following was observed (Fig. 3): LEF-1 did not interact with GST-LEF1 (lane 2), it did interact with GST-LEF2 (lane 3), and it did not interact with GST-LEF3 (lane 4). The interaction of LEF-2 (lane 3) appeared to be with the larger (full-length) species of LEF-1, whereas the smaller protein that is likely to be missing 13 to 14 aa from the N terminus did not interact with GST-LEF2.

Interaction of LEF-1 deletion clones with full-length LEF-2. To map the amino acid sequence involved in LEF-1 interaction with LEF-2, a set of *lef-1* deletion clones was constructed and cloned into the yeast-two-hybrid vector, pASI. These constructs were then transfected into yeast cells containing full-length *lef-2* cloned into pACTII, and the double-recombinant yeasts were tested for *lacZ* expression. The full-length LEF-1 and one C-terminal deletion clone (aa1-241) (Fig. 1, rows 1 and 3, respectively) interacted with full-length LEF-2. One N-terminal deletion clone (aa175-266) (row 12) gave some activity in the β-galactosidase liquid assay and grew slightly on plates lacking uracil. Similarly, two other N-terminal deletion clones (Fig. 1, rows 9 and 10) showed some limited growth on URA$^-$ plates. Two of these clones also showed some activity when cotransfected with pACTII alone, indicating that these constructs had intrinsic transactivation capabilities in this system (Fig. 1, rows 9 and 12). In a separate test, all of the LEF-1 deletion constructs were cloned into pACTII as GAL4 activation domain fusions and cotransfected with pASLEF2. The full-length LEF-1 and aa1-241 resulted in β-galactosidase activity, whereas those that showed slight positive reactions when cloned as DNA binding domain fusions (aa243-266, aa223-266, aa175-266) gave no activity (data not shown). All deletion clones expressed fusion proteins of the expected sizes as demonstrated by SDS-PAGE followed by Western blot analysis using a HA monoclonal antibody (data not shown).

Interaction of LEF-2 deletion clones with full-length LEF-1. In order to map the interaction domain of LEF-2, a set of LEF-2 deletion clones in pASI (GAL4 DNA binding domain) was constructed. Deletion clones aa1-96, aa1-60, and aa20-210 gave β-galactosidase activity when cotransfected with pACTLEF1 (GAL4 activation domain fusion) (Fig. 2, rows 2 to 4). These double recombinants were also able to grow on plates lacking uracil (Fig. 2). Deletion clones aa40-210 and aa60-210 resulted in no β-galactosidase activity or growth on plates lacking uracil when cotransfected with pACTLEF1 (Fig. 2, rows 5 and 6). Double recombinants with pASLEF2 clones and pACTII alone were negative, showing that the observed activities were due to interactions with pACTLEF1 and not the result of transactivation by these deletion clones (Fig. 2). These results indicate that a domain located between aa 20 and 60 of LEF-2 is essential for interaction with LEF-1.

Replication assays with LEF-1 and LEF-2 deletion clones. LEF-1 and LEF-2 are required for transient replication of an origin-containing plasmid (21). To determine if interaction of LEF-1 and LEF-2 was a prerequisite for their ability to function in baculovirus DNA replication, selected clones were tested in a transient-replication assay. Inserts from these clones were placed behind their native promoters (see Materials and Methods) and used in a transient DNA replication assay (21, 25). As shown in Fig. 4, both LEF-1 (lane 2) and LEF-2 (lane 8) are required for DNA replication. The LEF-1 clone encoding aa 1-241, which showed interaction with LEF-2 in the yeast-two-hybrid assay (Fig. 1, row 3), demonstrated the ability to support high levels of replication. The clone encoding aa 1 to 222, which demonstrated no interaction in the yeast-two-hybrid assay, allowed trace amounts of replication (Fig. 4, lane 4). The remaining LEF-1 deletions that showed no inter-

action in the yeast-two-hybrid assay were unable to support replication (Fig. 4, lanes 5 to 7).

Deletion clones of LEF-2 showed that although clones aa1-96 and aa1-60 are capable of interacting with LEF-1 (Fig. 2, rows 2 and 3), they are unable to support replication (Fig. 4, lanes 9 and 10). However, the clone containing aa20-210, which interacted with LEF-1 (Fig. 2, row 4), also supported replication (Fig. 4, lane 11) and clone aa40-210, which did not interact with LEF-1 (Fig. 2, row 5), failed to support replication (Fig. 4, lane 12).

These data showed that in every case, clones which failed to interact also failed to show high levels of replication. The very weak replication signal observed with LEF-1 aa1-222 may be due to a weak interaction between LEF-1 aa1-222 and LEF-2 which is not detectable in the yeast-two-hybrid system. The interaction domain of LEF-2 is located near the N terminus, as deletion of the first 40 aa (Fig. 2, row 5) abolished interaction. Deletion of this region also abolished replication (Fig. 4, lane 12). However, clones that contained the interaction domain but had major deletions of the C-terminal region (Fig. 4, lanes 9 and 10) did not support replication. These data indicate that regions of the LEF-2 open reading frame in addition to the oligomerization domain are required for DNA replication.

Primase domain mutation in LEF-1. Characterization of DNA replication in a variety of other viruses has led to the identification of a similar set of genes essential for DNA replication (26). One of the common components not yet identified as essential for baculovirus DNA replication is a primase. Therefore, we examined the LEF-1 and LEF-2 sequences to determine if they had features of this enzyme. A moderately conserved primase motif comprising invariant DXD residues separated by a single hydrophobic amino acid and preceded by three hydrophobic amino acids has been described in primases from a number of herpesviridae, bacteriophage T7, yeast, and mice (20). Similar motifs (WV[I/V]DAD) were also found in Ac*M*NPV and the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus LEF-1 sequence. A conserved aspartate-to-glutamine (IILDLD to IILQLD) change at aa 628 in herpes simplex virus type 1 (HSV-1) UL52 completely eliminated the primase activity associated with the HSV helicase-primase UL5-UL8-UL52 complex (20). We made a similar change in Ac*M*NPV LEF-1 whereby aa 76 was converted from aspartate to glutamine (WVVDAD to WVVQAD). This mutation did not prevent interaction with LEF-2 (Fig. 1, row 2), but it eliminated the ability of LEF-1 to function in the transient-replication assay (Fig. 5, lanes 7 and 8). The loss of ability to support replication was not caused by the insertion of the *Nco*I site at the start of the ORF, as the parent *Nco*I-containing clone supported replication at the wild-type level (Fig. 5, compare lanes 5 and 6 to lanes 1 and 2).

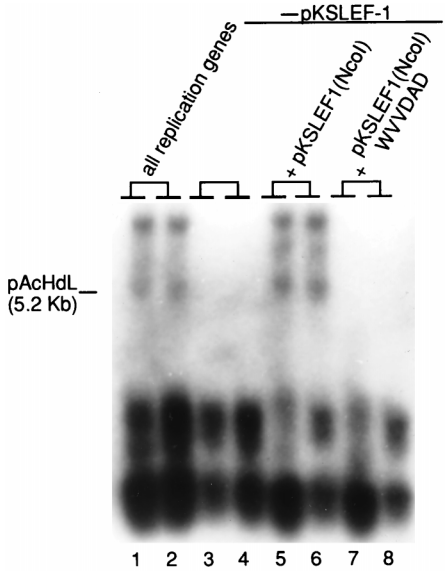

FIG. 5. Characterization of the ability of a LEF-1 mutation in the putative primase domain to support transient DNA replication. The figure shows results from two independent replication assays. Lanes 1 and 2, transfection with all replication genes (*lef-1*, *lef-2*, *lef-3* [SSB], DNA polymerase, *p143* [helicase], *ie-1*, *ie-2*, *pe-38*, and *p35*); lanes 3 and 4, all replication genes minus LEF-1; lanes 5 and 6, all replication genes minus LEF-1, plus LEF-1 with an *Nco*I site at the ATG [pKSLEF1(NcoI)]; lanes 7 and 8, all replication genes minus LEF-1, plus LEF-1 with a mutation in the putative primase domain.

DISCUSSION

We have demonstrated, using both yeast-two-hybrid assays and GST fusion affinity experiments, that the products of the Ac*M*NPV replication genes *lef-1* and *lef-2* form oligomers. We were able to map the interaction domain of LEF-2 to the region of aa 20 to 60. However, in LEF-1 the only deletion mutant that was able to interact with LEF-2 was one with a C-terminal deletion of 25 aa. These data suggest that the interaction domain in LEF-1 contains components of both the N- and C-terminal regions or that the deletions we examined disrupt secondary structure required for the interactions. By testing the mutants in a DNA replication assay, we found that clones that failed to interact also failed to support significant levels of DNA replication, suggesting that interaction of these two proteins is essential for DNA replication.

We identified a motif (WVVDAD) in the LEF-1 amino acid sequence that resembles a primase motif that is essential for DNA replication in other systems. When the first aspartate in this motif was changed to a glutamine, the resulting mutant was unable to substitute for LEF-1 in transient-replication assays. This mutant was still able to interact with full-length LEF-2 in yeast-two-hybrid assays. The interaction appeared weaker than that seen with wild-type LEF-1; however, it is difficult to correlate the results from yeast-two-hybrid liquid assays with binding strength (9).

In other well-characterized replication systems such as HSV-1, simian virus 40, T4, T7, ColE1 and *E. coli*, there is a core set of proteins involved in replication. These are DNA polymerase, helicase, primase, SSB, origin binding protein, and one or more accessory or processivity factors (26). In Ac*M*-NPV, genes with homology to DNA polymerase, helicase, and proliferating cell nuclear antigens are present but only DNA polymerase and helicase homologs are essential for DNA replication (21). In addition, LEF-3 has the properties of a single-stranded DNA binding protein (14) and IE-1 has been shown to bind to putative origins of DNA replication (6, 12, 27, 37). The presence of a primase motif in LEF-1 suggests that LEF-1 may be a primase and LEF-2 may be a primase accessory factor. Primases are often associated with one or more other proteins. For example, HSV primase (UL52) forms a complex with two other proteins, UL8 and UL52 (helicase), to form a helicase-primase complex (20). Future investigations undertaken to define the role of LEF-1 and LEF-2 in baculovirus DNA replication should consider these proteins together, as it is likely that in isolation they will not be functional. Further protein purification and biochemical characterization of LEF-1 is currently under way to determine the role that LEF-1 and LEF-2 play in baculovirus DNA replication.

ACKNOWLEDGMENTS

We thank Joel Funk for suggestions and criticisms of the manuscript and the Central Services Laboratory at OSU for assistance with this project. We also thank Bill Dougherty and Steve Elledge for providing the vectors used in this study. 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) and ACS (SG-208).

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