VIROLOGY 245, 360–371 (1998)
ARTICLE NO. VY989177

Vaccinia Virus Nucleoside Triphosphate Phosphohydrolase I Is an Essential Viral Early Gene Transcription Termination Factor

Linda M. Christen, Michelle Sanders, Christie Wiler, and Edward G. Niles*,[1]

*Departments of Biochemistry and Microbiology and the Center for Microbial Pathogenesis, State University of New York, Buffalo, New York 14214-3000

Received February 27, 1998; returned to author for revision March 20, 1998; accepted April 6, 1998

Deng and Shuman (*J. Biol Chem.* 271, 29386 (1996)) reported that an ATPase different from the known viral termination factor, VTF, is required for vaccinia virus early gene transcription termination. Properties of this ATPase were similar to those of a known vaccinia virus enzyme, nucleoside triphosphate phosphohydrolase I (NPH I) the product of gene D11L. Transcription-competent cell-free extracts were prepared from A549 cells infected with wild-type or mutant vaccinia virus harboring ts mutations in gene D11L. These extracts were employed to investigate the role of NPH I in early gene transcription termination. Extracts prepared under nonpermissive conditions from both wild-type virus and ts mutant virus-infected cells exhibited high levels of early and intermediate gene transcription activity but were incapable of supporting late gene transcription. ts mutant extracts lacked signal-dependent early gene transcription termination activity, which was restored by the addition of either free NPH I or a GST-NPH I fusion protein. A comparison of the NPH I amino acid sequence to the protein databases revealed the presence of a set of sequences characteristic of nucleic acid helicase superfamily II members. A series of site-specific mutations in the helicase motifs and N-terminal and C-terminal deletion mutations were expressed as GST fusion proteins and their activities assessed. Of the mutations in helicase motifs I to VI, alteration of all but motif III reduced the ATPase activity. Removal of as few as 24 amino acids from the N-terminal end eliminated ATPase activity, while deletion of 68 C-terminal amino acids exhibited only a modest decrease in ATP hydrolysis. Larger C-terminal deletions eliminated ATPase activity. Each deletion mutation, and site-specific mutations other than the motif III mutation, failed to support transcription termination *in vitro.* Mutations in motifs I, II, V, and VI inhibit wild-type NPH I transcription termination activity. However, deletion of up to 68 amino acids from the C-terminal end eliminates this inhibitory property. This observation is particularly interesting since these C-terminal deletions retain both ATPase activity and single-stranded DNA binding activity. Their failure to inhibit transcription termination suggests that these C-terminal deletion mutations eliminate a site required for a function other than from DNA binding or ATP hydrolysis.

INTRODUCTION

Double-stranded DNA containing poxviruses are unique in that they replicate in the cytoplasm of infected cells. In order to carry on this unusual life cycle, poxviruses encode the enzymes required for viral gene transcription, mRNA processing, and DNA replication (Moss, 1996). Viral genes can be sorted into three temporal classes. Early genes are transcribed in virus cores. Intermediate and late genes are transcribed on replicating DNA in the cytoplasmic replication factories. Each class possesses its own unique set of class specific transcription initiation factors (Moss *et al.,* 1991). Due to properties shared with both prokaryotic and eukaryotic transcription machinery (Broyles and Moss, 1986; Amegadzie *et al.,* 1992; Rosales *et al.,* 1994) a detailed investigation of poxvirus transcription promises to yield valuable insights into transcriptional mechanisms common throughout evolution.

Early viral genes are unique in that transcription terminates in a signal- and factor-dependent manner (Rohrman *et al.,* 1986; Yuen and Moss, 1986; Shuman *et al.,* 1987). Elongation proceeds beyond the sequence TTTTTNT in the nontemplate strand yielding UUUUUNU in the nascent mRNA which serves as a signal required for the termination event (Yuen and Moss, 1987; Shuman and Moss, 1989). Termination requires both the vaccinia termination factor (VTF) and ATP or dATP hydrolysis (Shuman *et al.,* 1987; Hagler *et al.,* 1994). VTF also serves both as the viral mRNA capping enzyme (Shuman *et al.,* 1987) and as an essential intermediate gene transcription initiation factor (Vos *et al.,* 1991). Recent evidence demonstrated that ATP hydrolysis required for termination is not catalyzed by VTF but rather by a different enzyme, termed Factor X (Deng and Shuman, 1996). Reconstitution experiments point to a nucleoside triphosphate phosphohydrolase activity with features similar to NPH I, the product of gene D11L (Rodriguez *et al.,* 1986; Broyles and Moss, 1987b), as the ATPase employed in transcription termination. During infection, transcription termination is restricted to early genes. *In vitro,* only RNA

[1] To whom reprint requests should be addressed. Fax: 1-716-829-2725. E-mail: eniles@buffalo.edu.

0042-6822/98 $25.00

polymerase capable of recognizing early promoters is subject to signal-dependent termination, suggesting that this form of RNA polymerase is uniquely termination competent (Condit *et al.,* 1996). Thus effective termination of early gene transcription requires the productive interplay of at least four factors: the virion RNA polymerase, the signal UUUUUNU in the nascent mRNA, VTF, a multifunctional mRNA processing enzyme, and the ATP hydrolyzing enzyme Factor X. A detailed investigation of the mechanism of viral early gene transcription termination promises insights both into poxvirus gene expression and into termination mechanisms employed in cellular systems.

A convenient scheme for analyzing viral transcription in extracts of virus-infected cells was described (Condit *et al.,* 1996). By preparing extracts from cells infected at the nonpermissive temperature with ts mutant virus, it was possible to generate extracts devoid of a single essential viral factor. Such null extracts are of general use in evaluating functions of viral proteins in viral gene expression *in vitro.* Three ts mutations, C50, C36, and E17, were mapped to gene D11L (Seto *et al.,* 1987). The altered bases in C50 and C36 were identified (Kunzi and Traktman, 1989; Kahn and Esteban, 1990). In this report, we show that infected cell extracts can be prepared lacking active vaccinia virus NPH I protein. Such extracts are defective in early gene transcription termination. Termination activity can be restored by the addition of NPH I expressed in and prepared from induced *Escherichia coli.* Analysis of a limited set of *in vitro* generated mutations demonstrates that NPH I ATPase activity is required for termination. Furthermore, four sequences identified as helicase superfamily II conserved regions (Gorbalenya and Koonin, 1993) are also essential both for ATPase and transcription termination activity. Carboxyl terminal deletion mutations which retain both ATPase and DNA binding activity fail to terminate transcription *in vitro,* demonstrating that this region of NPH I carries out some essential function other than ATP hydrolysis. Furthermore, these deletion mutations fail to serve as dominant negative effectors *in vitro,* suggesting that this region of NPH I interacts with some component involved in the termination reaction. Our observations both confirm and extend the recent report by Deng and Shuman (1998) in which they demonstrate a role for NPH I both in early gene transcription elongation and in termination.

RESULTS

In vitro transcription

Sedimentation of virus-infected cells after treatment with lysolecithin yields a transcription-competent cell-free extract (Condit *et al.,* 1996). Addition of plasmid DNA containing a G-less cassette downstream from a viral early, intermediate, or late gene promoter, along with ribonucleoside triphosphates, permits synthesis of RNA, which can be observed by gel electrophoresis. For a ts mutation in gene D12L, preparation of extract from cells infected at the nonpermissive temperature yielded extract deficient in both early gene transcription termination and intermediate gene transcription initiation activity which could be restored by addition of VTF synthesized in *E. coli.* This general approach provides a simple means of preparing specific null extracts useful for studies on viral protein function *in vitro.*

With an initial goal of evaluating the role of NPH I in early gene transcription termination, we sought to prepare a transcription competent extract from cells infected with ts virus altered in NPH I. To this end, three ts virus that map in gene D11L were employed, C50, C36, and E17 (Seto *et al.,* 1987). Early gene transcription requires late protein synthesis, while intermediate and late gene transcription requires synthesis of early and intermediate proteins, respectively. Since D11L ts mutations exhibit a defective late phenotype (Condit *et al.,* 1983), which is reduced intermediate and late gene expression at the nonpermissive temperature, extracts prepared from cells infected at 40°C would be expected to exhibit reduced early and late gene transcription. Since this was shown to be true it became necessary to develop an alternative approach to extract preparation. To permit the accumulation of intermediate and late viral gene products required for early and late gene transcription, viral infection was carried out initially at the permissive temperature. After 24 h, the temperature was shifted to 40°C with the hope of inactivating previously synthesized ts NPH I, and cycloheximide was added to prevent further protein accumulation. After an additional 24-h incubation, lysolecithin treatment was carried out and an infected cell extract was prepared.

To assess overall transcription activity in such extracts, transcription was assayed employing plasmid templates that possessed an early, intermediate, or late gene promoter upstream from a G-less cassette. Specific transcription of each plasmid yields a 380-nucleotide product. As a control, an extract was prepared from cells infected with wild-type virus at 40°C, as described by Condit *et al.* (1996). Alternatively, extracts were prepared from cells initially infected at 31°C, either with wild-type virus or with ts C50, and after 24 h shifted to 40°C and incubated for a further 24 h in the presence of cycloheximide. The wild-type virus-infected cell extract exhibits the expected levels of early, intermediate, and late gene transcription (Fig. 1). Extracts prepared from either wild-type or C50 virus-infected cells, experiencing further incubation in the presence of cycloheximide, yielded reduced but satisfactory levels of early and intermediate gene transcription. The reduced activity may reflect differences in length of incubation at 40°C or simply variability in extract preparation. However, late gene transcription activity is absent in the cycloheximide-treated infected cell extract. This observation suggests that one

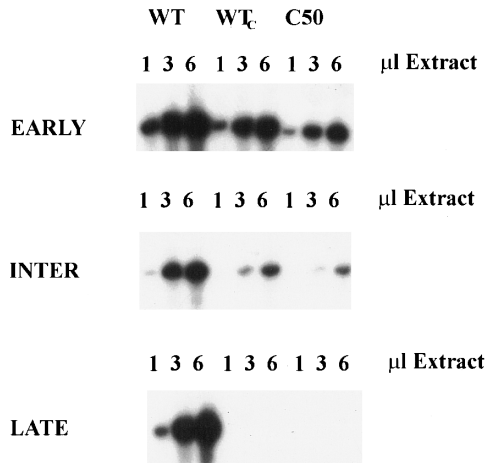

FIG. 1. Transcription activity of virus-infected cell extracts. A549 cells were infected with wild-type virus at either 31° or 40°C or with ts mutant C50 at 31°C, at an m.o.i. of 15. For the 40°C wild-type virus-infected cell sample, a cell extract was prepared after incubation for 24 h (WT). After incubation of the wild-type or C50 ts mutant virus-infected cell cultures for 24 h at 31°C, the temperature was raised to 40°C and 100 μg/ml cycloheximide was added. Extracts were prepared after incubation for an additional 24 h at 40°C (WT_c, C50). Transcription activity was measured at different extract concentrations using supercoiled plasmid templates that possessed a strong synthetic early promoter (Early), the gene G8R intermediate promoter (Inter), or late viral gene F17R promoter (Late), driving transcription through a 380-bp G-less cassette. Specific transcription of each template yields a 380-nucleotide RNA product that is visualized after electrophoresis in a 5% acrylamide, 8 M urea gel. The protein concentrations in each extract were WT, 12 mg/ml; WT_c, 10 mg/ml; C50, 0.7 mg/ml.

or more late transcription factors is unusually labile and unable to withstand the prolonged incubation at 40°C in cycloheximide. E17-infected cell extracts exhibited a similar level of total transcription, but C36-infected cell extracts lacked significant transcription activity and were not studied further. Additional investigations of possible variables in extract preparation were not conducted.

Western blot analysis was employed to assess the amount of NPH I in the wild-type and ts mutant virus C50- and E17-infected cell extracts, prepared from cells infected at 40°C and treated with cycloheximide. Different volumes of each extract were separated by gel electrophoresis, transferred to nitrocellulose, and probed with antibodies directed against either NPH I or the small subunit of the viral mRNA capping enzyme, D12L. This latter subunit serves as a control since the D12L subunit is known to be stable during infection (Niles *et al.,* 1989). In Fig. 2. it can be seen that the level of NPH I in the C50-infected cell extract is reduced in comparison to the wild-type virus-infected cell extract. However, the level of NPH I found in the E17-infected cell extract is barely detectable, indicating that the E17 NPH I is particularly unstable. It is not uncommon to find that vaccinia virus ts mutations exhibit reduced protein stability (Dyster and Niles, 1991). Since the extract prepared from C36-infected cells exhibited low transcription activity, it was not evaluated further. However, based on other observations (Diaz-Guerra and Esteban, 1993), one would expect that the ts C36 NPH I would be stable under these conditions.

To evaluate the ability of wild-type or ts mutant virus-infected cell extracts to support early gene transcription termination we next employed a supercoiled plasmid template, pSBterm, that possesses a G-less cassette harboring tandem termination signals downstream from an early viral promoter (Condit *et al.,* 1996). Read through transcription yields a 540-nucleotide product and signal-dependent termination produces a 450-base transcript. Extracts prepared from cells infected with ts C50 at 40°C were compared to wild-type virus-infected cells (Fig. 3). In the case of wild-type virus-infected cell extracts, extract alone exhibited mostly read through transcript with a low level of termination product. Addition of VTF alone enhances the production of the terminated transcript. The addition of NPH I alone had no effect on total RNA synthesis or on transcription termination in the wild-type virus-infected cell extract, in the presence or the absence of VTF. Transcription employing ts C50-infected cell extract alone yields only the read through product. Addition of VTF alone, however, does not enhance transcription termination, in contrast to the wild-type virus-infected cell extract. Addition of NPH I alone supports a small increase in termination but since VTF is limiting little termination product is observed. However, addition of both VTF and NPH I restores transcription termination, demonstrating that both proteins are essential termination cofactors. Both free NPH I and GST-NPH I exhibit similar activity in this assay, demonstrating that the N-terminal GST domain does not affect NPH I function (data not shown).

Helicase signature motifs in NPH I

A comparison of the NPH I amino acid sequence to the protein data bases identifies a series of proteins that

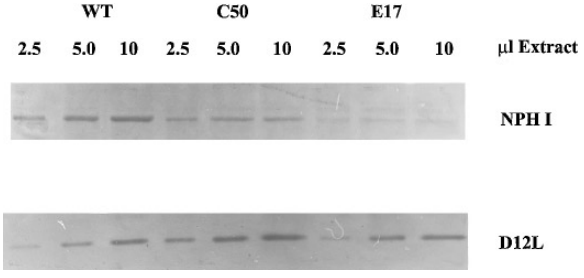

FIG. 2. Western blot analysis of virus-infected cell extracts. A549 cells were infected with wild-type virus or with ts mutants C50 or E17, at an m.o.i. of 15, at 31°C. After incubation for 24 h, the temperature was raised to 40°C and cycloheximide was added to 100 μg/ml. Infected cell extracts were prepared after incubation for an additional 24 h. Different volumes of extract were denatured, separated by gel electrophoresis, transferred to nitrocellulose, and treated with either anti-D11L or anti-D12L polyclonal antiserum. The positions of the bound antibodies were determined by alkaline phosphatase-linked second antibody.

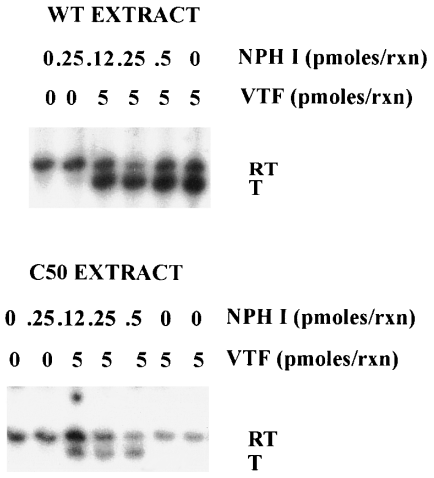

FIG. 3. Transcription termination activity of wild-type virus and ts mutant virus-infected cell extracts. Extracts of virus-infected cells were prepared as described in Fig. 2. A supercoiled plasmid template, pSBterm (Condit *et al.,* 1996), containing a G-less cassette harboring tandem termination signals downstream from a viral early promoter was employed. Read through transcripts synthesized in the presence of UTP, CTP, ATP, and 3′ *O* methyl GTP are 540 bases in length. Signal specific transcription termination yields a 450-base RNA product. The D11L protein (NPH I) was released from the GST-D11L fusion protein by thrombin treatment and separated from GST by passage through a glutathione Sepharose column. VTF (viral mRNA capping enzyme) was prepared from *E. coli* engineered to express both subunits (Higman *et al.,* 1992). RT, read through transcript; T, termination product.

exhibit significant sequence homology to NPH I. Prominent among these proteins are several known to belong to the helicase superfamily II (Gorbaleyna and Koonin, 1993). Seven degenerate sequence motifs have been associated with superfamily II helicases. Each motif contains a sequence consisting of highly conserved amino acids in addition to structurally homologous amino acids. NPH I possesses five perfect matches (Fig. 4). Possible

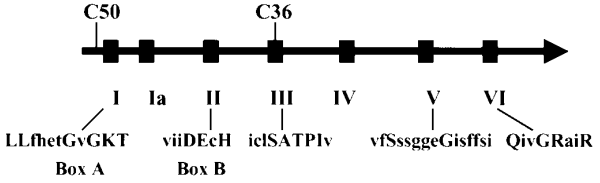

FIG. 4. NPH I possesses sequence motifs present in superfamily II helicases. Comparison of the NPH I amino acid sequence to the data bases reveals striking conservation of several motifs present in superfamily II helicases (Gorbaleyna and Koonin, 1993). The relative positions of seven putative motifs are denoted as boxes along an arrow which symbolizes the 631-amino-acid protein sequence. NPH I sequences corresponding to motifs I, II, III, V, and VI are written below each box. Five of the seven sequence motifs in NPH I contain both highly conserved amino acids (capital letters) and structurally homologous hydrophobic or hydrophilic amino acids (small letters). The five motif sequences exhibit a perfect match with the proposed consensus sequences. Motifs I and II correspond to the Walker A and B boxes (Walker *et al.,* 1982). Also noted are the positions of two ts mutations, C50 and C36.

TABLE 1

NTP I ATPase Activity (nmol/min/μg)

Enzyme	Amino acids	Experiment 1	Experiment 2
Wild type	1–631	30.1	41.6
5′ Δ1	25–631	0.09	
5′ Δ5	185–631	0	
5′ Δ6	225–631	0	
3′ Δ1	1–603	3.9	13.4
3′ Δ2	1–563	25	20.3
3′ Δ3	1–524	0.22	0.05
3′ Δ4	1–483	0.02	0.02
3′ Δ5	1–433	0.01	0.02

Motif				
m1	GVGKT	I	0	0
m2	DECH	II	0.21	0.09
m3	SATPI	III	36.9	46.7
m4	GGEG	V	0.39	0.84
m5	RAIR	VI	0.02	0.05

Note. ATPase activity measurements were carried out at different concentrations of wild-type or mutant GST-D11L at 10 mM ATP, 10 mM $MgCl_2$ (experiment 1) or 1 mM ATP, 1 mM $MgCl_2$ (experiment 2). Phosphate release was measured after incubation with enzyme for 10 min and phosphate concentration was calculated from a standard phosphate curve. The observed rate was plotted at different enzyme concentrations and the specific activity was calculated from the slope of the straight line and is presented as nmol/min/μg enzyme. The wild-type amino acid sequence for each helicase homology motif is presented for single site mutants m1 to m5. The mutations are as follows: m1, K61D; m2, E142K; m3, T185A, P186A; m4, G446R; m5, R476S, R479A.

candidates for the two remaining motifs can be found which possess one or two amino acid alterations. Of the motifs present in NPH I, two coincide with the Walker A and B box sequences (Walker *et al.,* 1982), proposed to be associated with ATP binding. To date, helicase activity has been demonstrated in only a small fraction of proteins identified as helicase superfamily members. Although the presence of helicase sequence motifs in NPH I does not demonstrate function, it is pleasing to speculate that NPH I might serve as a helicase in the termination process.

Initial mutagenic analysis of NPH I function

A set of N-terminal and C-terminal deletion mutations in NPH I were constructed in addition to site-specific mutations in five of the superfamily II helicase motifs (Table I). Each mutation was expressed as a GST-NPH I fusion protein and isolated from induced *E. coli.* For each mutant fusion protein, a prominent degradation product copurifies with the full-length product (Fig. 5). Since the 60-kDa truncated fusion proteins bind to glutathione Sepharose, each must be deleted at the carboxyl termi-

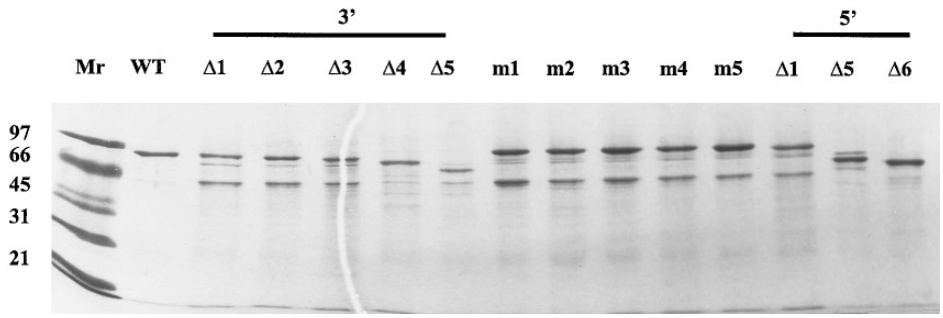

FIG. 5. Gel electrophoretic analysis of wild-type and mutant GST-D11L fusion proteins. Wild-type and mutant GST-D11L fusion proteins were purified from induced *E. coli* possessing pGEX 4T1-D11L plasmid derivatives. An S100 fraction was prepared from induced cells and passed sequentially over glutathione Sepharose and heparin agarose. Protein samples were denatured, separated in a 10% polyacrylamide gel, and stained with Coomassie blue. For the wild-type fusion protein, greater than 90% purity was routinely obtained. For the mutant fusion proteins, the level of purity varied from about 40 to 80%. The prominent contaminant in each case is a 60-kDa truncated fusion protein. Other minor bands also are derived from the full-length fusion protein.

nal end. As will be seen, such carboxyl terminal deletions lack both ATPase and single-stranded DNA binding activity and are unable to support transcription termination. Therefore, these truncated fusion proteins would be expected to have little effect on the set of analyses presented in this report. Passage of the protein over phosphocellulose at pH 6.8 increases purity of the GST-NPH I fusion protein. Although impure, each protein preparation was devoid of deoxyribonuclease and phosphatase activity. Some preparations had measurable ribonuclease activity but in all cases, the level of ribonuclease did not prevent their use.

Nucleoside triphosphate phosphohydrolase activity

ATPase activity was measured for both wild-type GST-NPH I and each mutant form. Measurements were conducted at either 1 mM ATP and $MgCl_2$ or 10 mM ATP and $MgCl_2$ and at saturating single-stranded DNA concentrations. ATPase activity was shown to be linear with respect to time and enzyme concentration (data not shown). Free NPH I exhibits the same specific activity as the GST fusion protein, demonstrating that the GST domain does not interfere with substrate or activator binding or with catalysis. For site-specific mutations in the helicase superfamily II motifs, alteration of motifs I, II, V, and VI severely reduced catalytic activity (Table 1). Motifs I and II correspond to the Walker A and B boxes (Walker *et al.,* 1982) and a significant reduction in ATPase activity would be expected. Based on this observation, a function for motifs V and VI in either ATP binding or catalysis would be anticipated. A double amino acid substitution in motif III, common to all superfamily II helicases, failed to affect ATPase activity, however. Deletion of as few as 24 N-terminal amino acids eliminated ATPase activity. If the putative Walker A and B boxes are employed in ATP binding, this would be expected. ts mutation C50 maps in the same area of NPH I (Fig. 4), but its effect on ATPase activity has not been measured (Kunzi and Traktman, 1989; Kahn and Esteban, 1990). Larger N-terminal deletions remove the putative Walker A and B boxes and lack ATPase activity, as well. Deletion of 68 amino acids from the C-terminus has little effect on ATPase activity; however, removal of an additional 39 amino acids reduces the ATPase activity more than 100-fold.

Transcription termination activity

The ability of each NPH I mutation to support transcription termination in C50-infected cell extracts was next assessed. Of the single site mutations, m1 to m5, only m3 permitted transcription termination (Fig. 6). This mutation possesses two amino acid alterations yet it retains full activity. Although highly conserved, this motif is not required for NPH I activity *in vitro,* either as an ATPase or as a termination cofactor. Among m1 to m5 mutations, the loss of ATPase activity correlates well with the loss of transcription termination activity. Each N-terminal deletion mutant lacks the ability to stimulate termination *in vitro,* in agreement with their loss of ATPase activity (Table 1). For the C-terminal deletion mutations, none retain the ability to support transcription termination, in spite of the fact that deletion of up to 68 amino acids permits retention of high levels of ATPase activity. Since the 3′ Δ1 and 3′ Δ2 mutations retain ATPase activity they are also likely to retain ssDNA binding. These observations suggest that the C-terminal region of NPH I may play a role in termination other than ATP hydrolysis.

Single-stranded DNA binding by wild-type and mutant NPH I

Single-stranded DNA binding by NPH I can be quantified by an electrophoretic mobility shift assay (Figs. 7A–7D). Binding yields two shifted complexes at each protein concentration tested for either free NPH I (Fig. 7B) or for the GST-D11L fusion protein derivatives (Fig.

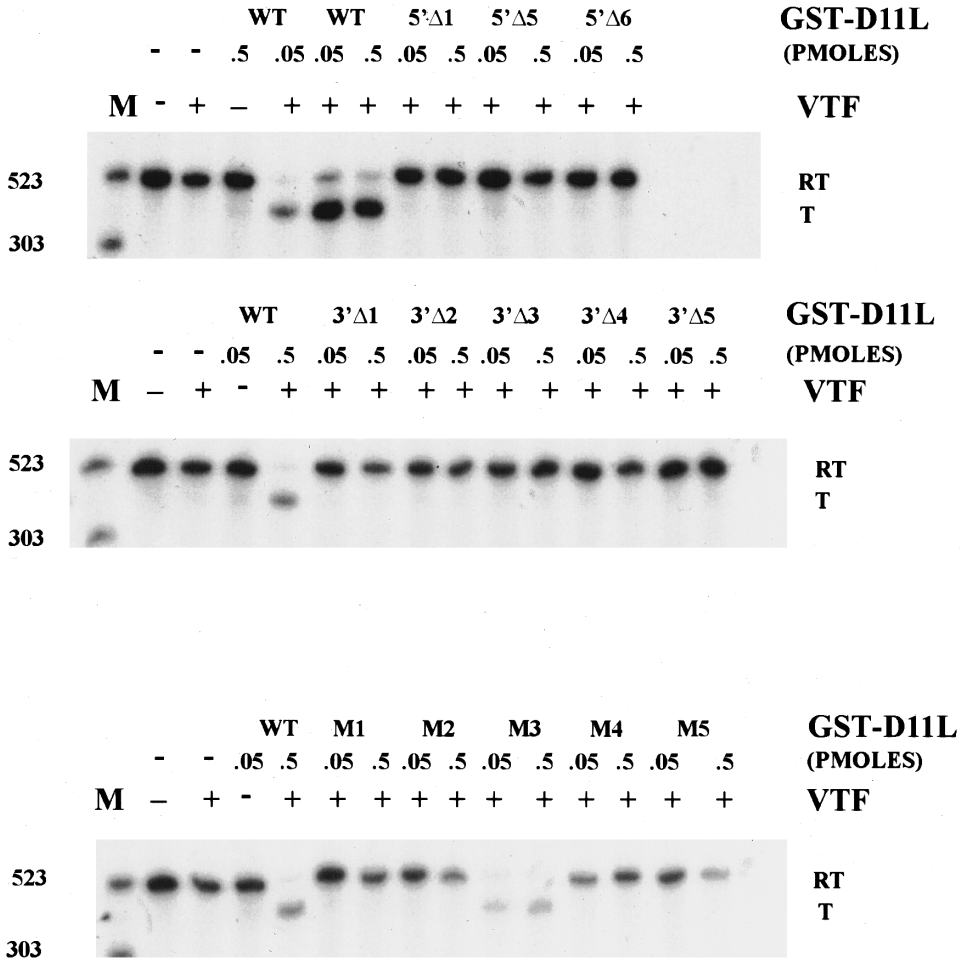

FIG. 6. Transcription termination activity of wild-type and mutant GST-D11L proteins *in vitro.* An extract prepared from ts C50-infected cells was employed in each reaction. Transcription was carried out in the presence or the absence of the indicated amounts of VTF and wild-type or mutant GST-D11L. RNA was isolated, separated by gel electrophoresis, and located by phosphorimaging. RT, 540-base read through product; T, 450-base terminated transcript.

7C). This behavior is consistent with the protein/DNA complex adopting two conformations. Both free NPH I and GST-D11L fusion protein exhibit equivalent affinity, demonstrating that the GST domain does not interfere with ssDNA binding. Binding is also dependent upon the length of the single-stranded DNA probe, with shorter DNA exhibiting weaker binding (data not shown). The DNA binding ability of the collection of GST-NPH I mutations was assessed using $ssDNA_{24}$ and similar concentrations of total GST-NPH I (Fig. 7A). Single site mutations, m2 to m5, bind as well as the wild-type GST-D11L protein. However, the m1 mutation exhibits significantly weaker binding. N-terminal deletion 5′ Δ1 displays substantially reduced binding, while 5′ Δ5 lacks measurable DNA binding activity. C-terminal deletion mutations 3′ Δ1 and 3′ Δ2 also exhibit high DNA binding. However, binding by larger carboxyl terminal mutations 3′ Δ3 and 3′ Δ4 is dramatically reduced and the longest mutation 3′ Δ5 lacks any binding activity. The reduction of ATPase activity observed in deletion mutants 3′ Δ3 to 3′ Δ5 might be a result of altered DNA binding rather than a direct effect on the ATPase active site. It is interesting to note that mutations 3′ Δ1 and 3′ Δ2 retain both ATPase activity and single-stranded DNA binding yet fail to support transcription termination.

Ability of NPH I mutants to act as dominant negative inhibitors of transcription termination *in vitro*

Altered forms of GST-D11L were analyzed in a competition experiment to assess whether the GST-D11L mutants possess the ability to inhibit transcription termination *in vitro.* Electrophoretic analysis of product RNA showed that mutant m2 inhibited transcription termination in a concentration-dependent fashion (Fig. 8A). Similar results were obtained for m1, m4, and m5 (data not shown). These results suggest that the site-specific mutations compete with the wild-type GST-D11L protein for binding to some essential termination component. N-terminal and C-terminal deletion mutations fail to inhibit

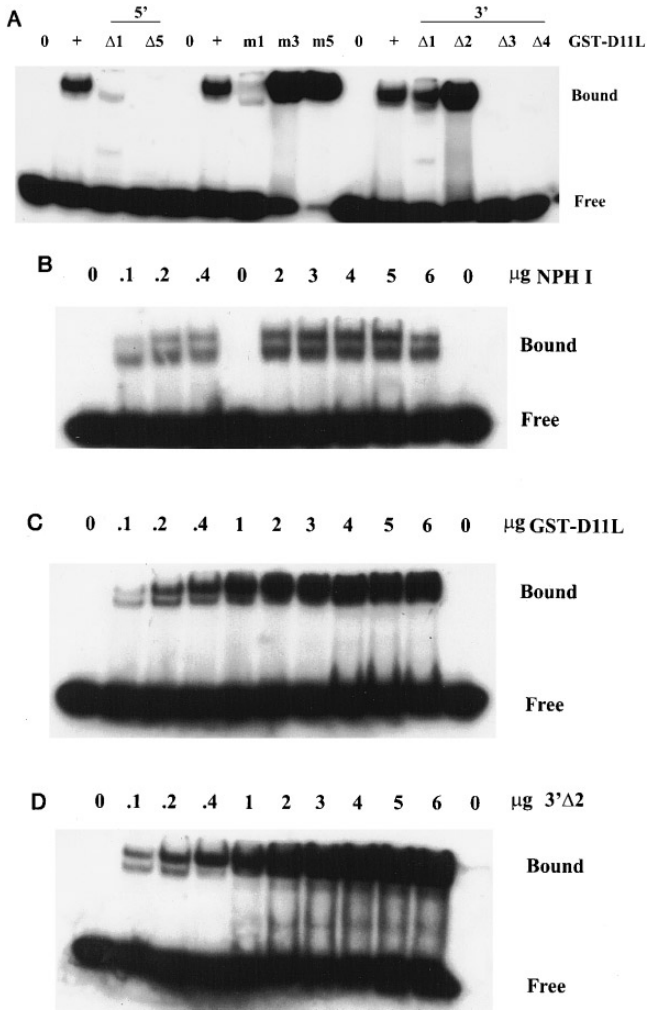

FIG. 7. EMSA analysis of wild-type and mutant GST-D11L proteins. (A) Incubations were set up on ice containing 10 fmol of 5′ end-labeled $ssDNA_{24}$ and 2 μg of wild-type or mutant GST-D11L (1 μg, approximately 10 pmol). Samples were separated in a 6-cm polyacrylamide gel, at 4°C, and after drying the gel, the positions of the ^{32}P-labeled $ssDNA_{24}$ were determined by phosphorimage analysis. (B–D) Titration of free NPH I, wild-type GST-D11L, and the 3′ Δ2 deletion mutation, respectively.

termination, however, indicating that they are unable to compete. Of particular interest are 3′ Δ1 and 3′ Δ2, which retain normal ssDNA binding and ATPase activity yet fail to support termination *in vitro.* The decrease in overall RNA synthesis observed at high GST-D11L is due to a low level of contaminating RNase. Since these mutations also fail to act as dominant negative effectors of termination it suggests that the carboxyl terminal end of NPH I interacts with one or more other components of the termination system.

N-terminal deletions also fail to act as inhibitors, which may indicate that this region of NPH I may also be important in such interactions. However, N-terminal mutations lack both ATPase activity and ssDNA binding. As a result, we have no means of assessing proper folding for these mutant protein. Therefore, we cannot be certain that their failure to inhibit isn't due simply to improper folding during synthesis.

DISCUSSION

Extracts can be prepared from lysolecithin-treated vaccinia virus-infected cells and employed in the analysis of viral gene transcription (Condit *et al.* 1996). For a ts mutation in the viral early gene D12L, a null extract lacking D12L was generated and proved useful in demonstrating that this protein is required for intermediate gene transcription initiation. In order to evaluate the role of NPH I in viral early gene transcription termination a similar approach was undertaken. However, since ts mutations in gene D11L exhibit reduced intermediate and late gene transcription (Kunzi and Traktman, 1989) and the two subunits of VETF, the viral early gene transcription initiation factor, and virion RNA polymerase subunits are synthesized late in infection, an alternate approach was undertaken. By initially infecting at the permissive temperature, normal viral protein synthesis takes place and late gene products accumulate in the

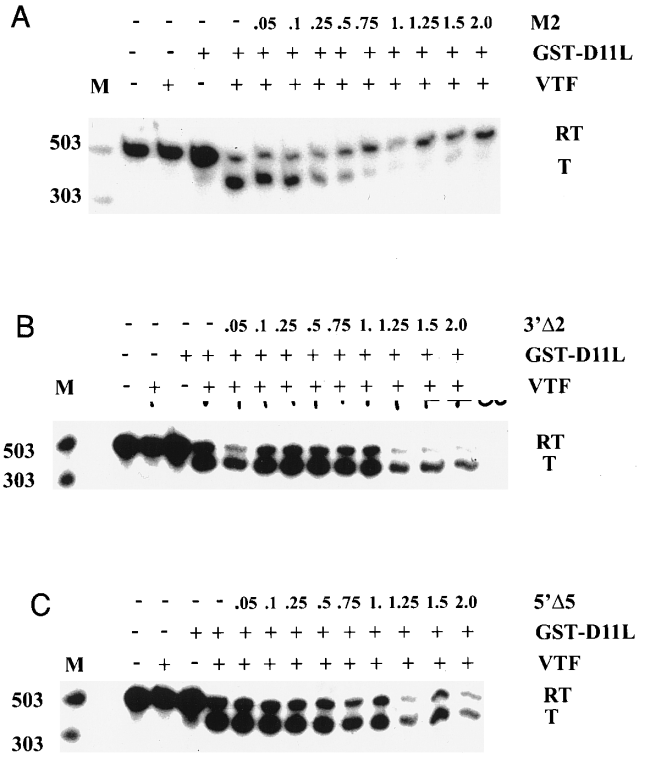

FIG. 8. Inhibition of transcription termination by mutant forms of GST-D11L. Transcription reactions were carried out employing an extract derived from ts C50 virus-infected cells including 5 pmol of VTF, 0.05 pmol of wild-type GST-D11L, and the indicated level of mutant GST-D11L (pmol). (A–C) Titration of the m2 mutation of GST-D11L, the 3′ Δ2 mutation of GST-D11L, and the 5′ Δ5 mutation of GST-D11L, respectively. Motif mutations m1, m4, and m5 exhibit inhibitory activity indistinguishable from that of m2. M, molecular weight markers.

infected cell. After shifting to the nonpermissive temperature, the ts mutant proteins are inactivated. Addition of cycloheximide inhibits further viral protein synthesis. If the normal viral proteins are stable at this temperature and the ts mutant protein is unstable, a null extract can be prepared. Analysis of total transcription activity showed that early and intermediate gene transcription is retained in these extracts but late gene transcription activity is lost. This observation suggests that one or more late gene transcription factors is unstable and inactivated by the extended incubation at 40°C. Although reduced early and intermediate gene transcription activity was observed in these extracts, they were shown to lack early gene transcription termination activity and proved to be useful for these studies. Western blot analysis demonstrated that the NPH I protein was retained in C50-infected cell extracts, albeit at a reduced level. In extracts prepared from E17-infected cells, little NPH I protein was found. Degradation of vaccinia virus ts mutant proteins is commonly observed (Dyster and Niles, 1991).

Analysis of transcription termination activity *in vitro* demonstrated that the ts mutant virus-infected cell extracts failed to terminate. Addition of VTF did not restore termination activity to the ts mutant virus-infected cell extracts, unlike extracts prepared from wild-type virus-infected cells. However, addition of either GST-D11L or free NPH I, in addition to VTF, restored termination activity, providing strong evidence that NPH I is an essential termination cofactor. This conclusion is supported by the observation that NPH I mutants lacking ATPase activity also fail to terminate *in vitro* and is in agreement with the results of Deng and Shuman, (1998).

A comparison of the NPH I amino acid sequence to the databases reveals a strong structural homology to the superfamily II helicases (Gorbalenya and Koonin, 1993). NPH I possesses five of the seven signature motifs with less-well-conserved matches to the other two. A series of N-terminal and C-terminal deletion mutations and several site-specific mutations were constructed and expressed as GST fusion proteins. Analysis of their ATPase activities demonstrated that the mutations altered in the helicase superfamily II motifs I, II, V, and VI lacked activity. A double mutation in motif III, however, retained activity *in vitro,* indicating that this sequence is not essential for ATP hydrolysis. Motifs I and II correspond to the well-known Walker box A and B sequences (Walker *et al.,* 1982), which are expected to be involved in ATP binding. It is not surprising that ATP hydrolytic activity would be lost in these mutations. The functions of motifs V and VI are not well known and their role in ATP hydrolysis should be investigated further. The shortest N-terminal deletion impinges upon motifs I and II and almost certainly would effect ATP binding. ts mutation C50, G44E, maps just to the left of the putative Walker A box which begins at amino acid G58, GVGKT. Altered ATPase activity would be anticipated in the C50 NPH I. The shortest C-terminal deletion mutations retain ATPase activity. In deletion 3′ Δ1, ATPase activity is somewhat variable, up to 30% of the wild-type enzyme in some preparations. 3′ Δ2 is consistently 60% or so of the normal ATPase activity. These results demonstrate that the C-terminal region is not essential for ATPase activity. Furthermore, since ATPase activity requires single-stranded DNA binding (Paoletti *et al.,* 1974), removal of the C-terminal 68 amino acids must not alter DNA binding. Larger C-terminal deletions, however, eliminate ATP hydrolytic activity.

Each single site mutation except m3, a double substitution in motif III, fails to support transcription termination *in vitro.* For the mutations that lack ATPase activity, this is not surprising and supports the notion that NPH I provides the ATPase activity employed in transcription termination. The m3, motif III, mutation is particularly interesting in that it retains both ATPase and transcription termination activity. However, in ts C36, Pro 186 is changed to Ser (Kahn and Esteban, 1990), indicating that this region of NPH I plays an essential role *in vivo.* Further biochemical analysis of C36 is required to define the biochemical basis of the ts mutation. A three-amino-acid substitution in the equivalent sequence in eIF-4A also retains ATPase activity yet lacks RNA unwinding activity in this well-known helicase (Pause and Sonenberg, 1992). A family of mutations in the herpes virus UL9 motif III sequence lack the ability to complement loss of function *in vivo,* indicating that this motif is essential for UL9 function (Martinez *et al.,* 1992). Both observations support an essential role for motif III in eIF4A and UL9 activity. Either motif III in NPH I is indeed nonessential for termination or our *in vitro* assay does not accurately reproduce its *in vivo* requirement. Analysis of additional motif III mutations will be conducted in order to resolve this issue.

Motif VI corresponds to a similar sequence in the vaccinia virus NPH II RNA helicase. Mutations in the NPH II sequences reduce both ATP hydrolysis and RNA unwinding without significantly reducing RNA binding (Gross and Shuman, 1996). These observations contrast with those reported by Pause and Sonenberg (1992) in which they attributed their loss of ATPase activity in a similar set of eIF4A mutations to reduced RNA binding. Differences in results obtained for mutant forms of various helicases may be a reflection on their varied subunit composition and biological functions. In regard to a putative helicase function for NPH I, although correlative, results from other systems have little direct bearing and only a successful demonstration of unwinding activity *in vitro* will permit defining this protein as a helicase. To date, attempts to demonstrate helicase activity in pure GST-D11L employing simple RNA/DNA helicase substrates have failed (Friedman and Niles, not shown). Perhaps one or more additional factors are required for

unwinding activity. Mutations in the NPH I helicase motifs II to VI exhibit normal single-stranded DNA binding in our analyses. However, a single substitution in motif I reduces DNA binding. The cause of this reduction is not obvious but is consistent with the reduction of DNA binding observed for the N-terminal deletion mutations.

Carboxyl terminal deletions 3′ Δ1 and 3′ Δ2 exhibit an interesting property. Each of these deletion mutations retains both ATPase activity and the ability to bind single-stranded DNA. However, each mutation lacks transcription termination activity. These observations suggest that the C-terminal region of NPH I is employed in some function other that ATP hydrolysis and DNA binding. Furthermore, neither deletion mutation is capable of acting as a competitive inhibitor of GST-D11L in an *in vitro* transcription termination assay. One simple model proposes that the C-terminal region of NPH I binds to some additional factor required for termination. Loss of the C-terminal 68 amino acids eliminates binding, which not only prevents termination but also prevents competition with wild-type GST-D11L. This putative NPH I binding partner could be VTF, a subunit of the virion RNA polymerase, or perhaps some as yet unidentified essential termination factor. Prior results support a direct interaction between NPH I and the virion RNA polymerase (Broyles and Moss, 1987a; Zhang *et al.,* 1994). Recent evidence demonstrates the presence of NPH I in a paused ternary complex (Deng and Shuman, 1998). N-terminal mutations also fail to inhibit GST-D11L *in vitro.* Unfortunately, due to the lack of any measurable activity, one cannot be certain that these deletion mutations folded properly. As a result, it is premature to propose an interaction between the N-terminal region and one or more partners at this time.

Any model attempting to explain poxvirus early gene transcription termination must take into account the following facts (Fig. 9). Termination requires the sequence UUUUUNU in the nascent mRNA (Rohrman *et al.,* 1986; Yuen and Moss, 1987; Shuman and Moss, 1989). The UUUUUNU containing mRNA must be tethered to the transcription complex yet UUUUUNU must be free, presumably to be available for interaction with some other component (Deng *et al.,* 1996). In addition, termination requires VTF, the viral multifunctional mRNA processing enzyme consisting of the products of genes D1R and D12L (Shuman *et al.,* 1987). The role of VTF is undefined but the enzyme is clearly capable of productive RNA binding. However, a strong preference for the sequence UUUUUNU has not been demonstrated to date (Luo and Shuman, 1993; Higman *et al.,* 1994). Termination is limited to the RNA polymerase that is capable of initiation at early gene promoters (Condit *et al.,* 1996). Although a specific interaction with the H4L subunit, unique to the virion RNA polymerase (Ahn and Moss, 1992), would be an attractive mechanism for restriction of termination to early genes, recent evidence argues against this model

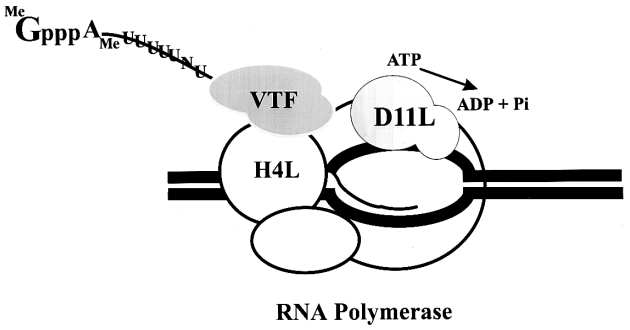

FIG. 9. A cartoon depicting a model of the vaccinia virus early gene transcription termination complex. Any mechanism considered for termination of early gene transcription must include the following observations. Termination requires the presence of the sequence UUUUUNU in the nascent mRNA. VTF, the viral mRNA capping enzyme, is an essential factor whose role in termination is undefined. Only RNA polymerase that recognizes early promoters is sensitive to signal-dependent termination. This form of virion RNA polymerase possesses the H4L subunit. Finally, a single-stranded DNA-dependent ATPase activity, NPH I, with helicase homology motifs, is employed as an energy transducing factor.

(Deng and Shuman, 1996). Finally, ATP hydrolysis by NPH I is required. Since NPH I must bind single-stranded DNA to stimulate ATPase activity, this requirement argues strongly that NPH I must be able to interact with single-stranded DNA during the termination reaction. Since the template strand is bound to nascent mRNA in the transcription bubble, the most likely source of single-stranded DNA is the free nontemplate strand, in the paused ternary complex. This fact supports a model in which the DNA binding site on NPH I interacts with the nontemplate strand, activating a cryptic ATPase active site. Furthermore, the inability of C-terminal deletion mutations to serve as dominant negative effectors of termination *in vitro* argues strongly that a productive interaction of the C-terminal region of NPH I with some component of the termination system is essential. Further genetic and biochemical investigations of the interplay among these termination cofactors promises to resolve the current ambiguities.

MATERIALS AND METHODS

Cells and virus

Wild-type vaccinia virus WR and ts mutant viruses C50, C36, and E17 (Condit and Motyczka, 1981; Condit *et al.,* 1983; Ensinger, 1982) were propagated on BSC40 African green monkey cells, at the permissive temperature, 31°C, as described (Condit and Motyczka, 1981). Crude virus containing extracts of infected cells were prepared by freeze/thaw, and infectious virus titers were determined on BSC40 cells at the permissive temperature, 31°C, and the nonpermissive temperature, 40°C.

Transcription

Extracts of virus-infected cells were prepared by lysolecithin treatment, as described (Condit *et al.,* 1996). A549 cells were infected with wild-type virus or ts mutants C50, C36, and E17, at a multiplicity of infection (m.o.i.) of 15, at the permissive temperature, 31°C. After 24 h, the medium was removed and replaced with 40°C medium containing 100 μg/ml of cycloheximide. After a further 24 h, cells were washed and treated with 250 μg/ml lysolecithin and extracts prepared. Transcription assays were carried out in 10 μl total volume containing 1 to 6 μl extract, 1 mM ATP, 1 mM UTP, 20 μM CTP, 2 μCi [α-^{32}P]CTP, 0.2 μg supercoiled plasmid DNA, 25 mM HEPES buffer, pH 7.4, 50 mM KCH_3COO, 5 mM $MgCl_2$, 1 mM dithiolthreitol, and 7.5% glycerol for 30 min at 30°C. Total early, intermediate, or late gene transcription activity was measured employing templates containing a strong artificial early promoter, an intermediate promoter derived from the G8R gene, or the late F17R gene, as described previously (Condit *et al.,* 1996). Termination of early gene transcription was monitored by using a plasmid template, pSBterm, which possess tandem termination signals within the G-less cassette. After proteinase K treatment, RNA was isolated by extraction with phenol/chloroform, precipitated with isopropanol, and resuspended in formamide. After heating at 90°C, samples were separated by electrophoresis in 5% acrylamide 8 M urea gels and the RNA were visualized by phosphorimage analysis or by autoradiography. Termination efficiency was calculated as the molar ratio of terminated RNA to the sum of read through and terminated RNA.

Expression and purification of GST-D11L

The entire coding region of gene D11L was amplified by PCR. The 5′ primer possessed a *Bgl*II site upstream from the translation initiation ATG and the 3′ primer included a single *Sal*I site. The PCR product was inserted into the *Sma*I site of pGEM 3 Zf^+, yielding pGEM 3 Zf^+-D11L, and the sequence of the D11L gene was determined to assess fidelity of amplification. The *Bgl*II to *Sal*I region was inserted in frame into pGEX 4T1 cut with *Bam*HI and *Sal*I and transformed into *E. coli* TB-1. Induction of mid-log phase cells yielded the synthesis of a 98-kDa GST-NPH I fusion protein. Large-scale induction was carried out at 20°C as described (Higman *et al.,* 1992). Cells were collected by centrifugation and stored at −70°C until used. The first several steps of purification, through the S100 step, were described for the mRNA capping enzyme (Higman *et al.,* 1992). GST-NPH I was purified from an S100 fraction of induced cells by sequential affinity chromatography on glutathione Sepharose and heparin agarose. NPH I was eluted from the heparin agarose column with a linear gradient from 0 to 0.5 M NaCl. In some instances, GST-NPH I was cleaved with thrombin and NPH I was separated from free GST by chromatography on glutathione Sepharose.

Mutagenesis

Both deletion mutations and site-specific mutations were constructed in the NPH I coding sequence. The 5′ and 3′ deletions were constructed by PCR employing primers that were complementary to various positions in the NPH I coding sequence. For the 5′ deletions, the 5′ primers were synthesized so that a single *Bgl*II site was incorporated upstream from the first NPH I codon such that the insertion of the NPH I sequence into the *Bam*HI site in pGEX 4T1 would generate a coding sequence in which the NPH I sequence would be in frame with the GST sequence. For 3′ deletions, the 3′ primers contained tandem translation termination signals adjacent to the final NPH I codon. Site-specific mutagenesis of D11L was conducted by the Kunkel method using the *dut/ung* system (Kunkel *et al.,* 1987) purchased in kit form from Bio-Rad. The D11L sequence in pGEM 3Zf^+-D11L was isolated as a single strand containing dUMP. Selected mutagenic primers were annealed and elongated and the double-stranded plasmids were transformed into *E. coli* TB-1. Plasmids were isolated from progeny colonies and the sequence of the D11L gene in the region of each mutation was determined. pGEX 4T1 −D11L plasmids containing mutant sequences were constructed by exchange of specific DNA fragments between pGEX 4T1-D11L and mutant pGEM 3Zf^+-D11L. Mutant GST-D11L fusions were isolated from induced *E. coli* as described for the wild-type fusion protein. In some instances further purification on phosphocellulose at pH 6.8 was included.

Immunological techniques

A trp E fusion plasmid (Dieckmann and Tzagoloff, 1985) was constructed capable of expressing a fusion protein containing the D11L coding sequence from amino acids 142 to 631 linked to the trp E protein, by insertion of a portion of the vaccinia virus *Hin*dIII D fragment from a *Bgl*II site at 10,822 to a *Bam*HI site at 12,838 (Niles *et al.,* 1986). Induction of *E. coli* TB-1 containing this plasmid yielded a fusion protein that was purified from inclusion bodies by preparative acrylamide gel electrophoresis and employed in the generation of a rabbit polyclonal antiserum containing antibodies directed against the D11L protein. Western blots were conducted as described (Niles and Seto, 1988) using either anti-D11L or anti-D12L antibodies. Anti-D12L antibodies are directed against the small subunit of the mRNA capping enzyme and serve as a control (Niles *et al.,* 1989).

Nucleoside triphosphatase (ATPase) assay

The release of inorganic phosphate from ATP was quantified by the colorimetric assay as described by Myette and Niles (1996). Standard reactions were carried out in a 100-μl total volume, for 10 min at 37°C, containing 100 mM MES, pH 7.0, 1 mM ATP, 1 mM $MgCl_2$, 5 mM

dithiolthreitol, and 5 μg heat-denatured calf thymus DNA (Paoletti *et al.,* 1974). Wild-type or mutant GST-D11L or free NPH I concentrations ranged up to 0.3μg per assay. For time course determination, 500-μl reactions were prepared. At times after enzyme addition, 100-μl aliquots were removed and quenched with 2.5 μl 250 mM EDTA and free phosphate was determined. GST-NPH I and free NPH I exhibited similar specific activities in this assay.

Mobility shift analysis

Single-stranded DNA (ssDNA) mobility shift analyses were conducted as described (Higman *et al.,* 1994). Reactions (20 μl) were prepared on ice containing 50 mM Tris HCl, pH 8.0, 10% glycerol, 10 mM β-mercaptoethanol, 10 fmol of 5′-labeled $ssDNA_{24}$, a 24-nucleotide DNA fragment whose sequence corresponds to the top strand of the pGEM3Zf$^+$ polylinker from nucleotides 5 to 28, and up to 5 μg protein. One microgram of GST-D11L is approximately 10 pmol of protein. Electrophoresis was conducted at 4°C in gels that were 1.5 mm thick and 6 cm long, for 45 min at 200 V. The migration position of the ssDNA was observed either by phosphorimage analysis or by autoradiography.

ACKNOWLEDGMENT

This work was supported by NIH Grant GM 54816.

REFERENCES

Ahn, B. Y., and Moss, B. (1992). RNA polymerase-associated transcription specificity factor encoded by vaccinia virus. *Proc. Natl. Acad. Sci. USA* 89, 3536–3540.

Amegadzie, B. Y., Ahn, B. Y., and Moss, B. (1992). Characterization of a 7-kilodalton subunit of vaccinia virus DNA-dependent RNA polymerase with structural similarities to the smallest subunit of eukaryotic RNA polymerase II. *J. Virol.* 66, 3003–3010.

Broyles, S. S., and Moss, B. (1986). Homology between RNA polymerases of poxviruses, prokaryotes, and eukaryotes: Nucleotide sequence and transcriptional analysis of vaccinia virus genes encoding 147-kDa and 22-kDa subunits. *Proc. Natl. Acad. Sci. USA* 83, 3141–3145.

Broyles, S. S., and Moss, B. (1987a). Sedimentation of an RNA polymerase complex from vaccinia virus that specifically initiates and terminates transcription. *Mol. Cell. Biol.* 7, 7–14.

Broyles, S. S., and Moss, B. (1987b). Identification of the vaccinia virus gene encoding nucleoside triphosphate phosphohydrolase I, a DNA-dependent ATPase. *J. Virol.* 61, 1738–1742.

Condit, R. C., and Motyczka, A. (1981). Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. *Virology* 113, 224–241.

Condit, R. C., Motyczka, A., and Spizz, G. (1983). Isolation, characterization, and physical mapping of temperature-sensitive mutants of vaccinia virus. *Virology* 128, 429–443.

Condit, R. C., Lewis, J. I., Quinn, M., Christen, L. M., and Niles, E. G. (1996). Use of lysolecithin-permeabilized infected-cell extracts to investigate the in vitro biochemical phenotypes of poxvirus ts mutations altered in viral transcription activity. *Virology* 218, 169–180.

Deng, L., and Shuman, S. (1996). An ATPase component of the transcription elongation complex is required for factor-dependent transcription termination by vaccinia virus RNA polymerase. *J. Biol. Chem.* 271, 29,386–29,392.

Deng, L., and Shuman, S. (1998). Vaccinia NPH-I, a DEXH-box ATPase, is the energy coupling factor for mRNA transcription termination. *Genes Dev.* 12, 538–546.

Deng, L., Hagler, J., and Shuman, S. (1996). Factor-dependent release of nascent RNA by ternary complexes of vaccinia RNA polymerase. *J. Biol. Chem.* 271, 19556–19562.

Diaz-Guerra, M., and Esteban, M. (1993). Vaccinia virus nucleoside trihposphate phosphohydrolyase I control early anb late gene expression by regulating the rate of transcription. *J. Virol.* 67, 7561–7572.

Dieckmann, C. L., and Tzagoloff, A. (1985). Assembly of the mitochrondrial membrane system. CBP6, a yeast nuclear gene necessary for synthesis of cytochrome b. *J. Biol. Chem.* 260, 1513–1520.

Dyster, L. M., and Niles, E. G. (1991). Genetic and biochemical characterization of vaccinia virus genes D2L and D3R which encode virion structural proteins. *Virology* 182, 455–467.

Ensinger, M. J. (1982). Isolation and genetic characterization of temperature-sensitive mutants of vaccinia virus WR. *J. Virol.* 43, 778–790.

Gorbalenya, A. E., and Koonin, E. V. (1993). Helicases: Amino acid sequence comparisons and structure-function relationships. *Curr. Opin. Struct. Biol.* 3, 419–429.

Gross, C. H., and Shuman, S. (1996). The QRxGRxGRxxxG motif of the vaccinia virus DExH box RNA helicase NPH-II is required for ATP hydrolysis and RNA unwinding but not forRNA binding. *J. Virol.* 70, 170–113.

Hagler, J., Luo, Y., and Shuman, S. (1994). Factor-dependent transcription termination by vaccinia RNA polymerase: Kinetic coupling and requirement for ATP hydrolysis. *J. Biol. Chem.* 269, 10050–10060.

Higman, M. A., Bourgeois, N., and Niles, E. G. (1992). The vaccinia virus mRNA (guanine-N7-)-methyltransferase requires both subunits of the mRNA capping enzyme for activity. *J. Biol. Chem.* 267, 16430–16437.

Higman, M. A., Christen, L. A., and Niles, E. G. (1994). The mRNA (guanine-7-)methyltransferase domain of the vaccinia virus mRNA capping enzyme: Expression in *Escherichia coli* and structural and kinetic comparison to the intact capping enzyme. *J. Biol. Chem.* 269, 14974–14981.

Kahn, J., and Esteban, M. (1990). Identification of the two point mutations in two vaccinia virus nucleoside triphosphate phosphohydrolase I temperature-sensitive mutants and role of this DNA-dependent ATPase enzyme in virus gene expression. *Virology* 174, 459–471.

Kunkel, T. A., Roberts, J. D., and Zakour, M. A. (1987). Rapid and efficient site specific mutagenesis without phenotypic selection. *Methods Companion Methods Enzymol.* 154, 367–382.

Kunzi, M. S., and Traktman, P. (1989). Genetic evidence for involvement of vaccinia virus DNA-dependent ATPase I in intermediate and late gene expression. *J. Virol.* 63, 3999–4010.

Luo, Y., and Shuman, S. (1993). RNA binding properties of vaccinia virus capping enzyme. *J. Biol. Chem.* 268, 21253–21262.

Martinez, R., Shao, L., and Weller, S. K. (1992) The conserved helicase motifs of the herpes simplex virus type 1 origin-binding protein UL9 are important for function. *J. Virol.* 66, 6735–6746.

Moss, B. (Ed.) (1996). Poxviridae: The viruses and their replication. *In* "Virology" (B. N. Fields, D. M. Knipe, R. M. Chanock, T. P. Monath, P. M. Howley, J. L. Melnick, B. Roizman, and S. E. Strauss, Eds.), Vol. 2. Lippincott–Raven. Philadelphia.

Moss, B., Ahn, B. Y., Amegadzie, B., Gershon, P. D., and Keck, J. G. (1991). Cytoplasmic transcription system encoded by vaccinia virus. *J. Biol. Chem.* 266, 1355–1358.

Myette, J. R., and Niles, E. G. (1996). Characterization of the vaccinia virus RNA 5′-triphosphatase and nucleotide triphosphate phosphohydrolase activities demonstrate that both activities are carried out at the same active site. *J. Biol. Chem.* 271, 11945–11952.

Niles, E. G., Condit, R. C., Caro, P., Davidson, K., Matusick, L., and Seto, J. (1986). Nucleotide sequence and genetic map of the 16-kb vaccinia virus *Hin*dIII D fragment. *Virology* 153, 96–112.

Niles, E. G., and Seto, J. (1988). Vaccinia virus gene D8 encodes a virion transmembrane protein. *J. Virol.* 62, 3772–3778.

Niles, E. G., Lee-Chen, G. J., Shuman, S., Moss, B., and Broyles, S. S. (1989). Vaccinia virus gene D12L encodes the small subunit of the viral mRNA capping enzyme. *Virology* 172, 513–522.

Paoletti, E., Cooper, N., and Moss, B. (1974). Regulation of synthesis of two immunologically distinct nucleic acid-dependent nucleoside triphosphate phosphohydrolases in vaccinia virus-infected HeLa cells. *J. Virol.* 14, 578–586.

Pause, A., and Sonenberg, N. (1992). Mutational analysis of a DEAD box RNA helicase: The mammalian translation initiation factor eIF-4A. *EMBO J.* 11, 2643–2654.

Rodriguez, J. F., Kahn, J. S., and Esteban, M. (1986). Molecular cloning, endoding sequence, and expression of vaccinia virus nucleic acid-dependent nucleoside triphosphatase gene. *Proc. Natl. Acad. Sci. USA* 83, 9566–9570.

Rohrmann, G., Yuen, L., and Moss, B. (1986). Transcription of vaccinia virus early genes by enzymes isolated from vaccinia virions terminates downstream of a regulatory sequence. *Cell* 46, 1029–1035.

Rosales, R., Harris, N., Ahn, B. Y., and Moss, B. (1994). Purification and identification of a vaccinia virus-encoded intermediate stage promoter-specific transcription factor that has homology to eukaryotic transcription factor SII (TFIIS) and an additional role as a viral RNA. *J. Biol. Chem.* 269, 14260–14267.

Seto, J., Celenza, L. M., Condit, R. C., and Niles, E. G. (1987). Genetic map of the vaccinia virus *Hin*dIII D Fragment. *Virology* 160, 110–119.

Shuman, S., Broyles, S. S., and Moss, B. (1987). Purification and characterization of a transcription termination factor from vaccinia virions. *J. Biol. Chem.* 262, 12372–12380.

Shuman, S., and Moss, B. (1989). Bromouridine triphosphate inhibits transcription termination and mRNA release by vaccinia virions. *J. Biol. Chem.* 264, 21356–21360.

Vos, J. C., Sasker, M., and Stunnenberg, H. G. (1991). Vaccinia virus capping enzyme is a transcription initiation factor. *EMBO J.* 10, 2553–2558.

Yuen, L., and Moss, B. (1986). Multiple 3′ ends of mRNA encoding vaccinia virus growth factor occur within a series of repeated sequences downstream of T clusters. *J. Virol.* 60, 320–323.

Yuen, L., and Moss, B. (1987). Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. *Proc. Natl. Acad. Sci. USA* 84, 6417–6421.

Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* 1, 945–951.

Zhang, Y., Ahn, B.-Y., and Moss, B. (1994). Targeting of a multicomponent transcription apparatus into assembling vaccinia virus particles requires RAP94, an RNA polymerase-associated protein. *J. Virol.* 68, 1360–1370.