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Differential Transcription of Baculovirus Late and Very Late Promoters: Fractionation of Nuclear Extracts by Phosphocellulose Chromatography

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An in vitro transcription system based on a cytidine-free cassette was developed for the late *39k* gene and the very late polyhedrin gene of *Autographa californica* nuclear polyhedrosis virus (AcNPV). Optimization of transcription conditions revealed that a preincubation step was not required for transcription of late and very late promoters, although preincubation was essential for efficient transcription from an early promoter. The *39k* and polyhedrin constructs were actively transcribed by nuclear extracts prepared from AcNPV-infected *Spodoptera frugiperda* cells at 12 or 36 h postinfection but not by nuclear extracts prepared from uninfected or infected cells harvested during the early phase of infection. Transcription from the very late polyhedrin promoter was fivefold higher than that from the *39k* late promoter with the nuclear extract prepared at 36 h postinfection. The 36-h extract was fractionated by phosphocellulose chromatography. Transcription activity eluted in two fractions, at 0.3 and 0.5 M KCl. Both the *39k* and polyhedrin constructs were transcribed by these fractions; however, the patterns of late and very late transcription were distinctly different. With the 0.3 M fraction, incorporation into the *39k* transcript was approximately 10-fold higher than incorporation into the polyhedrin transcript. Alternatively, with the 0.5 M fraction, transcription of the polyhedrin construct was twofold higher than transcription of the *39k* construct. These results indicate that this in vitro system will be useful for purification and identification of factors that discriminate between late and very late promoters.

Baculoviruses potentially express 150 proteins (2), including those responsible for regulating the temporal expression of viral genes. Different classes of viral genes have been defined on the basis of their expression in infected and uninfected cells. The early genes have promoters that are similar to typical eukaryotic promoters. These genes are transcribed by the host RNA polymerase II, and their transcription is regulated by host transcription factors and viral transactivators (5, 8, 13, 15). The structures of the late and very late promoters resemble the bacteriophage T7 late and mitochondrial promoters with respect to the fact that a short conserved sequence serves as both a promoter and an initiator element. The most conserved sequence element of *Autographa californica* nuclear polyhedrosis virus (AcNPV) late promoters is the transcription initiator (A/T/G)TAAG (3). Essential promoter elements for polyhedrin and p10, the two highly expressed very late genes, include the 12-bp initiator AATAAGTATTTT and a downstream A+T-rich region corresponding to the 5′ untranslated leader sequence of their mRNAs (22, 32).

The baculovirus late and very late promoters are transcribed by an α-amanitin-resistant virus-encoded or virus-modified RNA polymerase (11, 33). A transient expression assay has been used to identify proteins required for expression of late genes of AcNPV (16, 17, 21, 24–28). These factors include IE1, IE2, DNA polymerase, helicase, and late expression factors (LEFs) 1 to 10. Because of the nature of this assay, it is not possible to determine whether these proteins are directly involved in late gene expression or whether they are required for earlier events in the virus life cycle. IE1 and IE2 are known to transactivate early genes (5, 9), and DNA polymerase, helicase, and LEFs 1 to 3 are required for DNA replication (14). Thus, it is possible that these seven proteins have indirect effects on late gene expression. Little is known about the possible roles of most of the LEFs in late transcription. The predicted amino acid sequence of LEF 8 contains a motif that is conserved among RNA polymerases from various sources, suggesting that *lef-8* encodes the viral RNA polymerase (28).

In vitro transcription from baculovirus late promoters by using nuclear extracts prepared from infected *Spodoptera frugiperda* cells was recently reported (7). These experiments were based on a runoff transcription assay, which is commonly associated with high background problems. In the baculovirus system, this was further compounded by the presence of nucleases in infected cell extracts. Glocker et al. (7) greatly improved the in vitro system by using primer extension assays. However, these assays are too time-consuming to be useful for the purification of transcription factors. To improve the sensitivity of in vitro transcription assay using baculovirus promoters, we constructed cytidine-free cassettes for transcription from the late *39k* promoter and the very late polyhedrin promoter. Factors that differentially regulate the late and very late promoters were separated on phosphocellulose.

MATERIALS AND METHODS

Preparation of nuclear extracts. *S. frugiperda* (Sf9) cells were cultured and infected with the E2 strain of AcNPV as previously described (28). Nuclear extracts were prepared from uninfected or AcNPV-infected Sf9 cells at 8, 12, and 36 h postinfection (p.i.) as previously described (31), with minor modifications. All manipulations were carried out on ice or in a cold room, and all buffers, tubes, and centrifuge rotors were prechilled to 4°C. The infected cells were collected at the indicated times p.i. by centrifugation at 2,000 rpm for 10 min in a Beckman JA-10 rotor. The pellets were washed twice in phosphate-buffered saline by centrifugation. The final pellets were 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). 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

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by phase microscopy for complete breakage. 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 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, 3 μg of leupeptin per ml). 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 50.2 Ti rotor for 90 min at 4°C. The supernatant was collected, and globular proteins were precipitated for 20 min after dropwise addition of ammonium sulfate to 0.33 g/ml of supernatant. The precipitated proteins were collected by centrifugation at 30,000 rpm for 20 min in a Beckman 50.2 Ti rotor. The protein pellet was dissolved in 2 ml of dialysis buffer (50 mM Tris [pH 7.9], 1 mM EDTA, 100 mM KCl, 20% glycerol, 3 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride) per 10^9 cells. The extracts were then dialyzed four times for 2 h each with a 200-fold excess of dialysis buffer. After dialysis, the extract was cleared by centrifugation in a microcentrifuge for 5 min. The nuclear extracts were frozen in small aliquots at −80°C. After dialysis, the nuclear extracts were frozen in small aliquots in liquid nitrogen. Protein concentration of the nuclear extracts was measured by the Bradford method (4).

Site-directed mutagenesis of the AcNPV polyhedrin promoter and construction of reporter plasmids. For mutation of the cytidine residues within the polyhedrin leader sequence, pVL941CAT (18) was used as the parental plasmid. Five degenerate oligonucleotides were synthesized to mutate the five C residues to G, A, or T, using the method of Deng and Nickoloff (6): C_{-39} primer, 5′-AATAAGTATTTTANTGTTTTCGTAA-3′ (N = C, A, G, or T); C_{-32} primer, 5′-TTTTACTGTTTTNGTAACAGTTTTG-3′; C_{-27} primer, 5′-TGTTTTCGTAANAGTTTTGTAATAA-3′; C_{-9} primer, 5′-GTAATAAAAAAANCTATAAATATTC-3′; C_{-8} primer, 5′-TAATAAAAAAACNTATAAATATTCC-3′. The cytidine residues are numbered relative to the ATG codon for start of translation. A selection primer (5′-ATCGTTTACATCGGATCCGACTCTGCTG-3′) that changes the *Kpn*I site within the polyhedrin coding sequences to a *Bam*HI site was used. The correct sequence of each mutation was verified by DNA sequence analysis.

On the basis of the results of these experiments, an additional oligonucleotide that mutates all five C residues was constructed. All of the substitutions (underlined) in oligonucleotide Polh/CF1 are optimal (5′-AATAAATAAGTATTTTAGTGTTTTTGTAAAAGTTTTGTAATAAAAAAATTTATAAATATTCCGGAT-3′). The construct pPolh/CFcat was constructed from pVL941 by using the Polh/CF1 oligonucleotide and the selection primer described above.

Construction of C-free cassettes for in vitro transcription. To construct pPolh/CFS, we first used PCR to amplify a 191-bp fragment of the synthetic oligonucleotide in $p(C_2AT)$ (30). Plasmid $p(C_2AT)$ has a 380-bp C-free cassette that is not suitable for transcription analyses of AcNPV late and very late promoters because it contains two TAAG motifs. The primers used were the 17-mer M13 universal sequencing primer and an oligonucleotide (5′-GATAAGAATGAACCCGGGGAGGAAG-3′) that is complementary to an internal sequence of $p(C_2AT)$. The PCR product was purified by agarose gel electrophoresis and digested with *Sma*I and *Eco*RI. This fragment was cloned into the *Sma*I and *Eco*RI sites of pUC18, and the resulting construct was named pCFS. Then the 92-bp *Eco*RV-to-*Ssp*I fragment of pPolh/CF1cat was cloned into *Sma*I-digested pCFS. DNA sequencing was used to determine the correct orientation.

To construct a transcription template for the *39k* late promoter, p39LS-WT (8) was digested with *Afl*II, and the larger 5-kb fragment was purified and religated. Site-directed mutagenesis was performed on the resulting plasmid (p39KΔAflII) to generate an *Eco*RV site 4 bp downstream of the TAAG initiator by using the mutagenic oligonucleotide (5′-AAAAGAATATATAAGAGTTGATATCGGCCATTTCACAG-3′). Then the 191-bp *Sma*I-*Sac*I fragment of pCFS was cloned into the *Eco*RV and *Sac*I sites of the mutated *39k* plasmid to generate p39KL/CFS.

CAT assays. Plasmid DNAs were purified according to the boiling procedure (10), precipitated with polyethylene glycol (12), and purified on cesium chloride-ethidium bromide gradients (29). *S. frugiperda* cells (10^6) were cotransfected with 1 μg of AcNPV DNA and 2 μg of pVL941CAT or one of the cytidine substitution mutants of pVL941CAT. Transfections with plasmid DNAs were performed according to method I of Summers and Smith (31). After 48 h, cells were harvested and chloramphenicol acetyltransferase (CAT) assays were performed as previously described (9). The acetylated and unacetylated chloramphenicol species were separated by thin-layer chromatography, and the amount of radioactivity in each species was directly quantitated with a Betagen Betascope 603 blot analyzer. The results are expressed as the amount of radioactivity in the acetylated chloramphenicol spot divided by the total radioactivity recovered from each reaction. All transfections were performed in triplicate; the results reported here represent the mean values, and error bars indicate standard deviations. CAT assays were conducted under conditions in which less than 30% of the substrate was acetylated.

In vitro transcription assays. The standard conditions for optimal in vitro transcription from the polyhedrin promoter were determined empirically. Transcription reaction mixtures typically contain 50 μg of protein and the following components in a reaction volume of 50 μl: 25 mM Tris (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 1.5 mM DTT, 10% glycerol, 40 U of RNasin (Promega), and 1.5 μg of template. The nucleotide mixture was added immediately without a preincubation step. The final concentrations of the components in the nucleotide mix were 0.6 mM each ATP and UTP, 5 μM GTP, 10 μCi of [α-^{32}P]GTP (400 Ci/mmol; Amersham), and 0.1 mM 3′-deoxy-CTP. Reaction mixtures were incubated for 25 min at 30°C. Reactions were stopped by the addition of 350 μl of stop buffer (50 mM Tris [pH 7.5], 1% sodium dodecyl sulfate, 5 mM EDTA, 25 μg of tRNA per ml). Transcription reaction mixtures were extracted with phenol and chloroform. RNA in the aqueous phase was precipitated by the addition of 0.15 M sodium acetate and 2 volumes of ethanol. The RNA pellets were resuspended in 15 μl of loading buffer (80% formamide, 0.01% xylene cyanol, 0.01% bromophenol blue), and the RNAs were separated electrophoresis on 6% polyacrylamide–7 M urea gels. The dried acrylamide gels were directly quantitated with a Betagen Betascope 603 blot analyzer. The results are expressed as counts per minute incorporated into the bands corresponding to the C-free transcripts. The conditions for in vitro transcription from the *ie1* promoter have been described previously (34).

Phosphocellulose chromatography. A 2-ml phosphocellulose P11 column was equilibrated with dialysis buffer at 4°C. Nuclear extract (2 ml) prepared from baculovirus-infected cells at 36 h p.i. was loaded onto the column. The column was washed with 5 ml of dialysis buffer and then eluted with 5 ml of the same buffer containing 0.3, 0.5, 0.75, or 1.0 M KCl. The individual fractions were dialyzed against 200 ml of dialysis buffer for 2 h at 4°C. The samples were then frozen in liquid nitrogen. Fractions were assayed in the absence of a preincubation step and in the presence of 100 μg of α-amanitin per ml.

A. Polyhedrin C-free

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                »                                             Met
                      -39   -32  -27                 -9-8
WT          ATAAGTATTTTACTGTTTTCGTAACAGTTTTGTAATAAAAAACCTATAAATATG
VL941       .....................................................T
Polh/CFS    ............G......T....A.................AT.......(191 nt C-free)C
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B. *39k* late promoter C-free

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                           »              >>    >  >               Met
WT          GTATAAAAGAATATATAAGAGCTAATTTAGGCCATTTCACAGTAATCACCGACAAATG
39KL/CFS    .....................T.G..(191 nt C-free)C
```

FIG. 1. Construction of C-free cassettes for polyhedrin and *39k* promoters. (A) The nucleotide sequence of the polyhedrin leader region from start of transcription to start of translation. The conserved ATAAG motif found in the promoters of all baculovirus late genes is indicated by a double underline, and the transcription initiation site is shown by a double arrowhead. The ATG for start of translation is shown in boldface. The five C residues in the polyhedrin leader sequence are indicated (−39, −32, −27, −9, and −8). The substitutions in the C-free cassette are indicated. (B) Nucleotide sequence of the *39k* promoter. The ATAAG initiator for *39k* late expression is indicated by a double underline, and the initiation site is shown by a double arrowhead. The two TATA boxes that regulate early transcription are indicated by a single underline, and the conserved CAGT motif found at the transcription initiation site of many early genes in indicated by a dotted underline. The early transcription initiation sites are indicated by single arrowheads. The ATG for start of translation is shown in boldface. The sequence of the *39k* late promoter C-free construct is shown below the wt sequence; only the substituted residues are indicated.

RESULTS

Mutational analysis of the C residues in the AcNPV polyhedrin promoter. The sequences between the sites for initiation of transcription and translation of the polyhedrin gene are required for high-level transcription of the polyhedrin promoter (22). This region contains five cytidine residues (numbered relative to the ATG codon) that must be changed in order to construct a C-free cassette (Fig. 1). Therefore, we decided to separately mutate each cytidine to guanosine, adenosine, and thymidine. These substitutions were made in plasmid pVL941CAT so that the effects of each individual mutation on expression could be determined in CAT assays. Fifteen different cytidine substitution mutants were identified by DNA sequence analysis. Then Sf9 cells were cotransfected with AcNPV DNA and each mutant plasmid or wild-type pVL941CAT. After 48 h, the cells were harvested, and CAT assays were performed (Fig. 2). Two mutations had significant effects on polyhedrin expression. The substitution of A at −9 resulted in a twofold increase in CAT activity compared with the wild-type (wt) plasmid, while CAT activity was dramatically

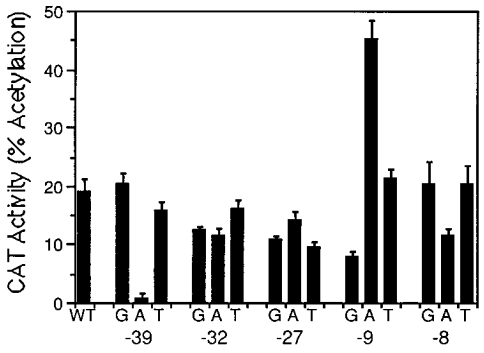

FIG. 2. Analysis of the five C residues in the polyhedrin promoter leader sequence by site-directed mutagenesis. *S. frugiperda* cells (10^6) were cotransfected with 1 μg of AcNPV DNA and 2 μg of each C-substitution mutant or wt (pVL941CAT) plasmid. After 48 h, cells were harvested and resuspended in 200 μl of 0.25 M Tris (pH 7.5) buffer. The same volume (20 μl) of each extract was used for CAT assays. Transfections were performed in triplicate; the results represent the mean values, and error bars indicate standard deviations. The cytidine residues are labeled as indicated in Fig. 1.

decreased as a result of a substitution of A at −39. Expression of plasmids −32G, −32A, −27G, −27T, −9G, and −8A was lower than wt expression, and the other seven mutations had no significant effect on polyhedrin expression.

In vitro transcription with pPolh/CFS. On the basis of the results of the experiment described above, a plasmid containing a mutated polyhedrin promoter, pPolh/CFcat, was generated from pVL941CAT by site-directed mutagenesis. In this plasmid, the five cytidine residues in the template strand were changed to G, T, A, A, and T, respectively. We compared the in vivo promoter activities of pPolh/CFcat and wt pVL941CAT by CAT assays. Figure 3A shows that the pPolh/CFcat construct was only slightly less active than the parental plasmid. In a control CAT assay conducted in the absence of added protein, there was only 0.65% acetylated chloramphenicol (data not shown).

A 92-bp fragment containing this mutated polyhedrin promoter was cloned into pCFS, and this plasmid was named

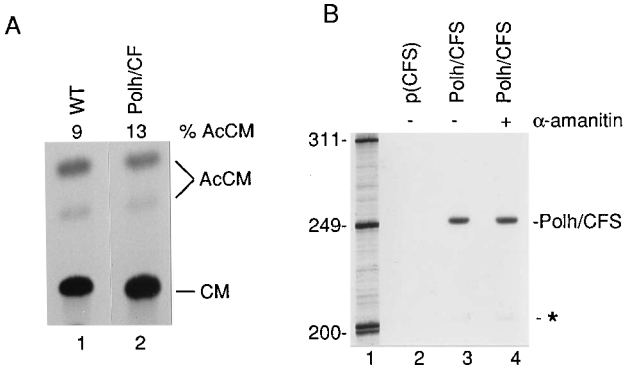

FIG. 3. Comparison of the activities of the wt and a C-free polyhedrin promoter in vivo and in vitro. (A) Transient expression of pVL941cat (WT) and pPolh/CFcat. Transfections and CAT assays were performed as described in the legend to Fig. 2. The positions of input chloramphenicol (CM) and acetylated chloramphenicol (AcCM) are indicated. Quantitation of the results is shown above each lane. (B) In vitro transcription reactions were performed by using standard reaction conditions with nuclear extracts prepared at 36 h p.i. from AcNPV-infected Sf9 cells. Lane 1, size marker, [^{35}S]dATP-labelled *Hin*fI-digested ϕX174 DNA fragments; lane 2, pCFS; lanes 3 and 4, pPolh/CFS. In lane 4, α-amanitin was added to the reaction mixture at a final concentration of 100 μg/ml before incubation. The expected position of the full-length Polh/CFS transcript is shown. The position of the premature termination/pause product is indicated by an asterisk. Sizes are indicated in nucleotides.

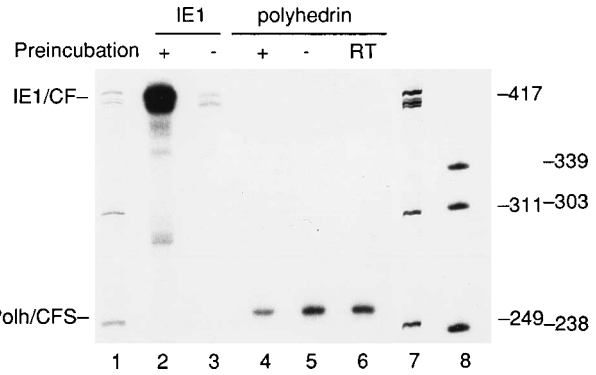

FIG. 4. Preincubation is not required for in vitro transcription from the polyhedrin promoter. Nuclear extracts prepared at 36 h p.i. were used for in vitro transcription from pPolh/CFS under standard conditions (lane 5). The reaction mixtures were preincubated on ice for 15 min (lane 4) or at room temperature for 15 min (lane 6). Plasmid pIE1/CF was transcribed with nuclear extracts prepared from uninfected Sf9 cells, using standard conditions of Yoo and Guarino (34) (lane 2) or without preincubation (lane 3). Lanes 1 and 7, *Hin*fI-digested ϕX174 DNA. Lane 8, RNA markers prepared by digestion of pGEM-1 with *Nae*I, *Nhe*I, or *Dde*I, followed by in vitro transcription with T7 RNA polymerase in the presence of [α-^{32}P]GTP. The positions of the IE1/CF and Polh/CFS transcripts are indicated on the left. The positions (in nucleotides) of DNA markers (right) and RNA markers (far right) are also shown.

pPolh/CFS (Fig. 1). When in vitro transcription assays are performed with pPolh/CFS in the absence of CTP, this template should direct the synthesis of a specific 243-nucleotide (nt) transcript whose 5′ and 3′ ends are determined by the transcription initiation site within the TAAG motif and the first C residue after the C-free cassette. In vitro transcription reactions were performed with nuclear extracts prepared from AcNPV-infected Sf9 cells at 36 h p.i. Analysis of the reaction products by acrylamide gel electrophoresis revealed a transcription product with a very clean background (Fig. 3B, lane 3). The expected size of the transcript is 243 nt. Because RNA migrates differently than DNA, the transcript does not appear to be the correct size in this figure. However, when RNA markers are used, the migration of the transcript corresponds exactly to the expected size (see, for example, Fig. 4). The only other transcription product observed was a band of approximately 200 nt that may represent premature termination or a pause site. Although this band cannot clearly be seen in Fig. 3, it is noted in order to compare this figure with later figures. No transcription products were detected with the parental pCFS as the template (Fig. 3B, lane 2). AcNPV late and very late genes are transcribed by an α-amanitin-resistant RNA polymerase (11), and, as expected, transcription was resistant to 100 μg of α-amanitin per ml (Fig. 3A, lane 4).

Preincubation is not required for in vitro transcription from the polyhedrin promoter. As suggested by Glocker et al. (7), we used a preincubation step (on ice for 15 min) in our initial experiments. However, when we attempted to optimize this step, we discovered that varying the preincubation time did not affect the level of transcription from the polyhedrin promoter (data not shown), suggesting that preincubation may not be required. To test this, we compared polyhedrin transcription levels under different conditions of preincubation: on ice for 15 min (Fig. 4, lane 4), no preincubation (lane 5), or at incubation at room temperature for 15 min (lane 6). We found that a preincubation step was not required for transcription from the polyhedrin promoter. In fact, the transcript levels were slightly higher in the absence of preincubation. The results obtained with the polyhedrin promoter contrast with those obtained with the pIE1/CF construct in which preincubation increased transcription from the immediate-early promoter 18-fold (Fig.

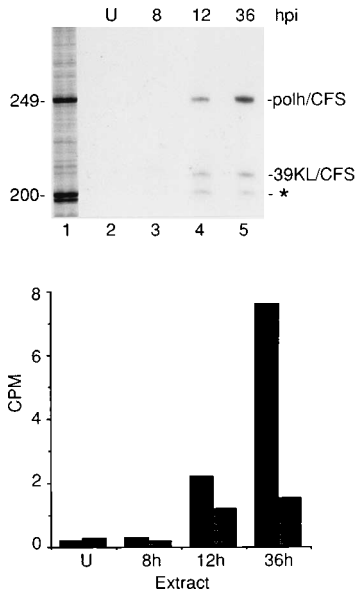

FIG. 5. In vitro transcription of pPolh/CFS and p39KL/CFS with different nuclear extracts. Nuclear extracts were prepared from uninfected (U; lane 2) or AcNPV-infected Sf9 cells and harvested at 8 (lane 3), 12 (lane 4), or 36 (lane 5) h p.i. Equal amounts of protein were used for in vitro transcription under standard reaction conditions. Each reaction mixture contained 0.75 μg each of pPolh/CFS and p39KL/CFS. The positions of transcripts initiating from the polyhedrin (Polh/CFS) and *39k* promoters (39KL/CFS) are indicated. The position of the premature termination/pause product originating from the polyhedrin promoter is indicated by an asterisk. A similar product was also observed with the *39k* promoter (not shown). Lane 1, *Hin*fI-digested ϕX174 DNA. Quantitation of the results for the polyhedrin promoter (solid) and the *39k* promoter (hatched) is shown below. Sizes are indicated in nucleotides on the left.

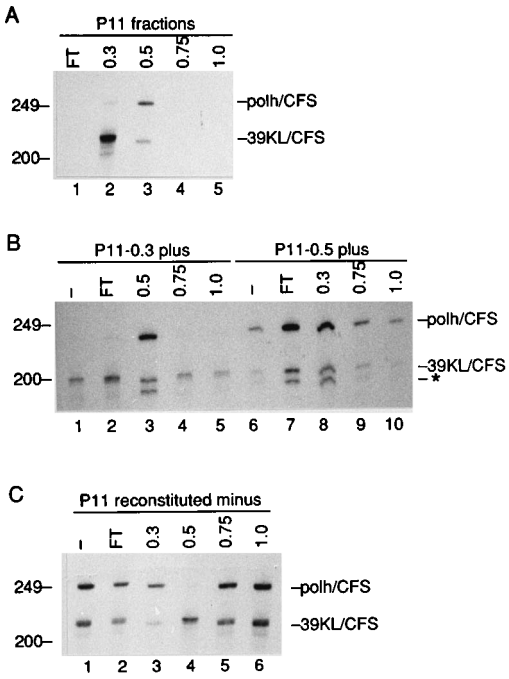

FIG. 6. Phosphocellulose fractionation of late and very transcription factors. (A) Elution profile of late and very late transcription activity. The fractions were tested separately, using 20 μl of each fraction and 1.5 μg of each template. (B) Mixing experiments. The 0.3 and 0.5 M KCl fractions (10 μl of each) were used in transcription reactions alone or in combination with 10 μl of the indicated fractions. (C) Reconstitution of transcription activity. Five microliters of each fraction was pooled and used for in vitro transcription (lane 1); the indicated fractions were omitted from the reconstitution (lanes 2 to 6). All transcription reactions contained 100 μg of α-amanitin per ml. Sizes are indicated in nucleotides on the left.

4; compare lanes 2 and 3). We have consistently observed high-level transcription of both the *39k* and polyhedrin constructs in the absence of preincubation with at least seven different preparations of nuclear extract (data not shown).

The C-free cassette can be used for the *39k* late promoter. We also tested whether the C-free cassette would be suitable for other late promoters, such as the *39k* late promoter. Previous mutagenesis experiments indicated that the sequences downstream of the TAAG element are not important for promoter activity (8); therefore, it was not necessary to include the nontranslated leader in the *39k* C-free cassette. Site-directed mutagenesis was performed to insert an *Eco*RV site 4 bp downstream of TAAG, and the 191-bp C-free cassette was cloned into this site (Fig. 1B). The resulting construct (p39KL/CFS) should direct transcription of a C-free cassette under the control of the *39k* late promoter. Most of the early *39k* promoter is deleted from this construct, and we do not detect activity with this construct under conditions optimized for transcription from the *ie1* promoter (data not shown).

We compared the activities of the two constructs, pPolh/CFS and p39KL/CFS, using nuclear extracts prepared from uninfected Sf9 cells or AcNPV-infected cells at 8, 12, and 36 h p.i. (Fig. 5). No transcripts were detected in nuclear extracts prepared from uninfected cells or infected cells harvested during the early phase of infection (Fig. 6, lanes 2 and 3). The 12- and 36-h-p.i. nuclear extracts supported specific transcription from both the polyhedrin and *39k* promoters. With the extracts prepared at 12 h p.i., incorporation into the polyhedrin-directed transcript was slightly higher than into the *39k* transcript, probably because the polyhedrin transcript is longer. However, with the extracts prepared at 36 h p.i., transcription from the polyhedrin promoter was fivefold higher than from the *39k* promoter, suggesting that the very late extract contained factors that specifically recognize the polyhedrin promoter (Fig. 5, lane 5).

Fractionation of late and very late transcription activities. The 36-h extract was fractionated by phosphocellulose P11 chromatography. Proteins that bound to P11 were eluted with a step gradient of KCl. The fractions were tested for transcription activity on the *39k* and polyhedrin promoters in the presence of 100 μg of α-amanitin per ml (Fig. 6A). Only the fractions eluted at 0.3 and 0.5 M KCl were active in transcription from baculovirus promoters. Both constructs were transcribed by these fractions; however, the patterns of late and very late transcription were distinctly different. With the 0.3 M fraction, incorporation into the *39k* transcript was approximately 10-fold higher than for the polyhedrin transcript. Alternatively, with the 0.5 M fraction, transcription of the polyhedrin construct was twofold higher than for the *39k* transcript. This result suggests that phosphocellulose chromatography can separate factors that preferentially activate transcription of late and very late promoters.

Mixing experiments were performed to identify transcription factors that eluted separately from the transcription activity (Fig. 6B). Supplementation of the 0.3 M fraction with the flowthrough (FT) fraction increased the transcription of both *39k* and polyhedrin by a factor of 3, indicating that this fraction contains a stimulatory factor. Addition of the 0.5 M fraction permitted transcription of the polyhedrin promoter, confirming results from Fig. 6A showing that this fraction contains a factor that permits transcription of the very late promoter. Supplementation of the 0.3 M fraction with the 0.75 M salt fraction resulted in a slight increase in activity, while the 1.0 M

KCl fraction produced a slight decrease in transcription from both promoters.

A mixing experiment with the 0.5 M fraction produced similar results. Supplementation with the FT fraction increased transcription of both the *39k* and polyhedrin constructs. The FT fraction did not significantly affect template preference, suggesting that the factor(s) in this fraction does not discriminate between late and very late promoters. Addition of the 0.3 M fraction produced results identical to those for the reciprocal mixture (compare lanes 3 and 8 in Fig. 6B). Supplementation with the 0.75 M fraction slightly increased transcription from both promoters, and addition of the 1.0 M salt fraction produced a slight decrease in transcription of both promoters, similar to results obtained with the 0.3 M fraction.

The mixing experiments were confirmed by a reconstitution experiment (Fig. 6C). When all five fractions were mixed, the transcription activity of the polyhedrin construct was twice that of the *39k* construct. If the FT fraction was omitted from the reconstitution, transcription from both promoters was reduced approximately twofold. This result is consistent with the previous experiments indicating that this fraction contains a factor which increases overall transcription activity. Omission of the 0.3 M fraction resulted in a transcription activity that preferentially transcribed the polyhedrin promoter. The transcription pattern with the reconstituted sample is similar to that observed with the 0.5 M KCl fraction. Reconstitution in the absence of the 0.5 M fraction yielded an activity that preferentially transcribed the *39k* promoter. This pattern is similar to that seen with the 0.3 M fraction alone. The results of these two reconstitutions confirm the data in Fig. 6A, indicating that the 0.3 and 0.5 M fractions contain factors that differentially transcribe late and very late promoters. In the absence of the 0.75 M fraction, transcription from both promoters was decreased 1.2-fold. Reconstitution in the absence of the 1.0 M fraction was essentially the same as that for all five fractions.

DISCUSSION

It has previously been reported that the sequences located between the start of transcription and translation are important for high-level expression of proteins linked to the polyhedrin promoter (19). The data of Ooi et al. (22) demonstrated that these sequences are required for efficient transcription initiation from the polyhedrin promoter. Therefore, we felt that it was essential to insert the C-free cassette downstream of −1 relative to the start of translation. This necessitated the mutation of five cytidine residues located in this region. Because substitutions in this region are known to affect transcription initiation, we separately mutated each cytidine residue to guanine, adenine, and thymidine in order to determine the optimal substitution for each residue. These mutations were made in plasmid pVL941CAT, which contains the CAT gene under the control of the polyhedrin promoter (18). This allowed us to analyze the effects of the substitutions by CAT assays in transient expression assays.

The results of this mutational analysis indicated that substitution of single residues had less of an effect on expression of polyhedrin than substitution of a 10-bp linker (22). For the most part, the single substitutions had minor effects on CAT expression. Only a substitution of adenine at −39 (+10 relative to start of transcription) dramatically decreased expression. This result is especially surprising considering that the very late p10 promoter has an adenine residue at this position. When adenine was substituted −9 (+41 relative to start of transcription), the CAT activity was increased twofold. Although we have not actually tested whether this substitution affects transcription, the results of Ooi et al. (22) showed that sequences even more distal to the polyhedrin initiation site affect transcription. Polyhedrin transcripts are relatively stable (22), so it is unlikely that this substitution increases their stability.

On the basis of the results of these experiments, plasmid pPolh/CFcat was constructed. This plasmid contains a mutated promoter that has optimal substitutions at all cytidine residues in the leader region. As expected, this promoter efficiently directs expression of CAT in baculovirus-infected cells. The C-free promoter was also used to construct a transcription template, and in vitro transcription reactions were performed with nuclear extracts prepared from baculovirus-infected Sf9 cells at 36 h p.i. Analysis of reaction products by gel electrophoresis revealed a band of the expected size with a clean background. As expected, transcription from the polyhedrin hybrid cassettes was resistant to α-amanitin and was detected only with nuclear extracts prepared from cells during the late phase of infection. These results indicate that this in vitro system can reproduce in vivo control mechanisms and will be useful in the identification, purification, and analysis of factors that regulate the transcription of polyhedrin.

We found that the *ie1* promoter was more efficiently transcribed than the polyhedrin promoter in the in vitro system. This result contrasts greatly with the in vivo situation. Although we have never directly compared the levels of *ie1* and polyhedrin transcripts in infected cells, we expect that the polyhedrin gene, which encodes a structural protein, is more highly transcribed than *ie1*, which encodes a transcription factor. It is important to note that different extracts were used for transcription of the two promoters. The *ie1* construct was transcribed in an uninfected cell extract, while polyhedrin was transcribed in an extract prepared from infected cells at 36 h p.i. As previously noted (7), we have found that late extracts contain DNase activity. Although the late transcription reactions were performed at 1 mM $MgCl_2$, the nuclease is still active, and this probably contributes to the low levels of transcription in this extract.

The C-free cassette was also shown to be useful for in vitro transcription from the *39k* promoter. A direct comparison of the activities of *39k* and polyhedrin C-free constructs showed that the two promoters were transcribed with equivalent efficiency in the 12-h-p.i. extract. In the 36-h extract, transcription of the polyhedrin construct was fivefold higher than from the *39k* promoter. The result obtained with the 36-h extract is consistent with the in vivo situation. At this time, the level of very late transcripts is higher than that of late transcripts. However, the 12-h result is different than we expected from the relative activities of late and very late promoters at this time in infected cells. Transcription of the very late genes is low during the late phase. Therefore, we expected the *39k* construct to be preferentially transcribed at this time. One possible explanation for this result is that the system was optimized for transcription of polyhedrin; therefore, it is possible that the conditions are suboptimal for transcription from the *39k* promoter. Alternatively, a level of control may be lost during the extract preparation. For example, a factor required for transcription of polyhedrin may be sequestered during the late phase; in the absence of this factor, polyhedrin transcription is very low. During the very late phase, the factor may become accessible, associate with the transcription machinery, and preferentially transcribe polyhedrin. In the preparation of nuclear extracts, this factor may be prematurely released, thereby permitting transcription of polyhedrin in vitro.

The observation that preincubation was not required for in vitro transcription from the polyhedrin promoter is consistent with other evidence indicating that late genes are not tran-

scribed by host RNA polymerase II (11, 33). A preincubation step is required for RNA polymerase II transcription because multiple transcription factors and RNA polymerase need to assemble on the template to form an active initiation complex (1). Our result suggests that transcription initiation by the α-amanitin-resistant viral RNA polymerase may be relatively simple and may not involve the assembly of multiple cofactors as does initiation by host RNA polymerase II. If this is true, it should be relatively simple to identify and purify the components of the late transcription system.

As an initial test of this hypothesis, we fractionated 36-h extracts by phosphocellulose chromatography. Proteins that bound phosphocellulose were separated into five fractions by elution with a step gradient. Transcription assays revealed that phosphocellulose is useful for the separation of factors that preferentially activate the *39k* and polyhedrin promoters. Two fractions were active in transcription when tested alone. One of these preferentially transcribed the *39k* promoter, and the other preferentially transcribed the polyhedrin promoter. An additional fraction had no transcription activity when tested alone but stimulated the activity of the two active fractions in mixing experiments. Currently, we do not know how many proteins or complexes are responsible for the activity in the different phosphocellulose fractions. The results of the preincubation experiment (Fig. 4) and preliminary fractionation data (not shown) indicate that the P11 0.5 M fraction contains a single transcription complex.

Our transcription and fractionation data support the hypothesis of Morris and Miller (20) on the mechanism for differential regulation of late and very late genes. The authors proposed that the polyhedrin promoter is transcribed at low levels during the late phase because it is an inherently weak late promoter. However, during the very late phase, the polyhedrin promoter is activated as a result of interactions of a specific activator with the DNA sequences between start of transcription and start of translation. Our fractionation experiment was able to separate transcription activity that preferred the *39k* construct by a factor of 10 over the polyhedrin promoter. This is similar to the in vivo pattern of transcription during the late phase. The transcription factors in this fraction could compose a basal transcription complex that primarily recognizes the sequences surrounding the TAAG motif of late promoters. A different phosphocellulose fraction preferentially transcribed the polyhedrin promoter, possibly because it contains the same basal transcription complex plus a factor that binds to the polyhedrin downstream sequence and directs baculovirus RNA polymerase to the polyhedrin promoter. Purification of the baculovirus RNA polymerase and associated transcription factors should help us to define the mechanism of differential transcription of baculovirus late and very late promoters.

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