Baculovirus Replication Factor LEF-1 Is a DNA Primase†
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The baculovirus replication factors LEF-1 and LEF-2 of the Autographa californica multinucleocapsid nucleopolyhedrovirus were overexpressed as fusions containing a hemagglutinin (HA) epitope and a HIS6 tag using recombinant baculoviruses. LEF-1 was purified to near homogeneity and found to have primase activity in an indirect assay employing Escherichia coli DNA polymerase I (Klenow enzyme) and poly(dT) template. The LEF-1 primase products were also directly characterized by electrophoresis in 20% polyacrylamide–8 M urea gels and agarose gels. Primer synthesis was time dependent, and products of several hundred nucleotides or more were observed from the M13 single-stranded DNA (ssDNA) template. The LEF-1 primase was absolutely dependent on divalent cations (Mg2+), and optimal activity was supported by 10 mM MgCl2. An alkaline pH (8.8 to 9.4) was optimal, whereas monovalent salt (KCl) was inhibitory. Mutation of an invariant aspartic acid in a putative primase domain caused LEF-1 activity to be abolished. Upon ultracentrifugation in glycerol gradients, LEF-1 was found to have a sedimentation coefficient of 38 that is consistent with its being present as a monomer. Elution profiles of LEF-1 and LEF-2 from ssDNA-cellulose and DEAE resin suggested that LEF-2 may bind to both DNA and LEF-1.

The Baculoviridae are a large and diverse family of rod-shaped, enveloped, occluded viruses that are pathogenic for invertebrates, particularly members of the Insecta. They have been reported from over 600 species, most of which are members of the Lepidoptera, Diptera, and Hymenoptera (34). Two genera of baculoviruses have been characterized, and they include the nucleopolyhedroviruses (NPVs) (47), which have numerous virions within large polyhedron-shaped occlusion bodies, and the granuloviruses (52), which commonly have a single virion within small granular occlusion bodies. Baculovirus genomes consist of double-stranded, circular, supercoiled DNA of 100 to 180 kb, depending on the strain of virus (20). Although evidence suggests that baculovirus genomes may replicate via a rolling-circle intermediate (31, 42), the mechanisms of initiation, elongation, processing, and maturation have not been determined.

Baculovirus DNA replication has been shown to be associated with discrete replication factories in the nuclei of infected cells (41). In addition, a conserved set of genes that are essential or highly stimulatory for transient DNA replication have been identified for Autographa californica multinucleocapsid NPV (AcMNPV) (26, 33), Orgyia pseudotsugata MNPV (OpMNPV) (reviewed in reference 1), and Lymantria dispar MNPV (44). These include genes encoding a DNA polymerase homolog, a DNA helicase homolog, ie-1, a transactivator of early gene transcription, and late expression factors (LEFs) encoded by lef-1, -2, and -3. The DNA polymerase and DNA helicase homologs were subsequently shown to have activities associated with these enzymes (35, 36). In addition, lef-3 encodes a product with the properties of a single-stranded DNA (SSB) binding protein (16, 37), interacts with helicase (14), and is required for transfer of helicase to the nucleus (53).

In addition to its ability to transactivate early gene transcription, the product of ie-1 has been demonstrated to bind to homologous regions which act as replication origins in transient assays (9, 46) and colocalizes with LEF-3 in nuclear replication factories (41). AcMNPV genes lef-1 and lef-2 potentially encode basic proteins with isoelectric points (pI) of 8.84 and 9.38, respectively, and calculated molecular masses of 30,780 and 23,926 Da, respectively (4). Possible roles for LEF-1 and LEF-2 have not been elucidated, although LEF-1 contains several motifs related to eukaryotic primases. When the putative primase domain in LEF-1, WVVQAD, was altered to WVVQAD, transient DNA replication was abolished (13). In addition, it was found in yeast two-hybrid analyses that LEF-1 and LEF-2 interact (13). These data suggested that LEF-1 may function as a primase and that LEF-2 may be associated with this activity.

In this report we describe the overexpression and purification of LEF-1 and demonstrate that it has DNA primase activity. The parameters of the LEF-1 primase activity were optimized, and the primase products were characterized by electrophoresis in polyacrylamide and agarose gels. We also discuss a possible role of LEF-2 in the baculovirus replication complex.

MATERIALS AND METHODS

Chemicals and enzymes. Nucleoside triphosphates (NTPs) and deoxy-NTPs (dNTPs) were purchased from Roche Molecular Biochemicals. 7-Deaza-2'-deoxyadenosine 5'-triphosphate and poly(dT) were from Amersham Pharmacia Biotech. Radiolabeled nucleotides [α-32P]ATP, [γ-32P]ATP, and [α-32P]dATP were from Perkin-Elmer. Escherichia coli DNA polymerase I (Klenow Exo−) and other modifying enzymes were from New England Biolabs.

Cells. Spodoptera frugiperda 9 (Sf9) cells were cultured in SF900II serum-free medium (Gibco-BRL) supplemented with 10% fetal bovine serum, penicillin G

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FIG. 1. Schematic diagram of plasmids used to construct recombinant baculoviruses expressing LEF-1 and LEF-2. The two oligomers used to engineer the plasmids pHSExpHisLef-1 and pHSExpHisLef-2 (45) are shown. They were inserted into a BglII site and resulted in the elimination of this site and the creation of an upstream BamHI site. The inserts were then removed with BamHI and NotI and inserted into pFastBac1 as shown. The orientation of a generic *lef* gene is indicated by the open arrow.

(50 U/ml), streptomycin (50 μg/ml; Whittaker Bioproducts), and Fungizone (amphotericin B, 375 ng/ml; Flow Laboratories) as previously described (17).

Transfer plasmids and virus construction. The transfer plasmids were constructed as follows. Plasmids pHSExpHisLef-1 and pHSExpHisLef-2 (45) were digested with BglII, treated with calf intestinal phosphatase, and modified using two oligonucleotides that were phosphorylated by treatment with polynucleotide kinase and ATP, gel purified, annealed (48), and inserted into the BglII site. The oligonucleotide names and sequences are 5′bamhahisc (GATCGGGATCC ACCATGAG CTCCCGGACG TGATGATG ATGGCGGGCG GTAATCAGTGGCG CATGTCATC ATGGTCG GATCCC) and 3′bamhahisc (GATCGGGATCC ACCATGAG CTCCCGGACG TGATGATG ATGGCGGGCG GTAATCAGTGGCG CATGTCATC ATGGTCG GATCCC). This resulted in the creation of an ATG translation start site followed by the HA epitope and HIS tag upstream of each open reading frame (ORF) but downstream of a new BamHI site (Fig. 1).

Clones were screened using BamHI digestion (the correct orientation is about 70 nucleotides [nt] smaller than the reverse orientation), and presumptive correct plasmids were sequenced to confirm the correct orientation of the insert. The primers used for sequencing were AcMNPV nt 10971 to 10990 (lef1-330, CGGTATACATGACTCTTGAC) and nt 3480 to 3462 (lef2-373, AACCTCT TCCTGTACATAC) (4). The ORFs were then removed by digestion with BamHI and NotI, gel purified, and inserted into a pFastBac vector (Gibco-BRL) digested with the same enzymes and gel purified. Recombinant baculoviruses were produced using the Bac-to-Bac baculovirus expression system (Gibco-BRL) following the manufacturer’s instructions.

A virus, vfbHAHISLef-ID76Q, in which the conserved primase motif WVV was altered to WVA and treated with the recombinant baculoviruses was coinfected with the recombinant baculoviruses was digested with the same enzymes and gel purified. Recombinant baculoviruses were produced using the Bac-to-Bac baculovirus expression system (Gibco-BRL) following the manufacturer’s instructions.

Assays for endonuclease and topoisomerase activity. Reactions were carried out in three mixtures of different composition. Reaction mixture 1 contained 25 mM Tris-HCl (pH 7.5), mixture 2 contained 25 mM Tris-HCl (pH 7.5) and 50 mM KCl, and mixture 3 contained 25 mM Tris-HCl (pH 8.8). All reactions contained 0.3 μg of DNA (replicative form [RF1]) of plasmid pSHELEF-1 (45), 10 mM MgCl2, 50 mM of bovine serum albumin (BSA) per ml, 1 mM DTT, and 1 mM ATP. *LEF-1 (0.1 μg in 3 μl of buffer D) was added to a final volume of 15 μl. After incubation for 1 h at 37°C, reactions were terminated by the addition of EDTA to 15 mM and SDS to 0.5%, and the samples were treated with proteinase K (100 μg/ml) for 30 min at 37°C. The samples were then analyzed by electrophoresis in a 0.7% agarose gel, followed by staining with ethidium bromide, and examined for the conversion of RF1 into RFII and RFIII or topoisomers.

(i) Direct assays. Reaction mixtures (10 μl) contained 25 mM Tris-HCl (pH 9.1), 10 mM MgCl2, 25 μg of poly(dT) per ml, 100 μg of BSA per ml, 1 mM DTT, 1 mM ATP, 2.5 μM 7-deaza-dATP, 2.5 μM [α-32P]dATP (10 μCi/ml), 5 μg of E. coli DNA polymerase I (Klenow Exo-) per ml, and various amounts of *LEF-1 added with 5 μl of buffer D. Reactions were carried out at 30°C for 60 min and were terminated by chilling on ice and adding 5 μl of 0.5 M EDTA saturated with sodium pyrophosphate. The samples were transferred onto pieces of Whatman 3MM paper, which were washed in four changes of cold 5% trichloroacetic acid–1% sodium pyrophosphate. The acid-insoluble radioactivity was measured by Cerenkov counting.

(ii) Indirect assays. Reaction mixtures (10 μl) contained 25 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 20 μg of poly(dT) per ml, 100 μg of BSA per ml, 1 mM DTT, 1 mM ATP, 2.5 μM 7-deaza-dATP, 2.5 μM [α-32P]dATP (10 μCi/ml), 5 μg of DNA polymerase I (Klenow Exo-) per ml, and various amounts of *LEF-1 added with 5 μl of buffer D. Reactions were carried out at 30°C for 60 min and were terminated by chilling on ice and adding 5 μl of 0.5 M EDTA saturated with sodium pyrophosphate. The samples were transferred onto pieces of Whatman 3MM paper, which were washed in four changes of cold 5% trichloroacetic acid–1% sodium pyrophosphate. The acid-insoluble radioactivity was measured by Cerenkov counting.
chilling on ice and adding 7 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% each bromophenol blue and xylene cyanol). In initial experiments, the reactions were inactivated by heating at 75°C for 5 min and treated with proteinase K (100 μg/ml) at 37°C for 30 min prior to the addition of the stop solution. We routinely omitted the proteinase K treatment because it did not affect electrophoretic pattern under subsequent analysis of the reaction products in a polyacrylamide gel.

After heat denaturation for 5 min at 75°C, a portion of each reaction was loaded onto a 20% polyacrylamide–8 M urea slab gel (17 by 14.7 by 0.08 cm). Electrophoresis was performed in TBE (Tris-borate-EDTA) buffer (48) at 600 V for 2 to 2.5 h until the bromophenol blue had migrated 3 to 4 cm above the bottom of the gel. Size standards, generated by partial alkaline hydrolysis of 5'-end 32P-labeled λDNA (12), were electrophoresed in a parallel lane on the same gel. The gel was transferred onto a polymer support and exposed to X-ray film at −80°C with an intensifying screen.

For analysis of RNA synthesized by *LEF-1 on single-stranded M13 DNA, the mixture containing 25 μg of M13mp9 ssDNA per ml, 25 mM Tris-HCl (pH 9.1), 10 mM MgCl2, 5 μM (100 μCi/ml) [α-32P]ATP, 50 μM each CTP, GTP, and UTP, 100 μg of BSA per ml, and 1 mM DTT was assembled on ice. *LEF-1 (1.3 μg in 39 μl of buffer D) was added to a final volume of 130 μl, and 30-μl portions were taken from the mixture immediately (time zero) or after incubation for 30, 60, and 120 min at 37°C. Reactions were terminated by the addition of EDTA to 15 mM and SDS to 0.5%, and the samples were treated with proteinase K (100 μg/ml) for 30 min at 37°C. An excess of unincorporated label was removed by spinning the samples in multisip tubes (AsyGen, Inc.) equipped with Bio-Gel P-4 medium (Bio-Rad Laboratories) according to the manufacturer’s instructions. Polynucleotides were precipitated in cold 75% ethanol, then dissolved in TE (Tris-EDTA) buffer, and analyzed by electrophoresis in a 1.2% neutral agarose gel in Tris-acetate buffer. The gel was transferred onto Whatman 3MM paper, dried under vacuum, and exposed to X-ray film as described above. Size standards (0.3 μg of M13mp9 DNA and 1.5 μg of a 0.24- to 9.5-kb RNA ladder [Gibco-BRL]) were electrophoresed in parallel lanes on the same gel and visualized by ethidium bromide staining.

**RESULTS**

**Purification of *LEF-1.** In order to facilitate the characterization of *LEF-1, the *LEF-1 ORF was fused at its N terminus with the HA epitope and a HIS6 tag and overexpressed in a recombinant baculovirus under the control of the polyhedrin promoter. The recombinant virus is called vfbHAHISLEF-1 (Fig. 1), and the recombinant protein HAHISLEF-1 is designated *LEF-1 in this report. The HA epitope allows monitoring of *LEF-1 by Western blot analysis, whereas the HIS6 tag allows purification of *LEF-1 with Ni-NTA resin. Evidence suggests that *LEF-1 may form a stable complex with another essential replication protein (LEF-2) in infected cells (13).

Association with LEF-2 may be required for *LEF-1 function as well as the proper processing of *LEF-1 during the infection cycle. Therefore, we also overexpressed *LEF-2 with a recombinant virus, vfbHAHISLEF-2, that was constructed in the same way as vfbHAHISLEF-1 (Fig. 1). For our studies, Sf9 cells were coinfected with both baculoviruses (vfbHAHISLEF-1 and vfbHAHISLEF-2), thereby ensuring expression of both *LEF-1 and *LEF-2.

Although infection with the recombinant viruses vfbHA HISLEF-1 and vfbHAHISLEF-2 induced accumulation of both proteins in infected cells, only a minor portion of the cellular pool of *LEF-1 and *LEF-2 appeared soluble upon extraction under nondenaturing conditions. An increase in KCl concentration in the lysis buffer up to 1.0 M elevated the yield of both proteins in the clarified extract (stage 1) but caused solubilization of chromatin, which markedly hampered further purification. We found that lysis buffer containing 0.2 M KCl was optimal for purification of *LEF-1.

The purification procedure was monitored by SDS-PAGE followed by Coomassie staining (Fig. 2A) and Western blotting with antibody HA.11 (Fig. 2B). Most contaminating proteins were removed from the extract by chromatography on an Ni-NTA column (stage 2) (Fig. 2A, lane 2). However, this fraction contained contaminating proteins and a prominent soluble *LEF-2 component. Further chromatography on a DEAE col-
umn caused the separation of the *LEF-1 sample into two fractions: *LEF-1 associated with *LEF-2 was retained on the column, whereas the *LEF-1 fraction free of *LEF-2 appeared in the flowthrough (stage 3) (Fig. 2B, lane 3).

Besides contaminating proteins, the DEAE column removed nucleic acids from the sample. After final chromatography on a heparin-Sepharose column (stage 4), *LEF-1 was essentially free of contamination (Fig. 2A, lane 4). SDS-PAGE followed by silver staining (Fig. 2C) confirmed that *LEF-1 was purified to near homogeneity. Western blot analysis did not reveal contamination of the *LEF-1 samples with traces of *LEF-2 (Fig. 2B, lanes 3 and 4). The *LEF-1 purification protocol requires 1 day and yields about 30 μg of pure protein from a 100-ml culture of infected cells.

At all stages of purification, *LEF-1 was present as a set of three polypeptides with apparent molecular masses of 32, 33, and 34 kDa (Fig. 2B, C, and D). These values are close to the calculated molecular mass of *LEF-1 (33.4 kDa). The reason for the observed microheterogeneity of *LEF-1 remains unclear. All three polypeptides were immunoreactive with antibody HA.11 (Fig. 2D). Therefore, they retained intact N termini with the HA epitope. Two smaller polypeptides were unlikely to have been produced by limited proteolysis of the largest one during purification because the extracts analyzed immediately after extraction of infected cells showed the same tripartite pattern of *LEF-1 as the purified samples. Treatment of samples with calf intestinal phosphatase had no effect on the electrophoretic pattern of *LEF-1 when it was analyzed by SDS-PAGE, suggesting that the heterogeneity was not due to phosphorylation (data not shown). However, this result does not completely exclude modification by phosphorylation, e.g., if the phosphates are inaccessible to the enzyme.

**Primase activity of *LEF-1.** Before purified *LEF-1 was assayed for primase activity, the *LEF-1 samples were tested for the presence of endonucleases and topoisomerases (see Materials and Methods). No conversion of RFI DNA into RFII and RFIII and no appearance of DNA topoisomers was observed after incubation of plasmid DNA with purified *LEF-1 (data not shown). This indicates that the *LEF-1 samples are essentially free of endonuclease and topoisomerase activity.

Purified *LEF-1 showed a primase activity in both the indirect and direct assays (Fig. 3). The indirect assay is based on the ability of primase to initiate DNA synthesis on an ssDNA template in the absence of exogenous primers. A typical reaction mixture contains Klenow enzyme, [α-32P]dATP, poly(dT) template, and ATP. There is no DNA synthesis and label incorporation in this system in the absence of primase. If RNA transcripts are synthesized by primase from ATP, they serve as primers for DNA polymerization by Klenow enzyme, thus resulting in the incorporation of [32P]dAMP into nascent DNA, which is monitored by trichloroacetic acid (TCA) precipitation and counting in a scintillation counter. The *LEF-1 fractions collected from a heparin-Sepharose column at the final stage in purification (Fig. 3A) were able to support DNA synthesis by Klenow enzyme on poly(dT) (Fig. 3B). The primase activity correlated well with the amount of *LEF-1 protein in the column fractions.

In the direct primase assay, the radioactive products synthesized by *LEF-1 in the presence of [α-32P]ATP and poly(dT) were analyzed by electrophoresis in a 20% polyacrylamide–8 M urea gel (Fig. 3C). *LEF-1 produced three radioactive products of different electrophoretic mobilities, called products I, II, and III (Fig. 3C). The amount of all three products correlated with the amount of *LEF-1 (Fig. 3A) and the primase activity (Fig. 3B) in the column fractions. Treatment with 0.1 N NaOH caused degradation of product III, confirming that it is RNA (data not shown). In contrast, products I and II were insensitive to alkali, and their structure is currently under investigation.

To find an optimum for the primase activity of LEF-1, we varied the pH and concentration of KCl and MgCl2 in the reaction mixture (Fig. 4A). *LEF-1 primase activity was highly...
dependent on pH and reached a maximum at alkaline conditions (pH 8.8 to 9.4) (Fig. 4A, lanes 2 to 8). The effect of KCl and MgCl₂ was assayed at pH 7.5, 8.3, and 9.1. The results obtained at pH 9.1 are shown in Fig. 4A (lanes 9 to 11 and 12 to 16). At all three pHs, KCl was inhibitory. The primase activity of *LEF-1 was absolutely dependent on the presence of divalent cations in the reaction mixture and reached a maximum at 10 mM MgCl₂. The magnesium dependence shown in Fig. 4A (lanes 12 to 16) clearly indicated that the optima for synthesis of products I and III are different. The amount of high-molecular-weight product III reached a maximum at 5 to 10 mM MgCl₂, whereas the amount of product I was maximal at 10 to 20 mM. At the optimum conditions, *LEF-1 remained enzymatically active during a 2-h incubation at 30°C (Fig. 4B).

The template dependence of *LEF-1 activity was analyzed in experiments with poly(dT) and ssDNA of phage M13mp9 (Fig. 5). The synthesis of products II and III by *LEF-1 was absolutely dependent on the presence of DNA template in the reaction mixture. In contrast, the synthesis of product I was highly stimulated in the presence of poly(dT), but was not absolutely dependent on the presence of DNA template (Fig. 5A and B, lane 1). The lack of strict template dependence for product I suggests that it might be synthesized by a mechanism other than the regular template-dependent polymerization. The synthesis of long RNA transcripts (product III) on poly(dT) and M13 DNA templates indicated that LEF-1 is capable of extensive nucleotide polymerization (Fig. 5A and B, lanes 3 and 4). However, the synthesis of nascent poly(A) chains on the poly(dT) template appeared to be less efficient than synthesis from M13 DNA templates. This may be because elongation of the poly(A) chain on the poly(dT) template can be blocked by the poly(dT) template’s folding back to form Hoogsteen base pairs in the transient triplexes (38). The trapping of nascent poly(A) in such triplex structures prevents efficient elongation of poly(A) chains by the enzyme. Natural single-stranded DNAs do not form regular triplexes with nascent RNA, although stable secondary structures in DNA may serve as physical obstacles for polymerization. In the presence of M13 DNA and only one precursor, [α-³²P]ATP, *LEF-1 synthesized the radioactive products I and II, but not product III (Fig. 5, lane 5). Addition of three other precursors, CTP, GTP, and UTP, allowed synthesis of long RNA polymers, some of which did not enter the 20% polyacrylamide gels that were employed in this assay (Fig. 5B, lane 1).
The long transcripts appeared to be a major product of the enzymatic activity of *LEF-1 in the complete system containing M13 DNA and four NTPs. To estimate the size of the RNA transcripts made on M13 DNA, the reaction products were subjected to electrophoresis in a 1.2% agarose gel (Fig. 6). Prior to thermal denaturation, the radioactive products synthesized by *LEF-1 were associated with M13 DNA (lanes 2 to 4). Heat treatment caused liberation of heterogeneous radioactive polymers mostly in a range of several hundred bases in size (lanes 6 to 8). Thus, *LEF-1 is capable of synthesis in vitro of long RNA transcripts that greatly exceed the size of primers synthesized by DNA primases inside the cell.

Primase domain mutation in *LEF-1. The ability to synthesize RNA transcripts on poly(dT) and natural ssDNA is in agreement with the primase function of LEF-1 predicted earlier (13) that was based on the presence of conserved primase motifs in baculovirus LEF-1 sequences. The conserved motif of Pri-type primases comprising two aspartate residues separated by a single hydrophobic amino acid and preceded by three hydrophobic amino acids (W[I/V][I/L/V][A/V/I/D]) is found in the LEF-1 sequence of AcMNPV and several other baculoviruses (Fig. 7).

To confirm that the enzymatic activity observed in *LEF-1 samples was actually dependent on the predicted primase domain, we made a conserved change from aspartate to glutamine at amino acid 76 (REWVV DAD to REWVV QAD) in *LEF-1 and expressed this construct in a recombinant baculovirus. In the previous study, we found that this mutation did not prevent interaction with LEF-2, but it eliminated the ability of LEF-1 to function in the transient-replication assay (13). The mutant *LEF-1(D76Q), fused at the N terminus with the HA epitope and HIS6 tag, was overexpressed in S9 cells and purified by the same method as the wild-type (wt) *LEF-1. When purified to near homogeneity, *LEF-1(D76Q) demonstrated three polypeptides with the same mobility as those from our wt *LEF-1 construct (Fig. 8A). However, the activity of the mutant *LEF-1(D76Q) in the indirect assay with Klenow enzyme was very low (Fig. 8B). In the direct assay, the mutant *LEF-1(D76Q) did not synthesize any of the three products, I, II, or III, typical of wt *LEF-1 (Fig. 8C). Thus, the enzymatic activity of LEF-1 requires the intact primase domain in the protein. These data confirm the dependence of DNA synthesis by Klenow enzyme in the indirect assay on RNA primers provided by *LEF-1. The minor stimulation of DNA synthesis caused by *LEF-1(D76Q) in the indirect assay may...
be due to a nonspecific stabilization effect of the added protein on Klenow enzyme.

**Sedimentation analysis of LEF-1 in glycerol gradients.** To elucidate the molecular structure of LEF-1, we analyzed the sedimentation of the purified protein in a 15 to 30% glycerol gradient under conditions that prevent nonspecific aggregation (Fig. 9). LEF-1 sedimented in the gradient slower than BSA (4.3S, 66 kDa) and had a sedimentation coefficient of about 4.8S.

**Fig. 7.** Sequence alignment of LEF-1 proteins of baculoviruses AcMNPV (ac) (4), OpMNPV (op) (2), Choristoneura fumiferana multinucleocapsid NPV (cf) (5), Lymantria dispar multinucleocapsid NPV (ld) (28), Spodoptera exigua multinucleocapsid NPV (se) (21), Heliothis armigera single-nucleocapsid NPV (8), Spodoptera exigua granulovirus (px) (18), Xestia c-nigrum granulovirus (xc) (19), and Culex nigripalpus granulovirus (cuni) (40). Putative primase domains are boxed. Invariant amino acids in the putative primase domains are in white letters against black, and invariant aspartates in these domains are indicated by asterisks. Identical amino acids are shown at the bottom of each alignment, and dots indicate conservative changes. The last line within the boxed domains shows invariant amino acids from selected archaeal and eukaryotic primases (3). Dashes indicate gaps in the alignment. The numbers on the left and right indicate the amino acid sequence coordinates. The alignment was produced using MacVector DNA analysis software.
This value suggests that the *LEF-1 samples consist of a mixture of protein monomers. The sedimentation rate of *LEF-1 in the glycerol gradients was much lower than that of AcMNPV RNA polymerase, another viral enzyme capable of synthesizing RNA products. In our experiments, the sedimentation pattern of purified AcMNPV RNA polymerase was consistent with a molecular mass of 300 to 400 kDa (data not shown), which is slightly less than has been reported previously (15).

Chromatographic behavior of LEF-2. LEF-2 presumably forms a functional complex with LEF-1 in infected cells (13), and it has been shown to be essential for plasmid replication in the transient-replication assay (26, 33). However, the function of LEF-2 in baculovirus replication is not known. The primase activity found in the purified *LEF-1 samples which were essentially free of contaminating *LEF-2 clearly indicated that LEF-2 is not required for the primase activity of LEF-1 in vitro. Some of our indirect data suggest that LEF-2 may serve as an accessory factor needed for incorporation of LEF-1 into replication complexes assembled on viral DNA.

We routinely overexpressed HIS-tagged proteins LEF-1 and LEF-2 together, and both proteins were expressed in Sf9 cells at almost equivalent levels. However, it was more difficult to solubilize *LEF-2 than *LEF-1, and thus the samples after passage through an Ni-NTA column appeared to enriched for *LEF-1 (Fig. 2B, lane 2). At the next chromatographic step, *LEF-2 bound tightly to a DEAE resin, whereas *LEF-1 appeared mostly in the flowthrough (Fig. 2B, lane 3). It was possible to elute *LEF-2 and a portion of *LEF-1 from DEAE-Toyopearl only by using buffers with relatively high concentrations of monovalent salt (0.3 to 0.5 M KCl) (data not shown). Optical densitometry revealed the presence of nucleic acids in this high-salt fraction. Because all free proteins were removed from the DEAE resin at lower salt concentration, this result suggested that *LEF-2 and *LEF-1 might be associated with traces of viral DNA.

In another experiment, the sample of *LEF-1 and *LEF-2 collected from the Ni-NTA column was subjected to chromatography on an ssDNA-cellulose column (Fig. 10). Stepwise elution resulted in the *LEF-1 pool's being divided in two fractions. One fraction of *LEF-1 was free of *LEF-2 and eluted at a relatively low salt concentration (110 to 170 mM KCl), whereas the other fraction contained both proteins and eluted at much higher salt concentrations (300 to 500 mM KCl). This result suggests that LEF-1 might associate with LEF-2 to form a stable complex with single-stranded DNA and probably with other replicative proteins associated with DNA.

**DISCUSSION**

The essential baculovirus replication protein LEF-1 was originally predicted to serve as a DNA primase, because a mutation in a conserved primase-like domain failed to support transient DNA replication (13). Therefore, we initiated a biochemical study of this protein to determine if it possesses primase activity. Upon testing a number of fractions obtained from AcMNPV-infected Sf9 cells in an indirect assay employing Klenow enzyme and poly(dT) template (see Materials and Methods), a strong primase activity was found associated with host cell DNA polymerase α (data not shown). However, we
were unable to detect a virus-induced DNA primase in extracts from infected cells.

To increase the LEF-1 level in infected cells, we overexpressed it in recombinant baculovirus under the control of the strong polyhedrin promoter. In this construct, the HA epitope and a HIS6 tag were fused to LEF-1 at the N terminus. A similar construct was found to retain its activity in transient replication and transcription assays (45). This construct allowed the rapid and efficient purification of the recombinant protein, *LEF-1, to homogeneity, as judged by SDS-PAGE (Fig. 2). Biochemical analyses revealed the presence of primase activity by *LEF-1, providing evidence for its role as a primase in baculovirus replication that had been predicted earlier. The list of virally encoded enzymes involved in baculovirus DNA replication now includes a DNA primase in addition to the DNA polymerase (35, 39, 50) and DNA helicase (32, 36) characterized previously. A set of similar viral enzymes are required for genome replication by the Herpesviridae, another family of large DNA viruses (7).

The identification of LEF-1 as a DNA primase is based on its ability to synthesize primers from a poly(dT) template, which then allows initiation of DNA synthesis by exogenous DNA polymerase (Klenow enzyme) (Fig. 3B). The synthesis of primers was confirmed in a direct assay involving examination of the products by gel electrophoresis (Fig. 3C). Furthermore, analysis of a mutant LEF-1 confirmed the dependence of its activity on an intact domain conserved in archaeal and eukaryotic primases (Fig. 7 and 8).

The *LEF-1 primase activity is absolutely dependent on divalent cations (Fig. 4A), which may be directly involved in catalysis (see below). The most striking property of *LEF-1 is its ability to synthesize in vitro polyribonucleotides of a thousand nucleotides or more on ssDNA of phage M13 (Fig. 6). In this respect *LEF-1 resembles DNA primase from the archaeon Pyrococcus furiosus (6). The RNA transcripts synthesized by *LEF-1 greatly exceed the size of primers synthesized by known DNA primases during DNA replication inside eukaryotic cells. If baculovirus replication is similar to that of other systems, some other viral or host cell factors may limit the size of RNA transcripts synthesized by LEF-1 to produce physiologically relevant RNA primers.

Two distinct types of primases that lack sequence or structural relatedness have been characterized. The Pri-type primases are found in eukaryotes and archaea, whereas the DnaG type are found in eubacteria (23). Baculovirus LEF-1 contains three motifs (for AcMNPV, residues 69 to 78, 114 to 139, and...
189 to 214) that are conserved in eukaryotic and archaeal primases (boxed in Fig. 7), suggesting that it is a Pri-type primase. These motifs presumably contribute to the structure of the major domain, which contains the primase active site (3). The lef-1 gene of AcMNPV encodes a protein of 266 amino acids with a calculated molecular mass of 30,780 Da. Other known lef-1 genes of baculoviruses encode proteins as small as 216 amino acids (Fig. 7). These represent the smallest DNA-synthesizing enzymes characterized so far. Due to its small size, baculovirus LEF-1 may be a valuable tool for structural research of Pri-type primases as well as for biochemical and mechanistic studies. The first conserved LEF-1 motif (REW[I/V][I/L/V][D][A/I/V][D]) contains two aspartate residues separated by a single hydrophobic amino acid (DXD) and preceded by three hydrophobic amino acids. This motif is also found in primases from a number of herpesviruses, phage T7, Saccharomyces cerevisiae, archaea, and mammals (3, 24). Experimental data suggest that the two proximal aspartates in this motif are important for enzymatic activity. In herpes simplex virus type 1 primase (UL52), a conserved aspartate-to-glutamine (III[D/D to III[Q/Q]) change at amino acid 628 completely eliminated the primase activity associated with the UL5-UL8-UL52 complex (25). In the catalytic subunit p49 of mouse primase, two aspartates (D109 and D111) of the respective domain and one distal aspartate (D306) are essential for primase activity and are thought to form the metal-binding core of the active site (10).

Aspartates D76 and D78 in the first conserved motif of AcMNPV LEF-1 and the distal invariant aspartate D193 in the third motif presumably play the same role. In agreement with this prediction, a conserved change from aspartate to glutamine at amino acid 76 (REW[V/V] to REW[V/Q]) in AcMNPV LEF-1 completely abolished the LEF-1 primase activity (Fig. 8). Because this mutation eliminated the ability of LEF-1 to function in transient-replication assays (13), the essential function of LEF-1 in baculovirus replication is directly connected with the primase activity. Interestingly, the three aspartates in mouse primase (D109, D111, and D306) align precisely with similar residues in the 31-kDa domain of DNA polymerase β, suggesting that Pri-type primases are probably members of the Pol X polymerase family (24). The three aspartates (D95, D97, and D280) in the archaeon Pyrococcus furiosus (Pfu) primase could be superimposed with the active-site residues of four different DNA polymerases, including human DNA polymerase β, indicating similar three-dimensional arrangements in all five structures (3). These data suggest that Pri-type primases including baculovirus LEF-1, use the common two-metal ion mechanism of DNA polymerases (49).

Another conserved motif in DNA primases is the Zn$^{2+}$ binding domain located near the active site. In bacterial DnaG-type primases, this domain is thought to play a role in template sequence recognition (43). The P$\beta$r primase structure confirms the presence of a Zn$^{2+}$ ion at a location quite close to the putative active site, suggesting that this ion may also play a role in the activity of Pri-type primases (3). However, the zinc binding site (C/HX2-5/C/HX1-13/C/HX2-5/C/H) and its derivatives are absent in some archaeal primases (3) and in baculovirus LEF-1. This raises doubts about whether the zinc ion is essential for the function of Pri-type primases.

Although *LEF-1 itself has relatively low affinity for ssDNA and was eluted from ssDNA-cellulose at 110 to 170 mM KCl, a portion of the cellular pool of *LEF-1 copurified with *LEF-2 as a fraction possessing a high affinity for ssDNA (Fig. 10). Since it has been demonstrated that LEF-1 interacts with LEF-2 (13), this observation suggests that LEF-2 may stabilize binding of LEF-1 to DNA template and probably to other baculovirus replication proteins. In this respect, LEF-2 resembles subunit p58 of eukaryotic DNA primase. Although p58 is not required for enzymatic activity of the catalytic subunit p49, it binds to ssDNA, tethers p49 to the 180-kDa subunit of DNA polymerase α, and stabilizes p49 (11).

All known DNA primases act in close association with either replicative DNA polymerases or DNA helicases. Both subunits of simian primase interact with host cell replication protein A and simian virus 40 large T antigen, which serves as the helicase in virus replication (51). E. coli primase (DnaG) interacts with the replicative DnaB helicase, SSB protein, and DNA polymerase III holoenzyme (27). In T7 phage, whose helicase and primase are homologous to the equivalent eubacterial proteins (22), the COOH-terminal region of the primase is directly linked to the NH$_2$-terminal region of the helicase, forming a single polypeptide. Replication of baculoviruses may proceed by a mechanism similar to that of herpesviruses. In herpesviruses, the 114-kDa primase subunit UL52 is tightly associated with UL5 and UL8, forming a heterotrimeric primosome possessing DNA-dependent ATPase, DNA helicase, and DNA primase activities (for a review, see reference 30). The functional partner of the LEF-1 and LEF-2 complex in the replicosome of baculoviruses is not known. Further analysis of the complexes of LEF-1 and LEF-2 with ssDNA observed in this study may provide a more detailed picture of the structure and organization of the replication machinery of baculoviruses.

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ADDENDUM IN PROOF

After this paper was submitted, Eugene Koonin informed us that his group had detected a low level of homology between baculovirus LEF-2 and the large subunit (p58) of eukaryotic DNA primases (E. V. Koonin, Y. I. Wolf, A. S. Kondrashov, and L. Aravind, J. Mol. Microbiol. Biotechnol. 2:509–512, 2000). We appreciate this information, which is in agreement with our suggestion that LEF-2 plays an accessory role in baculovirus DNA primase activity, analogous to that of p58 in eukaryotic primases.

REFERENCES


