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Guanylyltransferase Activity of the LEF-4 Subunit of Baculovirus RNA Polymerase

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The baculovirus *Autographa californica* nuclear polyhedrosis virus encodes a DNA-dependent RNA polymerase that transcribes viral late genes. This polymerase is composed of four equimolar subunits, LEF-4, LEF-8, LEF-9, and p47. Here we present data indicating that the LEF-4 subunit of RNA polymerase is a guanylyltransferase. Incubation of RNA polymerase in the presence of divalent cation and radiolabeled GTP resulted in the formation of a covalent enzyme-guanylate complex that comigrated with the LEF-4 subunit. The label transfer assay showed an absolute requirement for divalent cation which could be satisfied by either manganese or magnesium. The reaction was specific for guanine nucleotides, and GTP was more effective than dGTP in the formation of enzyme-guanylate complex. To demonstrate that LEF-4 was the guanylyltransferase, the single subunit was overexpressed in baculovirus-infected cells. The overexpressed protein was primarily cytosolic, indicating that other proteins in the RNA polymerase complex were responsible for nuclear targeting of LEF-4. LEF-4 alone was able to covalently bind GMP, although less efficiently than viral RNA polymerase.

Baculoviruses are unique among eukaryotic DNA viruses in that early genes are transcribed host RNA polymerase II (6, 12), while late genes are transcribed by a virus-encoded RNA polymerase (11). Baculovirus early genes are subdivided into two temporal classes, immediate-early and delayed-early. Differential transcription of these two subclasses is mediated by distinct promoter and enhancer motifs (8, 23). Transcription of the immediate-early genes, like *ie1*, does not require additional viral proteins or enhancer elements for high-level transcription (9). The delayed-early genes, however, are transcribed at basal levels in the absence of additional viral factors but require binding of the viral transactivator IE1 to viral enhancer elements for maximal levels of expression (7, 10). The late genes are also subdivided into two classes, and temporal expression of the late and very late genes is regulated by promoter elements that presumably affect binding of the viral RNA polymerase. Transcription of late and very late genes is absolutely dependent on a TAAG element at the start of transcription and is further regulated by a conserved 12-bp sequence surrounding TAAG (8, 25, 26). In addition, the very late genes show a requirement for A+T-rich region downstream of the start of transcription (19). This burst sequence is apparently responsible for temporal regulation of the very late genes, as construction of a chimeric promoter containing a late promoter element and a very late burst sequence produced a gene that was highly transcribed during both phases of infection (18).

Much of our understanding of the mechanisms that control the temporal expression of baculovirus genes is derived from in vitro transcription systems. We and others have shown that in vitro extracts faithfully reproduce the temporal progression of viral gene expression (12, 17, 31). Recently, we used an in vitro transcription assay to purify the RNA polymerase that transcribed late and very late baculovirus genes. This polymerase was unusual in that it contained both catalytic and specific promoter recognition activities. The polymerase was composed of four equimolar subunits, LEF-8, LEF-4, LEF-9, and p47 (11). All four of these proteins are encoded by viral genes, and each had previously been shown to be required for transient expression of viral late and very late genes (29). In addition, LEF-8, the largest of the four subunits, was predicted to encode an RNA polymerase subunit, based on the presence of a HGQKGV sequence near the C terminus of the protein (22). This motif is conserved among β subunits of RNA polymerases from a number of sources and is believed to form part of the catalytic site of the enzyme. LEF-9 also contains a sequence that matches the β′ motif NADFDGD in five of seven positions (14). These sequence homologies suggest that LEF-8 and LEF-9 may be the catalytic subunits of RNA polymerase.

Database searches of LEF-4 and p47 did not reveal any strong homologies to proteins with known functions. However, our identification of p47 and LEF-4 as essential components of the transcription apparatus is consistent with genetic information relative to their functions. Both the *p47* and *lef-4* genes were originally identified as the sites of temperature-sensitive mutations having a phenotype that suggested a role in transcription of late and very late genes (3, 4, 20). At the nonpermissive temperature, *p47* and *lef-4* mutant viruses were normal with respect to DNA replication but were defective in the release of infectious virus and expression of late proteins.

In building a model for the regulation of late gene expression, we felt it necessary to include pathways for posttranscriptional modifications. It has previously been shown that baculovirus late and very late mRNAs are capped and polyadenylated (24). In eukaryotic cells, both of these modifications are restricted to transcripts made by RNA polymerase II. mRNAs are capped with 7-methylguanosine (m7G) at the 5′ ends when they are less than 30 nucleotides in length, and this is mediated by specific interactions of capping enzymes with RNA polymerase II (4, 16). These observations suggest that either baculovirus RNA polymerase must interact with host capping enzymes in an analogous manner or the virus must encode its own capping enzymes. To test this hypothesis, we decided to assay for guanylyltransferase at all stages during the purification of baculovirus RNA polymerase. We found

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that the most purified fraction was active in the formation of enzyme-GMP complexes, which is the first step in the transfer of GMP to RNA. Furthermore, we identified the LEF-4 subunit as the guanylyltransferase and showed that the purified single subunit had guanylyltransferase activity.

MATERIALS AND METHODS

Construction of vLEF-4. The *Autographa californica* nuclear polyhedrosis virus (AcNPV) genomic clone pHindIII-C was digested with *Nar*I and *Xho*I and incubated with Klenow enzyme and deoxynucleoside triphosphates to fill in 5′ overhangs. A 1.6-kb fragment containing the complete *lef-4* open reading frame was purified by agarose gel electrophoresis and cloned into the *Sma*I site of pVL1393. Correct orientation of the insert was determined by restriction digest. The resulting plasmid pVL1393-LEF4, was cotransfected with *Bsu*36I-digested RP6-SC DNA into *Spodoptera frugiperda* cells. Recombinant viruses were plaque purified and amplified by standard protocols (28). One plaque isolate with the correct insert was named vLEF-4.

Purification of LEF-4 from baculovirus-infected cells. *S. frugiperda* cells grown in 1-liter spinner cultures were infected with vLEF-4 and harvested at 60 h postinfection. Cells were washed in phosphate-buffered saline, and resuspended in four times the packed cell volume of hypotonic buffer (10 mM Tris [pH 7.9], 10 mM KCl, 3 mM dithiothreitol [DTT], 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine, 3 μg of leupeptin per ml). The cells were allowed to swell on ice for 20 min and broken by homogenization in a glass Dounce homogenizer (B pestle). Cells were checked by phase microscopy for complete breakage, and then a 1/10 volume of restoration buffer (50 mM Tris [pH 7.9], 0.75 mM spermidine, 0.15 mM spermine, 10 mM KCl, 0.2 mM EDTA, 3 mM DTT, 67.5% sucrose) was added. The homogenate was layered over a 10-ml sucrose cushion (30% sucrose in hypotonic buffer) and centrifuged for 10 min at 3,000 rpm. The supernatant (cytosolic fraction) was saved, and the pelleted nuclei were resuspended in four times the packed-cell volume of nuclear extraction buffer (50 mM Tris [pH 7.5], 0.42 M KCl, 6 mM DTT, 0.1 mM EDTA, 10% sucrose, 5 mM $MgCl_2$, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride). The nuclei were then lysed by gentle rocking at 4°C for 30 min, and the lysate was centrifuged at 40,000 rpm in a Beckman Ti 50.2 rotor for 90 min at 4°C to remove the DNA. The protein components of cytosolic and nuclear fractions prepared from vLEF-4 and RP6-SC-infected cells were compared by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis. By this method, overexpressed LEF-4 was localized to the cytosolic fraction.

The LEF-4 cytosolic extract was precipitated with 40% saturated NH_4SO_4, resuspended in 8 ml of buffer A (50 mM Tris [pH 7.9], 0.1 mM EDTA, 1 mM DTT) containing 50 mM KCl, and dialyzed against 800 ml of the same buffer. Then the sample was applied at 1 ml/min to a 5-ml heparin (Bio-Rad) column connected to a Pharmacia FPLC system previously equilibrated with buffer A–50 mM KCl. The column was washed with 10 ml of loading buffer and eluted with a 20-ml linear gradient from 50 to 500 mM KCl. Samples were analyzed by SDS-PAGE. Peak fractions containing LEF-4 were pooled, dialyzed against buffer A–50 mM KCl, and the protein was applied to a Mono Q HR 5/5 column (Pharmacia) previously equilibrated with buffer A–50 mM KCl. The column was washed with 5 ml of loading buffer and then eluted with a 20-ml linear KCl gradient from 50 to 500 mM. Fractions that contained LEF-4 were concentrated to 200 μl and filtered through a Superdex 200 column in buffer A–100 mM KCl. Fractions (0.5 ml) were collected, individually frozen in liquid nitrogen, and stored at −80°C. The protein concentration of LEF-4 was determined by UV absorbance using a molar extinction coefficient of 57,800.

Assay of enzyme-GMP complex formation. Standard reaction mixtures contained 1 pmol of purified RNA polymerase or LEF-4, 1 mM $MnCl_2$, 5 mM DTT, and 5 μM [α-^{32}P]GTP in 25 μl. Samples were incubated for 15 min at 30°C and then stopped by the addition of 1% SDS. Samples were boiled and electrophoresed through an SDS–8% polyacrylamide gel. Gels were fixed, dried, and exposed to film.

RESULTS

Guanylyltransferase activity of RNA polymerase. Guanylyltransferases catalyze the transfer of GMP from GTP to a diphosphate-terminated RNA. This is a two-step reaction involving the formation of a covalent enzyme-guanylate intermediate in which GMP is linked by a phosphamide bond to a lysine residue in the enzyme (27). The first step in the guanylyltransferase reaction is routinely assayed by transfer of ^{32}P label from [α-^{32}P]GTP to the enzyme. Therefore, we used this assay to test for guanylyltransferase activity copurifying with baculovirus RNA polymerase.

AcNPV RNA polymerase was purified from *S. frugiperda* cells infected with a recombinant baculovirus that overexpresses all four RNA polymerase subunits as described previously (11). The purified RNA polymerase was filtered through a Superose 6 size exclusion column in 2 M KCl, and individual fractions were tested for RNA polymerase activity in our standard in vitro transcription assay (31). This assay uses two nucleoside-free templates that are separately linked to the late *39k* gene and the very late polyhedrin gene. After incubation at 30°C, samples were extracted with phenol and RNA products were analyzed by acrylamide gels in the presence of 8 M urea. As previously shown, the baculovirus RNA polymerase catalyzes the template-dependent synthesis of transcripts initiating at the baculovirus late promoters (Fig. 1A, lanes 2 to 7). The level of transcripts obtained was directly proportional to the amount of protein in each fraction, indicating that the enzyme was essentially homogeneous.

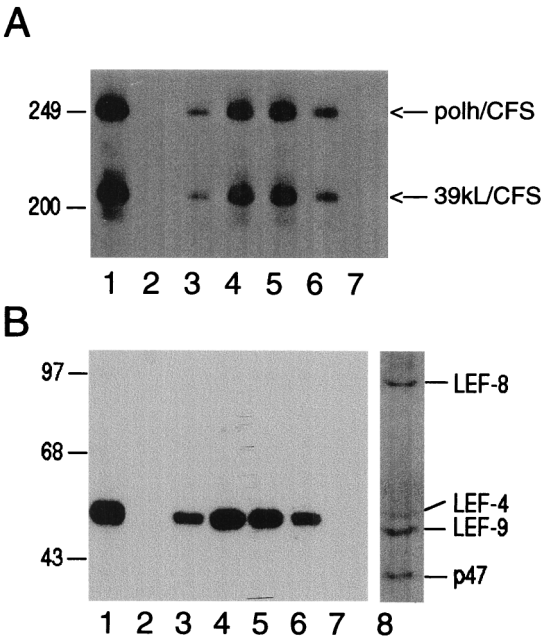

FIG. 1. Guanylyltransferase activity of baculovirus RNA polymerase. (A) Gel filtration chromatography of RNA polymerase. RNA polymerase was filtered through Superose 6 as previously described (11). Fractions corresponding to the peak of absorbance at 280 nm were assayed for in vitro transcription activity (lanes 2 to 7). A transcription assay of the material loaded onto the column is shown in lane 1. The transcripts corresponding to polydrin the (Polh/CFS) and 39k (39kL/CFS) promoters are indicated on the right. The sizes of relevant ϕX174-*Hin*fI molecular markers are shown on the left. (B) Guanylyltransferase assays. Proteins in the corresponding fractions were incubated with 50 mM Tris (pH 7.9), 2 mM DTT, 2 mM $MgCl_2$, and 1 μM [a-^{32}P]GTP. After incubation for 15 min at 30°C, samples were resolved by SDS-PAGE. Gels were dried and exposed to X-ray film Lane 8 shows the migration of the RNA polymerase subunits as detected by silver staining. The positions of the four polymerase subunits are indicated on the right. The positions of relevant molecular weight protein markers are shown in kilodaltons on the left.

The corresponding fractions were also analyzed for the formation of SDS-resistant GMP-protein adducts (Fig. 1B, lanes 2 to 7). A single radiolabeled protein that migrated as a 54-kDa species was detected in the autoradiographs of the resulting protein gels. The guanylyltransferase reactions contained only RNA polymerase, GTP, and divalent cation. Thus, the formation of the protein nucleoside was not dependent on the addition of DNA template, RNA product, or the other nucleotides. The amount of guanylate-enzyme (EpG) formed in each fraction was directly proportional to the amount of transcription activity (Fig. 1A, lanes 2 to 7). Physical association of the guanylyltransferase activity with RNA polymerase activity in 2

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      251                                                        310
AcNPV   SVKKWALKLDGMRGRGLFMRNFCIIQTDDMQFYKTKMANLFALNNIVAFQCEVMDKQKIY
BmNPV   SVKKWALKLDGMRGRGLFMRNFCIIQTDDMQFYKTKMANLFSLNNIVAFQCEVMDKQKIY
OpNPV   GVKKWAFKLDGVRGRGAFRRGYCLVQTDDMQLHAACISSPFGLNNVVTFQCEVV-ADKIF
Sce CE    YYVCEKTDGLR                                    TLLDGELV
             I                                             III

      311                                                        370
AcNPV   ITDLLQVFKYKYNNRTQYECGVNASYAIDPVTAIECINYMNNNVQSVTLTDTCPAIELRF
BmNPV   ITDLLQVFKYKYNNRTQYECGVNASYAIDPVTAIECINYMNSNVQSVTLTDTCPEIELRF
OpNPV   VTDLLQVFRYKYNNRTQYECNLHDAYPINADVAVECLNRLHCAVGSVPWPGLG---ELRF
Sce CE                RYLMFDCLAING
                        IIIa

      371                                                        430
AcNPV   QQFFDPPLQQSNYMTVSVDGYVVLDTELRYVKYKWMPTTELEYDAVNKSFNTLNGPLNGL
BmNPV   QQFFDPPLQQSDYMTVSVDGYVVLDTELRYVKYKWMPTTELEYDAVNNSFNTLNGPLNGL
OpNPV   QQFFDPPLAPTHYTTIPIDGYIVLDEQLQYAKYKWLPTVELEYDAPSGALHSIDGPLLGK
Sce CE                        DGLIF       LLKWKPEQENTVD
                               IV           V

      431                                  470
AcNPV   MILTDLPELLHENIYECVITDTTINVLKHRRDRIVPN
BmNPV   VILTNLPELLHENIYECVIADTTINVLKHRRDRIVPN
OpNPV   TVVADL-QLKHGAVYECAITDNAINVLKCRPDRIVPSKVC
Sce CE                          WEMLRFRDDK
                                 VI
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FIG. 2. Sequence of the LEF-4 guanylyltransferase domains. The C-terminal 220 residues of LEF-4 proteins from AcNPV, *Bombyx mori* nuclear polyhedrosis virus (BmNPV), and *Orgyia pseudotsugata* nuclear polyhedrosis virus (OpNPV) were aligned by using the GCG Pileup program (30). Residues corresponding to six sequence elements (designated motifs I, III, IIIa, IV, V, and VI) from the *S. cerevisiae* capping enzyme (Sce CE) (30) were aligned by eye with the corresponding regions in the baculovirus enzymes. Residues in the yeast capping enzyme that have been shown to be essential for function by alanine substitution (30) are underlined.

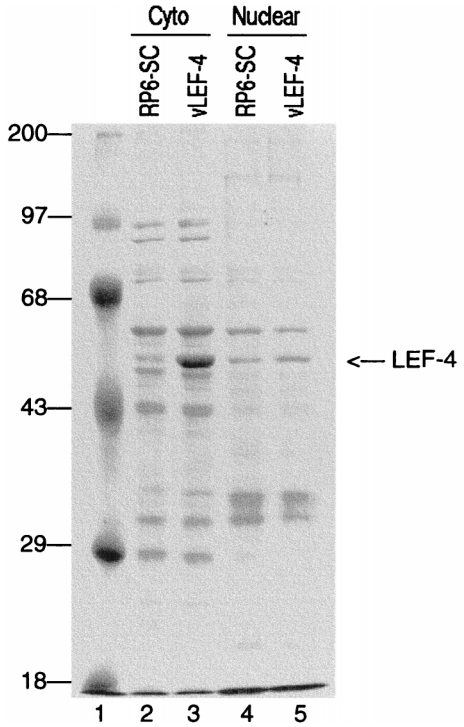

FIG. 3. Expression and localization of LEF-4 in baculovirus-infected cells. *S. frugiperda* cells were infected with vLEF-4 (lanes 3 and 5) or the parental virus RP6-SC (lanes 2 and 4) at a multiplicity of infection of 10. At 48 h postinfection, cells were harvested, washed in phosphate-buffered saline, and separated into nuclear and cytosolic (cyto) fractions. Equivalent amounts of protein in each fraction were separated on SDS-polyacrylamide gels and stained with Coomassie brilliant blue. Protein molecular weight markers were loaded in lane 1, and the sizes of the relevant proteins are shown in kilodaltons on the left. The position of LEF-4 is indicated on the right.

M salt strongly argues that guanylyltransferase activity was an integral component of the viral RNA polymerase complex.

The radiolabeled protein comigrated with the LEF-4 subunit of the polymerase complex (Fig. 1B, lane 8), suggesting that LEF-4 is the guanylyltransferase. Capping enzymes are members of a superfamily of nucleotidyltransferases, and members of this family bind nucleotides to an invariant lysine residue that is contained within a conserved KxDG motif. This motif is present in all three of the baculovirus LEF-4 proteins that have been sequenced (motif I in Fig. 2). Five additional motifs have been noted for the capping enzymes and ligases (30), and these sequences are conserved in the same order and with similar spacing in the baculovirus proteins. Sixteen amino acids in these six motifs have previously been shown to be essential for function of the *Saccharomyces cerevisiae* capping enzyme (30). Comparison of these residues (underlined in Fig. 2) with the corresponding sequences in the baculovirus LEF-4s reveals that 10 of them are identical while 8 have conservative substitutions. This sequence comparison strongly supports our biochemical data suggesting that LEF-4 is a guanylyltransferase.

Guanylyltransferase activity of LEF-4. To confirm that *lef-4* encodes a protein with guanylyltransferase activity, we constructed a recombinant baculovirus that overexpressed LEF-4 under the control of the polyhedrin promoter. Infected cells were harvested at 48 h postinfection and separated into cytosolic and nuclear fractions. To determine the subcellular localization of overexpressed LEF-4, nuclear and cytosolic fractions were analyzed by denaturing PAGE (Fig. 3). Staining of total proteins with Coomassie brilliant blue revealed that LEF-4 was strongly overexpressed in the recombinant virus compared to the parental control, RP6-SC. Overexpressed LEF-4 was predominantly found in the cytosolic fraction. It has previously been shown that LEF-4 is primarily localized in the nuclei of infected cells (5), as is the viral RNA polymerase (11). The failure of overexpressed LEF-4 to accumulate in the nucleus suggests that the LEF-4 subunit lacks a nuclear targeting signal and must rely on other components of the viral RNA polymerase for nuclear transport.

Cytosolic LEF-4 was purified by ion-exchange chromatography. LEF-4 bound to heparin-agarose at 50 mM KCl and eluted at 150 mM KCl. The peak fractions from heparin-agarose were dialyzed and loaded onto a Mono Q column at 50 mM KCl. LEF-4 bound to Mono Q and was eluted at 230 mM KCl. The Mono Q peak was nearly homogeneous with respect to the 54-kDa band, as judged by Coomassie brilliant blue staining of SDS-polyacrylamide gels (Fig. 3, lane 1). Approximately 340 mg was obtained from 1 liter of vLEF-4-infected cells.

The peak of LEF-4 protein from the Mono Q column was further purified by filtration through a Superdex 200 column at 100 mM KCl. A single UV-absorbing peak eluted at 13.6 ml (Fig. 4A). Fractions corresponding to the peak of A_{280} were analyzed by SDS-PAGE, and a 54-kDa band was observed in the Coomassie brilliant blue-stained gels (Fig. 4B). Comparison of the elution volume for LEF-4 relative to marker proteins filtered through the same column indicated a molecular mass of 114,200 for the native protein. At 400 mM KCl, LEF-4 eluted from the same column at 14.8 ml, consistent with a monomer molecular mass of 47,600 (data not shown). This finding suggests that the 54-kDa LEF-4 protein forms a dimer in solution at physiological salt concentrations. The dimers dissociate into monomers at higher salt concentrations indicating that the interactions at the dimer interface are relatively weak. The baculovirus RNA polymerase complex is also a

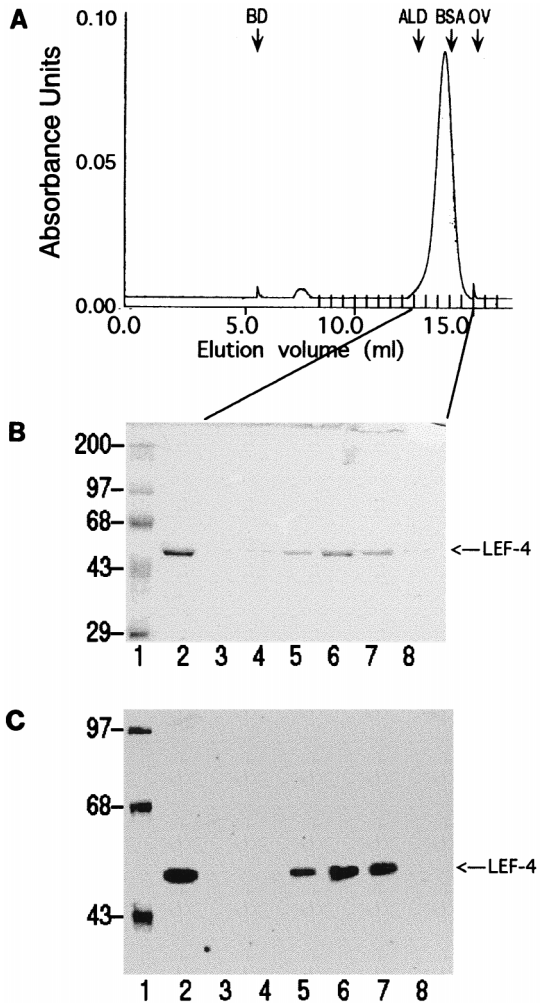

FIG. 4. Guanylyltransferase activity of LEF-4. (A) Gel filtration chromatography of LEF-4. The peak of LEF-4 protein from a Mono Q column was filtered through Superdex 200. Fractions (0.5 ml) were collected from 8.5 to 16 ml. Marker proteins used for calculation of the molecular mass of LEF-4 were aldolase (ALD), bovine serum albumin (BSA), and ovalbumin (OV). The exclusion volume was determined by gel filtration of blue dextran 2000 (BD). (B) SDS-PAGE analysis of Superdex 200 fractions. Fractions 9 to 14 from the Superdex column were separated on an SDS-polyacrylamide gel (lanes 3 to 8) and stained with Coomassie brilliant blue. Lane 2 shows the Mono Q peak fraction that was loaded onto the column. Protein molecular weight markers were loaded in lane 1, and the sizes of the relevant proteins are shown in kilodaltons on the left. LEF-4 is indicated on the right. (B) Guanylyltransferase assays. Proteins in the corresponding fractions were incubated with 50 mM Tris (pH 7.9), 2 mM DTT, 1 mM $MnCl_2$, and 1 μM [α-^{32}P]GTP. After incubation for 15 min at 30°C, samples were resolved by SDS-PAGE. An autoradiograph of the dried gel is shown. Protein molecular weight markers were loaded in lane 1, and the sizes of the relevant proteins are shown in kilodaltons on the left. The position of LEF-4 as judged by analysis of the Coomassie blue-stained gel is indicated on the right.

dimer of four subunits, but the polymerase dimer is stable at 2 M KCl (11).

The Superdex 200 gel filtration fractions were analyzed for guanylyltransferase activity by the GMP label transfer assay. A single radiolabeled protein that comigrated with LEF-4 was detected on autoradiographs (Fig. 4C). The peak of guanylyltransferase activity exactly coincided with the peak of LEF-4 protein. These data confirm that LEF-4 is the guanylyltransferase subunit of RNA polymerase.

Characterization of the guanylyltransferase reactions of RNA polymerase and LEF-4. With both purified LEF-4 and purified RNA polymerase, the amount of enzyme-guanylate formed was proportional to the amount of protein added (Fig. 5A). However, purified LEF-4 was not as active as the viral RNA polymerase in the guanylylation reaction. With purified RNA polymerase, approximately 15% of the protein was guanylylated in this experiment. This value varied from 7.5 to 20%, depending on the particular preparation. However, with the LEF-4 single subunit, we consistently observed that less than 1% of the enzyme was guanylylated, and only 0.84% of the input enzyme was radiolabeled in this experiment. It has been established in other systems that the activities of guanylyltransferases in vitro are limited by the number of open sites for guanylation (25). The K_ms for GTP are usually below the in vivo concentrations of GTP, and thus most enzymes are isolated in the guanylylated form. Guanylyltransferase reactions are reversible in the presence of PP_i, which is the product of the reaction and therefore shifts the equilibrium resulting in release of GTP from the enzyme. Thus, the addition of low levels of PP_i can stimulate the forward reaction by freeing occupied binding sites for subsequent reaction with radiolabeled GTP.

To test whether in vivo guanylylation was responsible for the low activity of the purified subunit, we performed pyrophosphate titration experiments with both enzymes (Fig. 5B). The addition of low levels of pyrophosphate increased the formation of radiolabeled EpG with the purified RNA polymerase. The amount of guanylate-enzyme formed increased linearly to 62 μM PP_i, and at this concentration 92% of the input enzyme was radiolabeled. The results of this experiment show that the transguanylylation reaction is freely reversible and that essentially all of the enzyme is catalytically active. Furthermore, this experiment serves to confirm that the LEF-4 subunit of purified polymerase is the guanylyltransferase, as it is the only protein of that size that is present in stoichiometric amounts. Minor contaminants, if present, would be unlikely to bind GTP at the molar amounts observed in this experiment.

The amount of EpG formed with the purified LEF-4 subunit was also increased in a linear fashion by the addition of low levels of PP_i (Fig. 5B). However, the increase was modest compared to RNA polymerase. In the presence of 62 μM PP_i 2.1% of the enzyme was guanylylated, only a 2.6-fold increase over that seen in the absence of pyrophosphate. This finding suggests that the low activity of the purified subunit compared to the holoenzyme is due to intrinsic differences between the two enzyme sources and not to preguanylylation of the enzyme in vivo.

Nucleotide specificity of the guanylyltransferase activity of RNA polymerase. The guanylylation reaction showed high specificity for [α-^{32}P]GTP (Fig. 6A). There was no label transfer to the LEF-4 subunit of RNA polymerase in the presence of [α-^{32}P]CTP, [α-^{32}P]UTP, or [γ-^{32}P]ATP. Also no radiolabeled product was formed with [γ-^{32}P]GTP, consistent with the formation of a GMP complex. Formation of protein-guanylate was detected from reactions containing [α-^{32}P]dGTP (lane 7). However, the amount of labeled enzyme formed at this substrate concentration was 30-fold lower than with [α-^{32}P]GTP, indicating that LEF-4 discriminates between ribose and deoxyribose sugars.

To further investigate the ribose specificity of LEF-4, a nucleoside triphosphate titration experiment was performed (Fig. 6B). With GTP, the yield of EpG increased linearly to 2.5 μM and reached saturation at 5 μM. Half-saturation was reached at 1 μM. With dGTP as the substrate, the reaction was linear to 5 μM, and at this concentration 10-fold less product was

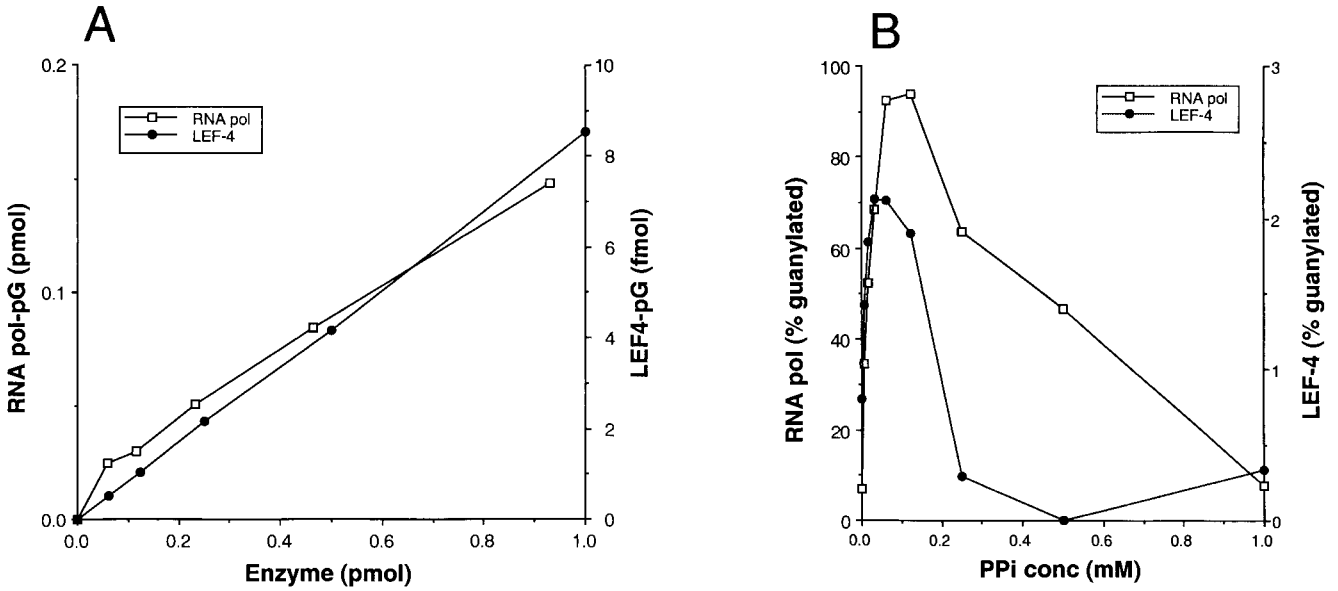

FIG. 5. Guanylyltransferase activity of purified LEF-4 and RNA polymerase. (A) Protein titration. Guanylyltransferase assays were performed with purified AcNPV RNA polymerase (pol) or with LEF-4 subunit. The reaction mixtures contained 50 mM Tris (pH 7.9), 5 mM DTT, 1 mM $MnCl_2$, 5 μM [α-^{32}P]GTP, and RNA polymerase or LEF-4, as indicated. Reaction products were quantitated by scanning the SDS-polyacrylamide gel in a PhosphorImager. The yield of guanylated RNA polymerase (pol-pG) is plotted on the left (in picomoles of radiolabeled protein), and the yield of guanylated LEF-4 is plotted on the right. (B) Inhibition of guanylyltransferase activity by PP_i. The reaction mixtures contained 50 mM Tris (pH 7.9), 5 mM DTT, 5 mM $MnCl_2$, 5 μM [α-^{32}P]GTP, 1 pmol of RNA polymerase or LEF-4, and $NaPP_i$ as indicated. The yield of guanylated RNA polymerase (expressed as percentage of input RNA polymerase radiolabeled) is plotted as a function of PP_i concentration on the left, and the yield of guanylated LEF-4 (expressed as percentage of input protein) is plotted on the right.

formed with dGTP than with GTP. The formation of LEF-4–dGMP continued to increased slowly up to 40 μM dGTP (Fig. 6B and data not shown), and at this concentration the amount of EpG was approximately 25% of that formed with saturating GTP. With dGTP, the reaction was half-maximal at 10 μM. These data confirm that RNA polymerase discriminates between the sugars and prefers ribose over deoxyribose.

Titration of GTP with the purified LEF-4 subunit revealed the explanation for the low activity of LEF-4 in the transguanylylation reaction. The formation of guanylate-enzyme was linear with respect to increasing GTP up to 500 μM and continued to increase slowly up to 5 mM GTP (Fig. 6C). At saturating levels of GTP, approximately 30% of the input enzyme was guanylated in vitro. At 5 μM GTP, the optimal concentration for the guanylyltransferase activity of RNA polymerase, the reaction with LEF-4 was only 2.5% of maximal. Titration of sodium pyrophosphate at 1 mM GTP increased the level of guanylation approximately threefold (data not shown), indicating that nearly all of the protein was catalytically active. This finding confirms that the transguanylylation reaction is fully reversible by the addition of pyrophosphate. Furthermore, these experiments indicate that the low activity of the single subunit is primarily due to the fact that LEF-4 by itself binds GTP poorly, which suggests that assembly of LEF-4 into the RNA polymerase complex lowers the K_m for GTP.

Cation dependence of the guanylyltransferase activity of RNA polymerase and of purified LEF-4. With both sources of enzyme, guanylyltransferase activity was dependent on the addition of a divalent cation (Fig. 7). For both LEF-4 and RNA polymerase, manganese was a more efficient cofactor than magnesium at concentrations below 10 mM, although the optimal concentrations differed for the two enzyme preparations. With RNA polymerase, activity was maximal between 0.6 and 10 mM, while the LEF-4 activity peaked at 5 mM. With both enzymes, activity declined between 10 and 20 mM. With magnesium as cofactor, the yield of guanylated RNA polymerase was proportional to the cation concentration between 0.2 and 1 mM $MgCl_2$ and was maximal between 5 and 10 mM. At 2 mM $MgCl_2$, the standard concentration for in vitro transcription, the yield of EpG was 90% of maximal. Magnesium was less efficient as a cofactor for LEF-4 than for RNA polymerase. The guanylyltransferase activity gradually increased as a function of magnesium concentration, and reached saturation between 20 to 40 mM (Fig. 7B and data not shown).

DISCUSSION

It is well established that the m7G cap at the 5′ end of mRNAs is recognized by proteins that catalyze polyadenylation, splicing, transport to the cytosol, and initiation of translation. In addition, the 5′ cap is a major factor in the regulation of mRNA turnover. With the exception of the *Baculoviridae*, all viruses that replicate in the nucleus depend on host capping enzymes which interact with RNA polymerase II, the polymerase of choice for most eukaryotic DNA viruses. Viruses that replicate in the cytosol have evolved a variety of different mechanisms to ensure that their RNAs are capped, including cap stealing for the influenza viruses and synthesis of virus-encoded capping enzymes for the poxviruses and reoviruses (2). Additional solutions are provided by the *Picornaviridae*, which have evolved an alternative mechanism for ribosome binding, and the double-stranded RNA viruses of yeast, which lack a 5′ cap but direct the synthesis of proteins that remove caps from host RNAs (15). Our identification of a virus-encoded RNA polymerase in baculovirus-infected cells suggested that the baculoviruses may also have evolved a novel solution to the capping problem.

We assumed that the baculovirus RNA polymerase would

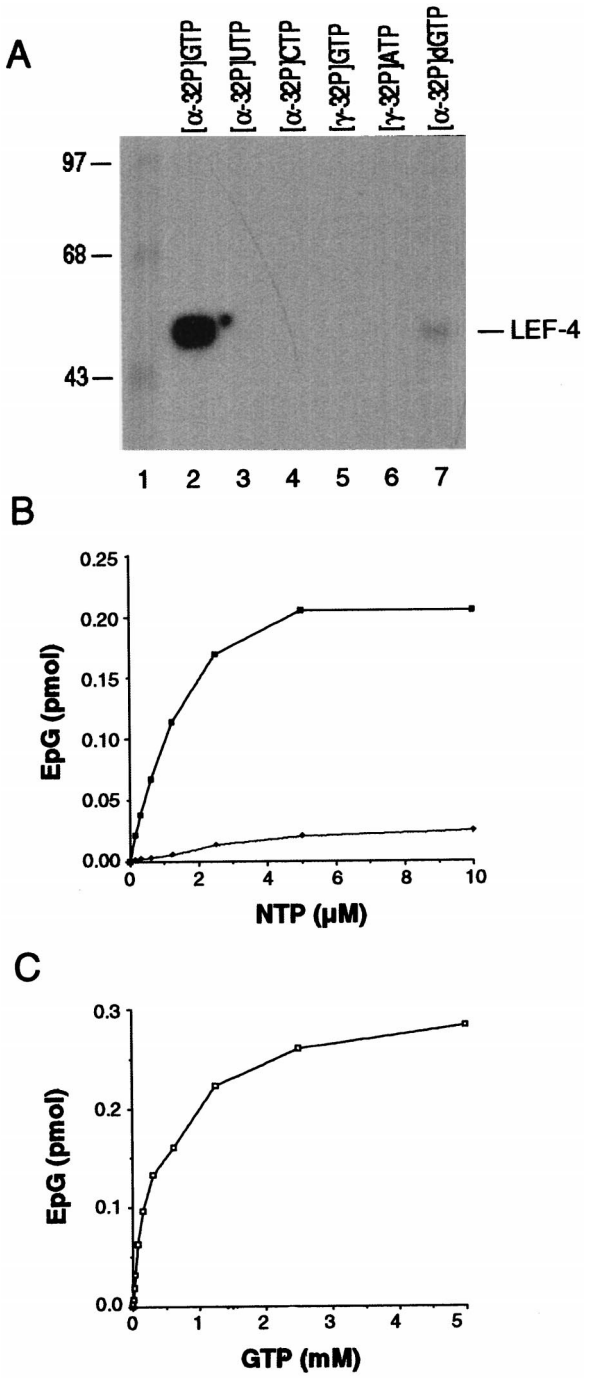

FIG. 6. Nucleotide and sugar specificity of the guanylyltransferase activity of RNA polymerase and purified LEF-4. (A) Nucleotide specificity. Incubations were performed with 0.2 μM indicated nucleoside triphosphate. An autoradiograph of the dried gel is shown. The position of LEF-4 is indicated on the right, and the migration of molecular weight standards is shown in kilodaltons on the left. Reaction mixtures contained 1 pmol of purified RNA polymerase, 50 mM Tris HCl (pH 7.9), 1 mM $MnCl_2$, 5 mM DTT, and the radiolabeled nucleotides as indicated. The specific activities of the nucleotides were as follows: [α-^{32}P]GTP, 5.9×10^5 cpm/pmol; [α-^{32}P]CTP, 5.3×10^5 cpm/pmol; [α-^{32}P]UTP, 4.0×10^5 cpm/pmol; [γ-^{32}P]GTP, 7.6×10^5 cpm/pmol; [γ-^{32}P]ATP, 4.0×10^5 cpm/pmol; and [α-^{32}P]dGTP, 5.5×10^5 cpm/pmol. (B) Nucleotide sugar specificity of guanylyltransferase of LEF-4. Reaction mixtures contained 1 pmol of purified RNA polymerase, 50 mM Tris HCl (pH 7.9), 1 mM $MnCl_2$, 5 mM DTT, and [α-^{32}P]GTP (squares) or [α-^{32}P]dGTP (diamonds). Samples were incubated at 30°C for 15 min. (C) GTP titration with purified LEF-4. Reaction mixtures contained 1 pmol of purified LEF-4, 50 mM Tris HCl (pH 7.9), 5 mM $MnCl_2$, 5 mM DTT, and [α-^{32}P]GTP.

interact either with host capping enzymes or with virus-encoded enzymes, as it is known that capping enzymes bind to their cognate polymerase (4, 16). Therefore, we decided to assay for guanylyltransferase activity during purification of viral RNA polymerase. We found that our most purified preparations of RNA polymerase contained the activity we sought. Incubation of enzyme with radiolabeled GTP and divalent cation resulted in the formation of a covalent protein-GMP complex. The 54-kDa radiolabeled protein comigrated with the LEF-4 subunit of viral RNA polymerase. Overexpression and purification of LEF-4 confirmed that the single subunit had guanylyltransferase activity. Analysis of the LEF-4 amino acid sequence revealed the presence of a KxDG motif, a conserved sequence element found in all members of the nucleotidyltransferases. These enzymes, which include guanylyltransferases and ligases, bind GMP or AMP to the lysine residue within this conserved motif. In the accompanying report (13), we present data showing that mutation of this lysine residue abrogates guanylyltransferase activity, consistent with its known function in related enzymes. In addition, we noted homologies between nucleotidyltransferases and three baculovirus LEF-4 proteins at five other motifs that are common to all viral and cellular guanylyltransferases (30).

Although we showed that both LEF-4 and RNA polymerase were active in the formation of a covalent nucleoside-protein complex, we were unable to demonstrate the transfer of GMP to diphosphate-terminated RNA with either enzyme preparation (data not shown). Attempts to show capping of in vitro-transcribed RNAs were also unsuccessful. However, we were able to calculate that the amount of radiolabel that would be incorporated into RNA caps was below our level of detection. This is a consequence of the relatively high concentrations of GTP required for in vitro transcription, the high percentage of RNA polymerase preguanylated in vitro, and the relatively low amount of RNA made in our in vitro transcription reactions. Lack of success with the purified LEF-4 subunit is due to the low affinity of LEF-4 for GTP and may also indicate that other components of the RNA polymerase complex are needed for RNA binding and the subsequent transfer reaction. Further experimentation will be required to address this issue.

Previous studies on the localization of LEF-4 in infected cells (5) showed that LEF-4 preferentially localized to the nucleus of infected cells, where it associated with the virogenic stroma. The virogenic stroma is the site of viral DNA replication, late gene expression, and packaging. Thus the stroma is the expected site for a component of the viral RNA polymerase. We have not localized the RNA polymerase complex by immunocytochemistry; however, cell fractionation data indicate that RNA polymerase is nuclear. Furthermore, immunoblot analysis of cytosolic and nuclear extracts with antiserum prepared against LEF-8 indicates that the large subunit of RNA polymerase is wholly nuclear (4a). Our finding of overexpressed LEF-4 in the cytosol indicates that the single subunit cannot direct its own nuclear targeting. This observation suggests that partial or complete preassembly of the RNA polymerase in the cytosol may be required for proper subcellular localization.

Identification of guanylyltransferase as a component of the viral RNA polymerase suggests that baculoviruses may also encode the other two enzymes required for the formation of the mRNA cap. RNA triphosphatase cleaves the 5′ triphosphate from the termini of primary transcripts to yield diphosphate-terminated RNAs, the substrate for guanylyltransferase. After transfer of GMP to the RNA substrate, RNA methyltransferase catalyzes the transfer of a methyl group from *S*-adenosylmethionine to the guanosine cap. In the accompany-

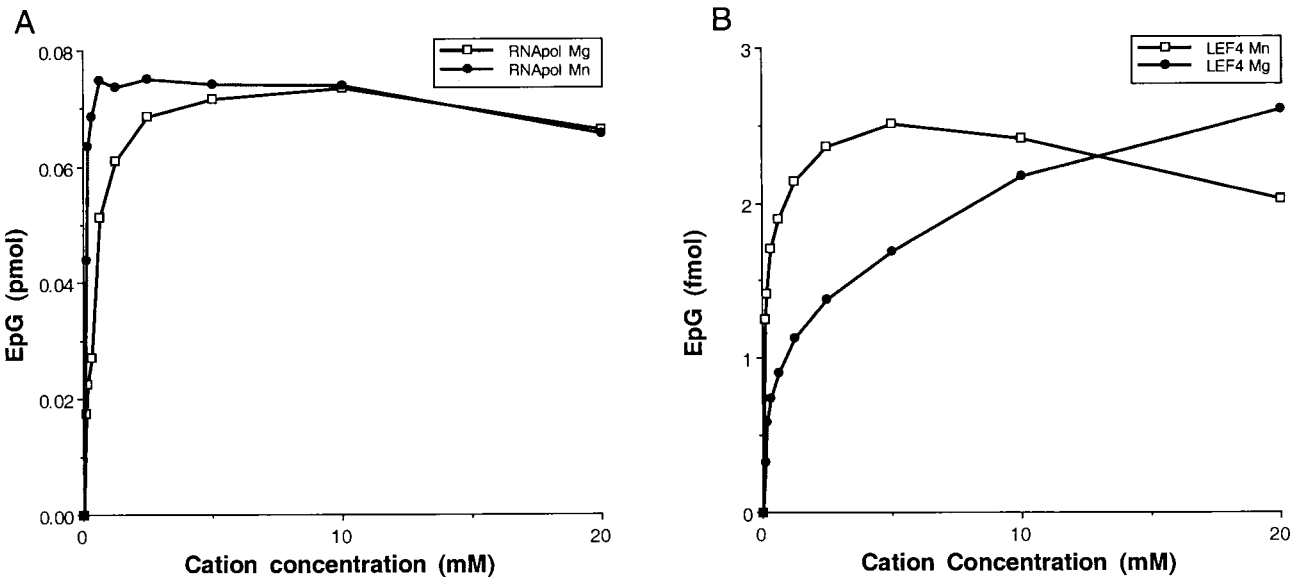

FIG. 7. Cation requirement of the guanylyltransferase activity of RNA polymerase (RNApol) (A) and purified LEF-4 (B). Reaction mixtures contained 50 mM Tris HCl (pH 7.9), 5 mM DTT, 5 μM [α-^{32}P]GTP, and divalent cation as indicated in 25 μl. Reaction mixtures were incubated at 30°C for 15 min and denatured in 1% SDS, and proteins were separated by PAGE. The yield of EpG is plotted as a function of magnesium or manganese concentration.

ing report (13), we present data showing that LEF-4 is a bifunctional protein and that RNA triphosphatase activity is localized in the N-terminal half of the protein. We have not yet identified a candidate for a virus-encoded methyltransferase. Presumably the host enzyme could catalyze this reaction, but it seems reasonable to propose that virus would also encode methyltransferase in addition to the other capping activities. The second step in the guanylyltransferase reaction is freely reversible until the cap is methylated. Therefore, efficient capping presumably requires specific protein-protein interactions between the capping enzyme and the RNA methyltransferase.

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