The Viral RNA Polymerase H4L Subunit Is Required for Vaccinia Virus Early Gene Transcription Termination

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Vaccinia virus early gene transcription is catalyzed by a multisubunit virion form of RNA polymerase that possesses a unique subunit, H4L. Prior studies from this laboratory showed that the NH2-terminal domain of H4L, containing amino acids 1–195, interacts with the COOH-terminal end of nucleoside triphosphate phosphohydrolase I (NPH I), an ATPase that is employed in early gene transcription termination. Carboxyl-terminal deletion mutations of NPH I lose both the ability to mediate transcription termination and binding to H4L, providing evidence that the interaction between NPH I and H4L is required for termination. In order to test this model further, antibodies raised against segments of H4L were tested for their ability to inhibit transcription termination in vitro. A bead-bound template was employed in these studies, which permitted us to separate transcription initiation from elongation and termination. Antibodies raised against H4L amino acids 1–256 inhibited termination in an in vitro assay using virus-infected cell extracts lacking NPH I, but antibodies raised against H4L amino acids 568–795 did not. Preincubation of anti-H4L1–256 antibodies with H4L fragments 1–256 or 1–195 prevented antibody inhibition of termination, demonstrating that inhibition was mediated by antibody binding to one or more epitopes in the NH2-terminal end of H4L. Antibody inhibition of termination is reduced in wild type virus-infected cell extracts containing NPH I. Furthermore, preincubation of a NPH I minus cell extract with NPH I prior to antibody addition, or readdition of NPH I to isolated ternary complexes prepared in the absence of NPH I, prevented antibody inhibition of transcription termination. These data show that NPH I and the inhibitory antibodies compete for a binding site(s) on H4L, providing further evidence that the H4L subunit of the vaccinia virus RNA polymerase plays a direct role in transcription termination.

Vaccinia virus, the prototypic poxvirus, possesses a double-stranded DNA genome of 191,686 base pairs (1) capable of encoding ~200 proteins. Poxviruses, which replicate within the cytoplasm of the infected cell, evolved to encode the enzymes required to carry out viral DNA replication, transcription, and mRNA processing (2). Studies with vaccinia virus revealed three temporal stages of gene expression: early, intermediate, and late. Capped and polyadenylated early mRNAs are synthesized within the virus particle immediately after infection (3–5). Following early gene expression and the onset of DNA replication, intermediate genes are transcribed on a replicating template. Late gene transcription follows that of intermediate genes and employs a similar template. Transcription of each gene class requires class-specific transcription initiation factors and employs class-specific promoters. Host factors are also required for both intermediate (6) and late (7, 8) mRNA synthesis.

Initiation of early vaccinia virus transcription requires the heterodimeric early transcription factor VETF1 (9) and the virion RNA polymerase possessing the RNA polymerase-associated protein RAP94, the product of H4L gene (10, 11). Initiation is coupled to ATP hydrolysis by VETF (12), which binds to the promoter sequence and recruits RNA polymerase to the template (13). Only the virion RNA polymerase molecules containing RAP94 exhibit VETF-dependent transcription of a double-stranded DNA template possessing a viral early promoter (14). Unlike the other subunits of vaccinia virus RNA polymerase, RAP94 is present in submolar amounts and is synthesized exclusively late in infection, whereupon it is packaged into nascent virions (10). Free H4L protein was not detected in virion extracts (10) and was not dissociable from purified viral polymerase even under denaturing conditions (14).

Early viral genes are unique in that transcription terminates in a signal- and factor-dependent manner (15–17). Elongation proceeds through the sequence TTTTTNT in the nontemplate strand, yielding UUUUUNU in the nascent mRNA, which serves as a signal required for the termination event (18). Termination requires both the vaccinia termination factor (VTF; also serves as viral mRNA capping enzyme) (16) and nucleoside triphosphate phosphohydrolase I (NPH I), the product of gene D11L, as the ATPase employed in transcription termination (19, 20). During infection, transcription termination is restricted to early genes. In vitro, only RNA polymerase capable of recognizing early promoters is subject to signal-dependent termination, demonstrating that this form of RNA polymerase is uniquely termination-competent (21).

Prior work demonstrated a physical interaction between the COOH-terminal end of NPH I and the NH2-terminal end of the H4L subunit of the virion RNA polymerase (22). Furthermore, COOH-terminal NPH I mutants failed both to bind to H4L (22)

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and to support transcription termination (20) and transcript release (22), indicating that the interaction between NPH I and H4L is needed for these processes. The requirement for NPH I/H4L binding would explain the known restriction of transcription termination to early genes, since the H4L subunit is only found on the form of RNA polymerase that transcribes early genes.

In order to test this model by a different approach, we evaluated the effect of region-specific H4L antibodies on early gene transcription termination, in vitro. We show that antibodies directed against the NH2-terminal region of H4L (representing amino acids 1–256) inhibit early gene transcription termination in an extract prepared from cells infected at the nonpermissive temperature with C50, a temperature-sensitive mutant virus. This extract lacks NPH I activity (20). Preincubation of the antibodies with H4L fragments representing amino acids 1–256 or 1–195 prevented the antibody inhibition. Interestingly, antibody inhibition of transcription termination is reduced in a wild type virus-infected cell extract containing NPH I. Moreover, addition of NPH I to a C50 mutant virus-infected cell extract, lacking NPH I, or to a ternary complex isolated from a C50 mutant virus-infected cell extract, prevented antibody inhibition of termination. These results demonstrate that the amino-terminal end of H4L is required for early gene transcription termination and confirm the importance of the H4L/NPH I interaction in this process.

EXPERIMENTAL PROCEDURES

Cells and Viruses—Wild type (WT) vaccinia virus strain WR and the temperature-sensitive (ts) mutant virus, C50 (23, 24), were propagated in BSC40 African green monkey cells at 37 °C, or the permissive temperature for ts mutants, 31 °C, respectively, as described (23). Crude extracts of virus-infected cells were prepared by freeze/thaw, and infectious virus titer was determined by plaque assay on BSC40 cells at the permissive temperature, 31 °C, and the nonpermissive temperature, 40 °C.

Purification of Anti-H4L Antibodies—Polyclonal rabbit antisera were prepared against segments of the H4L subunit of the virus RNA polymerase, representing amino acids 1–256, 258–556, and 568–795. The IgG fraction of each antiserum was isolated using Affi-gel Protein A MAb II kit (Bio-Rad) following the manufacturer’s instructions. Protein A-agarose was packed in a column (10-mL Poly-Prep column, Bio-Rad) and washed with the binding buffer provided with the kit. One mL of antiserum was diluted 1:1 with the binding buffer and loaded onto a Protein A-agarose column. The unbound fraction of the serum was washed off the column, and the bound fraction (IgG) subsequently eluted from the column with 2% (w/v) elution buffer. The eluates were then dialyzed in 1-mL fractions in tubes containing 200 mL of 0.1 M Tris-HCl, 0.0, 8.0, to neutralize the eluates. The column effluent was monitored spectrophotometrically at 280 nm in order to detect the protein peaks. Fractions were pooled, aliquoted, and stored at −80 °C.

Western Analysis—Proteins were resolved in a 12.5% polyacrylamide SDS gel and transferred to a nitrocellulose membrane. The blot was blocked with 3% gelatin and probed with the IgG fractions (diluted 1:500 in 1% gelatin) raised against MalE fusion proteins containing amino acids 1–256 or 568–795 of the H4L subunit of virion RNA polymerase. Following several washes, the membranes were incubated with goat anti-rabbit IgG (diluted 1:500 in 1% gelatin) conjugated to alkaline phosphatase. The alkaline phosphatase conjugate substrate (Bio-Rad) was then used to detect the development of immunoblots.

Immunoprecipitation—For each immunoprecipitation, 5 mL of the IgG fraction (0.7 mg/mL) was employed with 1 mL of the 35S-labeled transcription translation product in a total incubation of 200 mL. The reaction was incubated at 4 °C overnight with constant rocking. The antibodies were collected by the addition of 20 mL of Protein A-agarose, and the precipitate was washed four times as described (25). The final pellet was resuspended in sample buffer, boiled, and applied to an SDS-polyacrylamide gel. The gel was then soaked in 1 mL of cold Tris-bufferED, and fluorography was then performed at −80 °C.

Transcription Extracts—Extracts of virus-infected cells were prepared by lysosolichitin treatment, as described (21). A549 cells were infected with either wild type or ts mutant viruses at a multiplicity of infection of 15, at 37 °C or 31 °C, respectively. In the case of the ts mutant virus, after 24 h, the medium was replaced and replaced with 40 °C medium containing 100 μg/mL cycloheximide. After an additional 24 h at 40 °C, cells were washed and treated with 250 μg/mL lysosolichitin and extracts prepared. This procedure permits the initial synthesis of active NPH I, which is required for intermediate and late gene expression. After switching to 40 °C, the endogenous NPH I is inactivated and cycloheximide prevents the synthesis of new protein (20).

Transcription Termination Assay—The plasmid template, pSBtern (21), possesses tandem termination signals within the G-less cassette. Transcription reactions were carried out in a 20-μL total volume containing 6 μL of extract, 1 mM ATP, 0.1 mM UTP, 2 mM GTP, 4 μCi of [35S]CTP (800 Ci/mmol), 0.2 μg of supercoiled plasmid DNA, 20 mM Tris-HCl buffer, pH 8.0, 6 mM MgCl2, 2 mM dithiothreitol, and 8% glycerol for 30 min at 30 °C. When proteinase K treatment, RNA was isolated by extraction with phenol/chloroform, precipitated with isopropanol, and resuspended in formamide dye solution. Samples were heated at 90 °C and separated by electrophoresis in 5% acrylamide, 8 μm gels, and the RNA was visualized by autoradiography. Termination efficiency was calculated as the molar ratio of terminated RNA to the sum of read-through and terminated RNA.

Construction of the G21(TER29)A78 plasmid containing a vaccinia virus early promoter was described by Deng et al. (26); this plasmid was generously provided by Dr. Stewart Shuman. The prototype G21(TER29)A78 transcription unit consists of a synthetic early promoter coupled to a G-less cassette, which is a run of three G residues at positions +21 to +23. A 57-nucleotide A-less cassette lies downstream of the G-less cassette and flanked at its 3' end by four A residues at positions +78 to +81. A termination signal, TTTTTTTTTT, lies within the A-less cassette, spanning position +29 to +37. The biotinylated 324-base pair DNA template was amplified by polymerase chain reaction employing a 5' biotin tag on the upstream primer and isolated and ligated by preparative agarose gel electrophoresis. The purified DNA fragment was then immobilized to streptavidin-coated magnetic beads (Dynabeads M280; Dynal) as described (27). The bead-bound (B) template (typically, 100 fmol) was first incubated with 6 μL of C50 or WT virus-infected cell extracts, in the presence of 1 mM ATP, 10 μCi of [35S]CTP (800 Ci/mmol), 0.1 mM UTP, and 0.625 mM 35S CTP (22, 23). This ternary complex was isolated, washed, and incubated in the presence or absence of the IgG fractions, or NPH I. When indicated, the IgG fractions were precipitated with H4L fragments for 20 min on ice, prior to incubation with the ternary complexes. The ternary complexes were collected by centrifugation and resuspended, and termination was then assessed after elongation in the presence of 1 mM UTP, 1 mM CTP, 1 mM GTP, and 1 mM TTP and the presence of T7 DNA polymerase. The addition of the nascent RNA chains beyond the arrest site at G21 depended on removal of the blocking 3'OmeGMP moiety by the hydrolytic activity intrinsic to the vaccinia RNA polymerase elongation complex (28). Elongation of the transcript beyond the G21 position, in the presence of all four NTPs, would yield a transcript of about 177 bases in length. Greater than 90% of the isolated ternary complexes were routinely elongated in the second RNA synthesis reaction. Signal-dependent termination would be expected to produce a family of RNA products about 70 bases in length. Termination efficiency was calculated as the molar ratio of terminated RNA to the sum of read-through and terminated RNA.

Plasmids—pCITE-4a-H4L plasmid containing full-length H4L was constructed by inserting an Ncol-SalI DNA fragment derived from pCITE-4a-H4L (obtained from Dr. Stewart Shuman), containing the coding sequence of H4L, into the Ncol-SalI digested pCITE-4a. H4L retains a 5' HIan tag encoded in pET-14a. pCITE-4a-H4L1–195 was constructed by digesting the pCITE-4a-H4L plasmid with AccI restriction enzyme and religation of the digested construct. pET-30a-H4L1–195 was constructed by excising the DNA fragment corresponding to amino acids 1–195 from pCITE-4a-H4L1–195 construct, using Ncol and Xhol restriction enzymes, and inserting it into pET-30a (22).

In Vitro Transcription/Translation—Novagen Single Tube Protein system 3 (STP3) was used for the in vitro synthesis of 35S-labeled proteins directly from DNA templates containing T7 RNA polymerase promoter. The DNA template (typically 0.5 μg) was transcribed in 10 μL at 30 °C for 15 min, followed by the addition of 40 μL of translation mix, and incubated for another 60–90 min. pCITE-4a-derived recombinant plasmid was used.

RESULTS

Characterization of Anti-H4L Antibodies—A recent report by Mohamed and Niles (22) showed that the NH2-terminal end of...
Antibodies Directed against the NH2-terminal Region of H4L

The H4L subunit of the virion RNA polymerase can interact with the COOH-terminal end of NPH I, a single-stranded DNA-dependent ATPase. In vivo, monoclonal antibodies were raised in rabbits against MalE fusion proteins containing segments of H4L, representing amino acids 1–256, 258–556 and 568–795 (Fig. 1A). The IgG fraction of each antiserum was isolated by adsorption to Protein A-agarose and subsequently eluted using a low pH elution buffer. The relative affinity of each of these IgG fractions to the H4L protein was examined in both immunoprecipitation and Western blot analyses. Antibodies were resolved on a 12.5% polyacrylamide SDS gel, transferred to a nitrocellulose membrane, and probed with the IgG fractions raised against MalE fusion proteins containing amino acids 1–256 or 568–795. The relative affinity of each of these IgG fractions was determined by immunoprecipitation of 35S-H4L with H4L, H4L1–256 and H4L568–795. For each immunoprecipitation, 5 µl of the IgG fraction (0.7 mg/ml) was employed with 1 µl of the 35S-labeled translation product in a total incubation of 200 µl. Input, 100% of the input radioactivity; I, IgG from immunized rabbit serum; PI, preimmune rabbit serum. C, Western blot analysis of wild type vaccinia virus and C50 mutant virus-infected cell extracts using H4L1–256 and H4L568–795. Proteins were resolved on a 12.5% polyacrylamide SDS gel, transferred to a nitrocellulose membrane, and probed with the IgG fractions raised against MalE fusion proteins containing amino acids 1–256 or 568–795 of the H4L subunit of the virion RNA polymerase. H4L, migration position of full-length H4L protein.

Inhibit Early Gene RNA Synthesis—Prior work by Deng and Shuman (11), using an anti-H4L antibodies directed against the NH2-terminal 364 amino acids of H4L, showed that the NH2-terminal region of H4L is required for early gene transcription initiation. In confirmation of these results and in an attempt to evaluate our antibody reagents, the following in vitro analyses were conducted. Transcription-competent extracts were prepared from cells infected with either wild type virus or C50 mutant (lacking NPH I) virus. To test the ability of the virus-infected cell extracts to mediate early gene transcription termination, the pSB24-term plasmid template (21) is shown. Synthesis of a transcript that extends from the initiation site to the end of the G-less cassette would yield a product of about 540 bases in length. Signal-dependent termination would be expected to produce a family of RNA products about 450 bases in length. FL, full-length transcript; Term, terminated transcript; P, promoter; T, terminator. B, transcription was carried out employing a wild type virus-infected cell extract, without or with preincubation with anti-H4L antibodies αH4L1–256, αH4L568–795, and αH4Lγ58–795 (in the presence or absence of the indicated amounts of VTF). C, transcription was carried out employing a C50 mutant virus-infected cell extract, which lacks NPH I activity, without or with preincubation with anti-H4L antibodies αH4L1–256, αH4L568–795, and αH4Lγ58–795 (in the presence or absence of the indicated amounts of VTF and wild type NPH I). The percentage of RNA synthesis and transcription termination (indicated below the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager.

FIG. 2.

Antibodies directed against the NH2-terminal region of H4L inhibit early gene RNA synthesis. A, a diagram of the pSB24-term plasmid template (21) is shown. Synthesis of a transcript that extends from the initiation site to the end of the G-less cassette would yield a product of about 540 bases in length. Signal-dependent termination would be expected to produce a family of RNA products about 450 bases in length. FL, full-length transcript; Term, terminated transcript; P, promoter; T, terminator. B, transcription was carried out employing a wild type virus-infected cell extract, without or with preincubation with anti-H4L antibodies αH4L1–256, αH4L568–795, and αH4Lγ58–795 (in the presence or absence of the indicated amounts of VTF). C, transcription was carried out employing a C50 mutant virus-infected cell extract, which lacks NPH I activity, without or with preincubation with anti-H4L antibodies αH4L1–256, αH4L568–795, and αH4Lγ58–795 (in the presence or absence of the indicated amounts of VTF and wild type NPH I). The percentage of RNA synthesis and transcription termination (indicated below the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager. FL, 540-base full length transcript; Term, 450-base terminated transcript.
anti-H4L antibodies, transcription of pSB24-term was carried out in the presence or absence of exogenously added VTF, and the RNA products were analyzed by gel electrophoresis (Fig. 2B). In the absence of any added factor, a 55% termination efficiency was achieved. Addition of VTF enhanced the level of termination to about 78% without affecting total RNA synthesis. However, preincubation of the extract with antibodies directed against the NH₂-terminal region of H4L (1–256) inhibited total RNA synthesis, whereas preincubation with antibodies directed against other regions of H4L did not diminish total RNA synthesis and had no effect on termination (Fig. 2B). A similar pattern was obtained using C50 mutant virus-infected cell extract, which lacks NPH I (Fig. 2C). As reported previously, synthesis of only the read-through product was seen in the C50 extract (20). Addition of VTF alone did not significantly stimulate termination since NPH I was missing. However, addition of both VTF and NPH I enhanced the level of termination to about 66% (Fig. 2C). Preincubation of this extract with antibodies directed against the NH₂-terminal region of H4L (1–256) inhibited total RNA synthesis, whereas antibodies directed against other regions of H4L did not alter total RNA synthesis. These results are in agreement with that obtained by Deng and Shuman (11), showing that antibodies raised against the NH₂-terminal region of H4L inhibited viral RNA synthesis, further limiting the essential region to the first 256 amino acids. However, one important difference can be observed. Although αH4L1–256 inhibits RNA synthesis in both wild type virus and C50 mutant virus-infected cell extracts, in the C50 extract, termination is inhibited as well (Fig. 2, B and C).

To further evaluate the effect of αH4L1–256 on transcription termination, it was necessary to separate transcription initiation from elongation and termination. To do this, a bead-bound template was employed in the following studies. The prototype G21(TER29)A78 transcription unit (26) consists of a synthetic early promoter fused to a 20-nucleotide G-less cassette, which is flanked by a run of three G residues at positions +21 to +23. A 57-nucleotide A-less cassette was inserted downstream of the G-less cassette and flanked at its 3’ end by a run of four A residues at positions +78 to +81. A termination signal, TTTTTTTTTT, was placed within the A-less cassette, spanning position +29 to +37. Arrows represent the products produced by the various reaction conditions. FL, full-length; P, promoter; Term, termination product. B, ternary complexes containing the G21 transcript were synthesized in a C50 virus-infected cell extract, lacking NPH I. The ternary complexes were then isolated and the nascent transcript was initiated in a C50 mutant virus-infected cell extract, lacking NPH I. The G21 ternary complex with H4L Fragments—

Antibodies Directed against the NH₂-Terminal Region of H4L Inhibit Early Gene Transcription Termination in a C50 Mutant Virus-Infected Cell Extract—In order to evaluate the role of H4L in early gene transcription termination, the effect of the purified antibodies, αH4L1–256 and αH4L568–795, on transcription termination was assessed using the bead-bound template. The template-engaged 32P-labeled G21 RNA product was synthesized employing a C50 mutant virus-infected cell extract, lacking NPH I. The ternary complex containing the 32P-labeled G21 product was washed and preincubated for 30 min on ice in the presence or absence of increasing amounts of IgG antibodies, directed against either H4L 1–256 or H4L 568–795. The ability of the antibody-treated ternary complexes to terminate transcription was then evaluated by an additional incubation in all four NTPs, in the presence or absence of the indicated amounts of VTF and NPH I. The percentage of transcription termination (indicated below the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager. P denotes the migration position of paused transcripts.
Vaccinia Virus Transcription Termination

**FIG. 4. Antibody inhibition of transcription termination.** Ternary complexes containing the G21 transcript were synthesized in a C50 virus-infected cell extract, lacking NPH I. The ternary complexes were isolated and preincubated for 30 min on ice in the presence or absence of increasing amounts of anti-H4L antibodies, αH4L1–256 (lanes 4–6) and αH4L568–795 (lane 7). The G21 ternary complexes were then elongated with all four NTPs, in the presence or absence of the indicated amounts of VTF and NPH I. The percentage of transcription termination (indicated below the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager. FL, full-length; Term, termination product.

Recently, the interaction between the H4L subunit of the virion RNA polymerase and NPH I, a single-stranded DNA-dependent ATPase, was shown to be required for early gene transcription termination and transcript release (22). This interaction was mapped to the NH$_2$-terminal 195 amino acids of H4L. The ability of the H4L 1–195 to prevent the antibody inhibition of transcription termination (Fig. 6, lane 4) preincubated with increasing amounts of H4L fragments 1–256 or 1–195. The preincubated G21 ternary complexes were then elongated with all four NTPs, in the presence or absence of the indicated amounts of VTF and NPH I. In the absence of any added factor, synthesis of only the major read-through product was seen (Fig. 6, lane 1). Addition of VTF alone did not stimulate termination; however, addition of both VTF and NPH I enhanced the level of termination to about 61% (Fig. 6, lanes 2 and 3, respectively). Preincubation of the G21 ternary complex with αH4L1–256 inhibited transcription termination (Fig. 5A, lane 4). Preincubation of αH4L1–256 with H4L 1–256 prevented the antibody inhibition of transcription termination (Fig. 5A, lanes 5–8).

The template-engaged 32P-labeled G21 RNA product was synthesized employing a wild type virus-infected cell extract. The G21 ternary complexes were then incubated for 30 min on ice, in the presence or absence of 250 ng of αH4L1–256. Preincubation with the αH4L568–795, directed against the COOH-terminal region of H4L, was used as a negative control. The preincubated G21 ternary complexes were then elongated with all four NTPs, in the presence or absence of VTF and NPH I. In the absence of any added factor, synthesis of the major read-through product was seen (Fig. 6, lane 1). Addition of VTF enhanced the level of termination to about 61% (Fig. 6, lane 2). Addition of extra NPH I to the isolated ternary complexes had little effect on the level of transcription termination observed (Fig. 6, lane 3). In contrast to the C50 mutant virus-infected cell extract, preincubation of the G21 ternary complexes synthesized in wild type virus-infected cell extract with αH4L1–256 exhibited minor inhibition of transcription termination (Fig. 6, lane 4), which was prevented by addition of NPH I to the ternary complexes (Fig. 6, lane 5). Preincubation with αH4L568–795 had no effect on termination (Fig. 6, lane 6). These results indicate that the presence of NPH I in the ternary complex prevents antibody inhibition of transcription termination. Prior work from this laboratory showed that NPH I is not an integral component of the ternary complex, but rather that a reversible interaction between NPH I and the ternary complex occurs (22). Addition of NPH I to ternary complexes synthesized in wild type virus-infected cell extract had little effect on termination (Fig. 6, lane 3), but NPH I addition prior to the antibody exhibited an additional protection from antibody inhibition of transcription termination (Fig. 6, lane 5). These data indicate that both NPH I and αH4L1–256 compete for the same site(s) on H4L.

**Addition of NPH I to a C50 Mutant Virus-infected Cell Extract, Lacking NPH I, Reduces Antibody Inhibition of Transcription Termination—If NPH I and αH4L1–256 compete for
the same site(s) on H4L, addition of NPH I to a C50 mutant virus-infected cell extract should prevent antibody inhibition of transcription termination. To test this hypothesis, the template-engaged[^13]P-labeled G21 RNA product was synthesized in a wild type virus-infected cell extract. The isolated G21 ternary complexes were preincubated in the presence or absence of NPH I, prior to the antibody addition (lane 5). The G21 ternary complexes were then incubated for 30 min on ice, in the presence or absence of 250 ng of aH4L1–256 (lanes 4 and 5) or aH4L568–795 (lane 6). The preincubated G21 ternary complexes were then elongated with all four NTPs, in the presence or absence of the indicated amounts of VTF and NPH I. The percentage of transcription termination (indicated below) was quantified by scanning the autoradiograph with a PhosphorImager. FL, full-length; Term, termination product.

**DISCUSSION**

Signal-dependent transcription termination is restricted to early poxvirus genes (29), whose transcription is catalyzed by the virion form of RNA polymerase (10). Effective termination of early gene transcription requires the productive interplay of at least four factors: the virion RNA polymerase (21), the signal UUUUUUU in the nascent mRNA (15, 18, 30), VTF, a multifunctional transcription factor and mRNA processing enzyme (16), and the ATP-hydrolyzing enzyme NPH I (19, 20). It is clear that only the form of RNA polymerase that recognizes an early promoter is sensitive to signal-dependent termination (21). The H4L protein, RAP94, is an integral RNA polymerase subunit found only in the virion form of RNA polymerase that
Antibodies directed against the NH2-terminal region of H4L ternary complex prepared in the absence of NPH I with the action between NPH I and H4L to the NH2-terminal 195 amino terminal domain of H4L is required for early gene transcription inhibition of transcription termination. C50 mutant virus-infected cell extract prevents the antibody addition by antibody binding to one or more epitopes in the NH2-terminal 1–256, demonstrating that the inhibition was mediated by antibody to H4L. These data demonstrate that the NH2-terminal domain of H4L, an integral RNA polymerase subunit found in the virion form of RNA polymerase that recognizes and initiates at early gene promoters, is required for termination (20, 27). Therefore, in the terminal complex, NPH I must have access to single-stranded DNA to nascent RNA in the transcription bubble, the most likely source for single-stranded DNA is the free non-template strand in the paused ternary complex. Therefore, the role of H4L in early gene transcription termination could be simply a docking site for NPH I, which would permit NPH I association with the ternary complex, and provide access to the non-template strand when termination occurs. Alternatively, H4L may play an active role in termination yet to be described, in addition to its role in recruiting NPH I. Further genetic and biochemical studies are under way to evaluate the exact role of H4L in termination.

Prior work from this laboratory demonstrated a physical interaction between the COOH-terminal end of NPH I and the NH2-terminal end of the H4L subunit of the virion RNA polymerase (22). This observation correlated with NPH I mutants that failed both to bind to H4L (22) and to support transcription termination (20) and transcript release (22), indicating that the interaction between NPH I and H4L may be required for these processes. The essential interaction of NPH I and H4L provided an explanation for the observed restriction of UUUUUNU-dependent transcription termination to early genes, where only the H4L-containing RNA polymerase would be able to terminate. This interaction also defined H4L as a termination cofactor, recruiting NPH I to the ternary complex. However, this observation conflicted with a prior report (32) indicating that H4L was not required for NPH I-mediated transcription termination in vitro. Therefore, further experiments were needed to resolve this discrepancy and to evaluate the requirement of H4L for early gene transcription termination.

Antibodies directed against H4L were tested for their ability to inhibit transcription termination in vitro. Preincubation of a ternary complex prepared in the absence of NPH I with the antibodies directed against the NH2-terminal region of H4L (1–256) specifically inhibited transcription termination. Inhibition was blocked by preincubation of αH4L1–256 antibodies with H4L 1–195, demonstrating that the inhibition was mediated by antibody binding to one or more epitopes in the NH2-terminal end of H4L. These data demonstrate that the NH2-terminal domain of H4L is required for early gene transcription termination.

Prior results from this laboratory mapped the site of interaction between NPH I and H4L to the NH2-terminal 195 amino acids of H4L (22). Therefore, we investigated whether NPH I and αH4L1–256 compete for the same site(s) on H4L. Preincubation of the G21 ternary complexes synthesized in a wild-type-infected cell extract with αH4L1–256 exhibited reduced inhibition of transcription termination. Furthermore, preincubation of a NPH I minus virus-infected cell extract with NPH I prior to antibody addition, or readdition of NPH I to isolated ternary complexes prepared in the absence of NPH I, prevented antibody inhibition of transcription termination, indicating that NPH I binds to the site on H4L containing the epitopes that bind to the inhibitory antibodies.

One can propose three models to explain the mechanism of antibody inhibition of NPH I binding to H4L. In the simplest and least likely model, the antibody binding site and the NPH I interaction site are the same. Binding is mutually exclusive. In other models, NPH I and the antibody bind to different sites on H4L. In one case, binding of either antibody or NPH I blocks binding of the other protein. Alternatively, binding of one protein to H4L could change the conformation of H4L, weakening the binding of the other protein. In the absence of additional data, direct competition is the favored model. These results demonstrate that inhibition of termination requires antibody binding to one or more epitopes in the NH2-terminal end of H4L between amino acids 1 and 195. These data also confirm the prior conclusion (22) that the NH2-terminal region of H4L, containing amino acids 1–195, is required for transcription termination. The finding that the NH2-terminal domain of H4L, an integral RNA polymerase subunit found only in the virion form of RNA polymerase that recognizes and initiates at early gene promoters (10, 11, 31), is required for termination provides an explanation for the known restriction of transcription termination to early genes (29).

It is known that NPH I must bind single-stranded DNA to reveal a cryptic ATPase activity (33, 34) and that ATP hydrolysis is required for termination (19, 20, 27). Therefore, in the termination complex, NPH I must have access to single-stranded DNA. Since much of the template strand is annealed to nascent RNA in the transcription bubble, the most likely source for single-stranded DNA is the free non-template strand in the paused ternary complex. Therefore, the role of H4L in early gene transcription termination could be simply a docking site for NPH I, which would permit NPH I association with the ternary complex, and provide access to the non-template strand when termination occurs. Alternatively, H4L may play an active role in termination yet to be described, in addition to its role in recruiting NPH I. Further genetic and biochemical studies are under way to evaluate the exact role of H4L in termination.

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