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# Interaction between Nucleoside Triphosphate Phosphohydrolase I and the H4L Subunit of the Viral RNA Polymerase Is Required for Vaccinia Virus Early Gene Transcript Release*

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**Signal-dependent termination is restricted to early poxvirus genes whose transcription is catalyzed by the virion form of RNA polymerase. Two termination factors have been identified. Vaccinia termination factor/capping enzyme is a multifunctional heterodimer that also catalyzes the first three steps of mRNA cap formation and is an essential intermediate gene transcription initiation factor. Nucleoside triphosphate phosphohydrolase I (NPH I) is a single-stranded DNA-dependent ATPase. COOH-terminal deletion mutations of NPH I retain both ATPase and DNA binding activities but are unable either to terminate transcription or to act as dominant negative mutants *in vitro*. One appealing model posits that the COOH-terminal region of NPH I binds to one or more components in the termination complex. In an attempt to identify NPH I-related protein/protein interactions involved in transcription termination, a series of pull-down experiments were done. Among several vaccinia virus proteins tested, the H4L subunit, unique to the virion form of RNA polymerase, was shown to bind glutathione *S*-transferase (GST)-NPH I. To further confirm this interaction in virus-infected cells, we constructed recombinant vaccinia virus, vNPHINGST, that expresses GST-tagged NPH I. The H4L subunit of virion RNA polymerase specifically co-purified with GST-NPH I, consistent with a physical interaction. Through the analysis of a series of $NH_2$- and COOH-terminal truncation mutations of H4L, the NPH I interaction site was localized to the $NH_2$-terminal 195 amino acids of the H4L protein. The H4L binding site on NPH I was mapped to the COOH-terminal region between 457 and 631. Furthermore, COOH-terminal deletion mutations of NPH I failed to bind the $NH_2$-terminal region of H4L, explaining their inability to support transcription termination. The COOH-terminal end of NPH I was also shown to be required for transcript release activity and for dominant negative inhibition of release. The requirement for an essential interaction between NPH I and H4L provides an explanation for the observed restriction of transcription termination to early viral genes.**

Vaccinia virus, the prototypic poxvirus, possesses a double-stranded DNA genome of 191,686 base pairs (1) capable of encoding approximately 200 proteins. Poxviruses replicate within the cytoplasm of the infected cell. Their independence from host cell nuclear functions is aided by a distinctive replication and transcription apparatus encoded by viral genes (for a review, see Ref. 2). Virion enzymes produce mature viral mRNA with eukaryotic features, including a 5′ cap and a 3′ poly(A) tail. Vaccinia virus genes are expressed in a cascade that is divided into three temporal classes: early, intermediate, and late. Despite its complexity, the viral RNA polymerase requires separate and nonoverlapping sets of auxiliary proteins to initiate transcription of each gene class. Transcription of early genes occurs in the cytoplasm within the infecting core structure. The translation products of viral early mRNAs include RNA polymerase subunits and factors needed for intermediate gene transcription, which occurs after the onset of DNA replication. Late transcription follows intermediate and requires the synthesis of transcription factors encoded by viral intermediate genes. Host factors are also employed in both intermediate (3) and late (4, 5) mRNA synthesis.

Initiation of early vaccinia virus transcription requires the early transcription factor VETF (6) and virion RNA polymerase possessing the RNA polymerase-associated protein RAP94, the product of the *H4L* gene (7, 8). Only the virion RNA polymerase molecules containing RAP94 can functionally interact with VETF to transcribe a double-stranded DNA template possessing a viral early promoter (9). Unlike the other subunits of vaccinia virus RNA polymerase, RAP94 is present in submolar amounts and synthesized exclusively late in infection, whereupon it is packaged into nascent virions (7).

Early viral genes are unique in that transcription terminates in a signal- and factor-dependent manner (10–12). 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 (13, 14). Termination requires both the vaccinia termination factor (VTF)[1] (also serves as viral mRNA capping enzyme) (11) and nucleoside triphosphate phosphohydrolase I (NPH I), the product of the *D11L* gene, as the ATPase employed in transcription termination (15, 16). During infection, transcription termination is restricted to early genes. *In vitro*, only RNA polymerase

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[1] The abbreviations used are: VTF, vaccinia termination factor; CE, capping enzyme; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; NPH I, nucleoside triphosphate phosphohydrolase I; vTF7–3, recombinant vaccinia virus expressing T7 RNA polymerase; vNPHINGST, recombinant vaccinia virus expressing GST-tagged NPH I; MPA, mycophenolic acid; m.o.i., multiplicity of infection; ssDNA, single-stranded DNA; WT, wild type; ts, temperature-sensitive.

capable of recognizing early promoters is subject to signal-dependent termination, suggesting that this form of RNA polymerase is uniquely termination-competent (17).

Prior work indicates the presence of a multicomponent virion transcription complex. Broyles and Moss (18) showed that activities corresponding to two enzymes, vaccinia termination factor (VTF/capping enzyme) and nucleoside triphosphate phosphohydrolase I (ssDNA-dependent ATPase; NPH I), partially co-sedimented with the virion RNA polymerase complex that specifically initiates and terminates early gene transcription. Moreover, Zhang *et al*. (19) showed that nascent RAP94-deficient core particles exhibit low or undetectable amounts of the viral RNA polymerase, capping enzyme/termination factor, poly (A) polymerase, DNA-dependent ATPase, RNA helicase, and topoisomerase. The presence of these unpackaged viral enzymes in the cytoplasm indicated that RAP94 is required for targeting a complex of functionally related proteins involved in early gene transcription. Previous work from our laboratory showed that short COOH-terminal deletion mutations of NPH I, which retain ATPase activity, failed to terminate or to inhibit wild type NPH I-dependent transcription termination activity *in vitro* (16). One appealing model proposes that the COOH-terminal end of NPH I binds to one or more components in the termination complex. One likely candidate is RAP94, which is unique to the virion form of RNA polymerase capable of terminating early gene transcription.

In this report, we show that NPH I (D11L) binds to the H4L subunit of virion RNA polymerase (RAP94). The interaction site is mapped to the COOH-terminal 175 amino acids of NPH I and the $NH_2$-terminal 195 amino acids of H4L. Carboxyl-terminal deletion mutations of NPH I that retain both ATPase and DNA binding activities fail to bind H4L. COOH-terminal deletions also fail to mediate transcript release or to inhibit wild type NPH I's transcript release activity. These data provide an explanation for the observation that UUUUUNU-dependent transcription termination is restricted to early genes, whose transcription is catalyzed by the H4L-containing virion RNA polymerase.

## EXPERIMENTAL PROCEDURES

*Cells and Viruses*—Wild type (WT) vaccinia virus strain WR and the temperature-sensitive (ts) mutant virus, C50, (20, 21) were propagated in BSC40 African green monkey cells at 37 °C or the permissive temperature for ts mutants, 31 °C, respectively, as described (20). Crude virus-containing extracts of 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. BSC1 cells (ATCC CCL6) were used for plaque purification and guanine phosphoribosyl transferase selection (22). Propagation of recombinant virus vNPHINGST was carried out in BSC1 cell monolayers at 37 °C, in the presence of mycophenolic acid (MPA; 25 $\mu$g/ml). Recombinant vaccinia virus, vTF7–3 (23), expressing T7 RNA polymerase, was propagated in BSC40 cells at 37 °C.

*Generation of Recombinant Vaccinia Viruses*—BSC40 cells were infected with WT virus at a multiplicity of infection (m.o.i.) of 0.2/cell, at 37 °C. Following incubation for 2 h, the infected cells were transfected with 1.7 $\mu$g of pTM3GST/NPHI mixed with $CaCl_2$ (125 mM final) for 15 min at room temperature. Medium was then added to the infected cells, and incubation was continued at 37 °C. After 4 h, the medium was replaced, and cells were incubated for 2–3 days until they exhibited complete cell killing. The infected cells were then scraped, pelleted, and resuspended in 300 $\mu$l of PBS (170 mM NaCl, 3.35 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$). BSC1 cells were pretreated with MPA (25 $\mu$g/ml), xanthine (0.25 mg/ml), and hypoxanthine (15 $\mu$g/ml) and used for plaque purification and guanine phosphoribosyl transferase selection (22). Recombinant vaccinia virus with a GST tag at the $NH_2$ terminus of the gene encoding NPH I was isolated by plaque purification and named vNPHINGST. Each plaque was subjected to three rounds of purification. A combination of GST and T7 terminator primers was employed in a diagnostic polymerase chain reaction, in order to distinguish between crossing over into thymidine kinase *versus* WT NPH I loci.

*Purification of NPH I from Virus-infected Cells*—Approximately $1 \times 10^7$ BSC1 cells were co-infected with 5 m.o.i./cell of vTF7–3 (23) and vNPHINGST viruses. As a negative control, BSC1 cells were also infected with each of these viruses separately. The infected cells were incubated for 8 h at 37 °C in a regular medium. Following incubation, the regular medium was removed, and the cells were washed with methionine-free medium. The cells were then pulse-labeled for 1 h at 37 °C with 65 $\mu$Ci/ml of [$^{35}$S]methionine in 2.0 ml of methionine-free medium. After the labeling period, the isotope was removed, and the monolayers were washed twice with PBS. The pulse-labeled cells were harvested and lysed by the addition of 1.5 ml of radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 8.0, 0.1% SDS, 140 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 0.025% sodium azide). The lysed cells were frozen at −20 °C, and after thawing, the cell debris was transferred to an Eppendorf microcentrifuge tube. The cell extracts were cleared by centrifugation for 15 min in a microcentrifuge at 4 °C. The supernatants were then incubated with 200 $\mu$l (bed volume) of glutathione-Sepharose resin (Amersham Pharmacia Biotech). Resins were then washed four times in buffer A (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol (w/v), 50 mM NaCl), and the resin-bound proteins were resolved on SDS-PAGE for evaluation.

*Western Blotting Analysis*—Proteins were resolved on a 12.5% polyacrylamide SDS gel, and transferred to a nitrocellulose membrane. The blot was blocked with 3% gelatin and probed with rabbit polyclonal antibodies (diluted 1:1000 in 1% gelatin) raised against a pATH fusion protein containing amino acids 202–631 of NPH I or the $NH_2$-terminal 256 amino acids of H4L subunit of virion RNA polymerase. Following several washes, the blot was incubated with goat anti-rabbit IgG (diluted 1:20,000 in 1% gelatin) conjugated to horseradish peroxidase. The Supersignal West Pico chemiluminescent substrate (Pierce) was then used for the development of immunoblots followed by autoradiography.

*Transcription Extracts*—Extracts of virus-infected cells were prepared by lysolecithin treatment, as described (17). A549 cells were infected with either wild type or ts mutant viruses at an m.o.i. of 15, at 37 or 31 °C, respectively. In the case of the ts mutant virus, after 24 h, the medium was removed and replaced with 40 °C medium containing 100 $\mu$g/ml cycloheximide. After a further 24 h, cells were washed and treated with 250 $\mu$g/ml lysolecithin, and extracts were prepared.

*Transcript Release Assay*—Construction of the $G^{21}$(TER29)$A^{78}$ plasmid containing a vaccinia early promoter was described previously (24). The prototype $G^{21}$(TER29)$A^{78}$ transcription unit 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 21–23. A 57-nucleotide A-less cassette was inserted downstream of the G-less cassette and flanked at its 3′ end by four A residues at positions 78–81. A termination signal, TTTTTTTTT, was placed within the A-less cassette, spanning positions 29–37. The biotinylated 324-base pair DNA template was polymerase chain reaction-amplified employing a 5′ biotin tag on the upstream primer and isolated by preparative agarose gel electrophoresis. The purified DNA fragment was then immobilized to streptavidin-coated magnetic beads (Dynabeads M280; Dynal) as described (25).

The bead-bound (B) template (typically, 100 fmol) was first incubated with 6 $\mu$l of C50 or WT virus-infected cell extracts, in the presence of 1 mM ATP, 4 $\mu$Ci of [$\alpha$-$^{32}$P]CTP (800 Ci/mmol), 0.1 mM UTP, and 0.625 mM 3′-OMeGTP to synthesize the $G^{21}$ transcript. The ternary complex was then isolated, and the nascent transcript was extended through the A-less cassette, in the presence of 1 mM UTP, 1 mM GTP, 4 $\mu$Ci of [$\alpha$-$^{32}$P]CTP, and 1 mM cordycepin triphosphate (3′-dATP) to yield a bead-bound ternary complex containing the $A^{78}$ transcript. Elongation of the nascent chains beyond the arrest site at $G^{21}$ depended on removal of the blocking 3′-OMeGMP moiety by the hydrolytic activity intrinsic to the vaccinia RNA polymerase elongation complex (26). The ternary complexes were collected by centrifugation and resuspended, and transcript release from the paused ternary complex was then assessed (24) in the presence or absence of VTF, WT NPH I, and NPH I COOH-terminal deletion mutations (3′Δ1 and 3′Δ2) or Walker Box B motif-specific mutation of NPH I, M2 (16). After incubation for 10 min at 30 °C, the bound transcript was separated from the free by centrifugation and analyzed by gel electrophoresis. The percentage of RNA released was quantified by scanning the autoradiogram with a PhosphorImager.

*Plasmids*—pGEX 4T1-D11L plasmids containing either full-length or truncated D11L (NPH I) coding sequence were described (16). pET-30a-D11L-(457–631) was constructed by excising the COOH-terminal coding region from pGEX 4T1-D11L by restriction digestion using

*Eco*RV and *Sal*I restriction enzymes and then inserting it into pET-30a vector digested with the same enzymes. pCITE-4a-H4L plasmid containing full-length H4L was constructed by inserting a *Nco*I–*Sal*I DNA fragment derived from pET-14a-H4L (obtained from Dr. Stewart Shuman), containing the coding sequence of H4L, into the *Nco*I–*Sal*I-digested pCITE-4a. A series of H4L truncation mutations in pCITE-4a were constructed by restriction digestion of the original pCITE-4a-H4L construct with *Acc*I, *Bgl*II, *Hinc*II, *Spe*I, or *Msc*I–*Sna*BI restriction enzymes and religation of the digested construct. This gave rise to a series of H4L COOH- and $NH_2$-terminal truncations representing amino acids, 1–195, 1–288, 1–338, 1–577, and 235–795, respectively. pET-30a-H4L-(1–195) was constructed by excising the DNA fragment corresponding to amino acids 1–195 from pCITE-4a-H4L-(1–195) construct, using *Nco*I and *Xho*I restriction enzymes, and inserting it into pET-30a. Plasmid pTM3GST is a derivative of pTM-3 (27), which allows high level of expression of GST-tagged protein. pTM3GST was constructed by insertion of the *Nco*I–*Bam*HI GST-coding sequence, derived from pTM1GST (obtained from Dr. Michael Merchlinsky), into the *Nco*I–*Bam*HI-cleaved pTM-3 vector. The plasmid used to obtain recombinant vaccinia virus expressing GST-NPH I (pTM3GST/NPHI) was constructed by inserting a *Bgl*II–*Sal*I DNA fragment representing full-length NPH I coding sequence into the *Bam*HI–*Sal*I-cleaved pTM3GST.

*Resin Preparation*—Large scale induction of GST fusion of wild type as well as truncation mutations of NPH I was carried out at 20 °C as described previously (28). Cells were collected by centrifugation and stored at −80 °C until used. GST fusions were then purified from an S100 fraction of the induced cells by batchwise affinity to glutathione-Sepharose. In the case of both D11L-(457–631) and H4L-(1–195), the $His_6$-tagged fusions were isolated by batchwise affinity to nickel-agarose. The protein-bound resins were tested by SDS-PAGE, and the volume used was adjusted, whenever required, by dilution with the respective resin to yield equivalent amounts of each protein. Resins were kept as 50% slurry at −20 °C.

*In Vitro Transcription/Translation*—Novagen Single Tube Protein system 3 was used for the *in vitro* synthesis of $^{35}$S-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 continued incubation for 60–90 min. Both pCITE-4a- and pET-30a-derived recombinant plasmids were used.

*In Vitro Protein/Protein Interaction Assay*—The proteins were labeled *in vitro* with [$^{35}$S]methionine by the STP3 *in vitro* translation system, from Novagen. One μl of the translation mix was incubated with 25 μl of glutathione-Sepharose or nickel-charged His-bind resins coupled to 1 μg of the protein of interest at 4 °C overnight in binding buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol (w/v), 50 mM NaCl). After binding, the resin was washed four times, each with 500 μl of binding buffer. In case of nickel-charged His-bind resins, 50 mM imidazole was included in the buffer during the wash step. The washed resins were then boiled in 1× SDS-loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.72 M β-mercaptoethanol) for 5 min and analyzed by SDS-PAGE. The gel was then soaked in 1 M salicylic acid for 30 min and dried, and autoradiography was then performed at −80 °C.

## RESULTS

*Interaction between NPH I and H4L Subunit of Virion RNA Polymerase*—We employed a GST pull-down approach to evaluate possible interactions between NPH I, an early gene transcription termination factor (15, 16), and other components in the transcription termination complex. To this end, glutathione-Sepharose coupled with GST-NPH I was mixed with an $^{35}$S-labeled, *in vitro* synthesized H4L protein. As a negative control, glutathione-Sepharose resin coupled with GST was mixed with an equal amount of $^{35}$S-H4L. The interaction between the two subunits of the heterodimeric poly(A) polymerase, J3R and E1L (29, 30), was employed as a positive control to provide a measure of the efficiency of a strong protein/protein interaction in this assay (Fig. 1*A*, *top*). Compared with a resin-linked GST, GST-NPH I bound to H4L (Fig. 1*A*, *bottom*), showing an interaction between NPH I and the H4L subunit of virion RNA polymerase. In order to confirm this interaction in virus-infected cells, we constructed recombinant vaccinia virus, vNPHINGST, that expresses GST-tagged NPH I, under the control of T7 RNA polymerase. BSC1 cells were co-infected with 5 m.o.i./cell of recombinant vaccinia virus expressing T7 RNA polymerase, vTF7–3 (23), and vNPHINGST virus. As a negative control, BSC1 cells were also infected with each virus separately. The infected cells were lysed in radioimmunoprecipitation assay buffer, the lysates were incubated with glutathione-Sepharose and washed, and the resin-bound proteins were then examined for the presence of both NPH I and H4L by Western blot analysis using chemiluminescent substrate (Fig. 1*B*). The H4L subunit of virion RNA polymerase was detected in the resin-bound proteins prepared from extracts infected with both viruses but not in the single infection controls. Although this test does not prove a direct interaction between NPH I and H4L, this observation is consistent with the GST pull-down results in Fig. 1*A*.

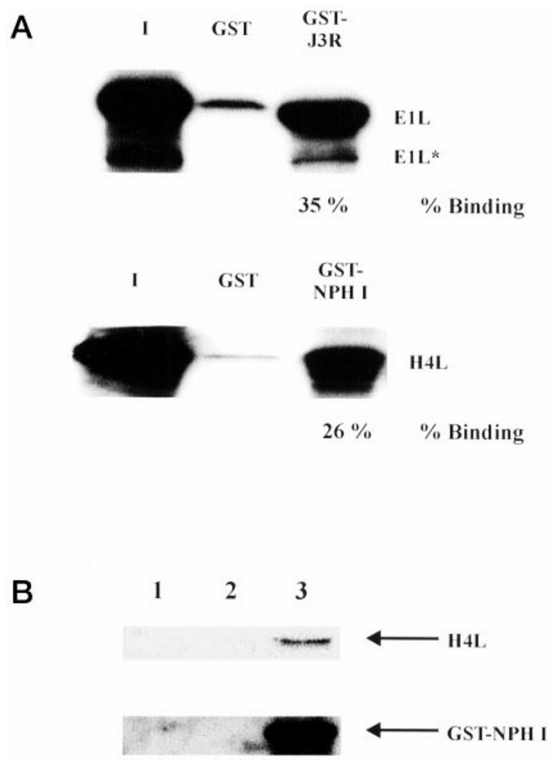


FIG. 1. **GST-NPH I interacts with H4L.** *A*, a coupled transcription/translation system was employed to make $^{35}$S-H4L or $^{35}$S-E1L. GST-NPH I and GST-J3R were purified from the S100 fraction of the induced cells by batchwise affinity to glutathione-Sepharose. One μl of the translation mix was incubated with 25 μl of glutathione-Sepharose resin (50:50 slurry) coupled to 1 μg of GST fusion protein of interest at 4 °C overnight in binding buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol (w/v), 50 mM NaCl). The resin was washed and analyzed by SDS-PAGE followed by x-ray autoradiography. The *top panel* shows the association of the large subunit of poly(A) polymerase, $^{35}$S-E1L, with the small subunit of poly(A) polymerase, GST-J3R, evaluated as a positive control. E1L and E1L* denote the migration position of the large subunit protein and a translation truncation produced *in vitro*. The *bottom panel* shows the association of $^{35}$S-H4L with GST-NPH I. H4L denotes the migration position of the H4L subunit. The percentage binding (indicated *below* the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager. *I*, 50% of the input radioactivity; *GST*, resin-bound GST. *B*, association of H4L with NPH I in virus-infected cells. BSC1 cells were co-infected with 5 m.o.i./cell of vTF7–3 (23) and vNPHINGST virus (*lane 3*). As a negative control, BSC1 cells were also infected with vNPHINGST (*lane 1*) or vTF7–3 (*lane 2*) separately. The infected cells were incubated for 8 h at 37 °C and then pulse-labeled with 65 μCi/ml of [$^{35}$S]methionine for 1 h. The pulse-labeled cells were harvested and lysed in radioimmunoprecipitation assay buffer. The supernatants were incubated with glutathione-Sepharose, the resins were washed, and the resin-bound proteins were examined in Western blot analysis using chemiluminescent substrate. The *top panel* shows Western blotting using antibodies to H4L subunit of virion RNA polymerase, while the *bottom panel* shows Western blotting using antibodies to NPH I.

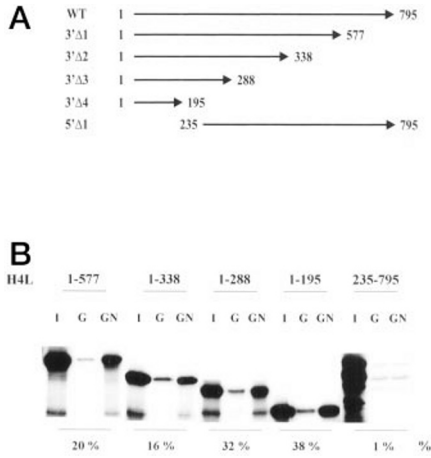


FIG. 2. **NPHI binds to the $NH_2$-terminal end of H4L.** *A*, a series of $NH_2$- and COOH-terminal truncation mutations of H4L was constructed and expressed *in vitro* in a coupled transcription/translation system. *Numbers* denote the H4L amino acids present in each protein fragment. *B*, the association of GST or GST-NPH I with a set of $^{35}$S-H4L truncation mutations was evaluated. Resin-bound protein was separated by gel electrophoresis, and H4L proteins were identified by an autoradiography. A prominent degradation product in each of the H4L COOH-terminal deletions bound to GST-NPH I. The $NH_2$-terminal truncation mutant of H4L-(235–795) failed to bind GST-NPH I. The percentage binding (indicated *below* the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager. *I*, 50% of the input radioactivity; *G*, resin-bound GST; *GN*, resin-bound GST-NPH I.

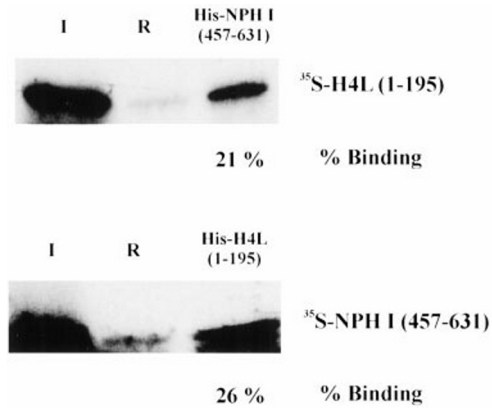


FIG. 3. **COOH-terminal region of NPH I (residues 457–631) binds to the $NH_2$-terminal region of H4L (residues 1–195).** A coupled transcription/translation system was employed to make the $^{35}$S-H4L $NH_2$-terminal fragment (residues 1–195) and $^{35}$S-NPH I COOH-terminal fragment (residues 457–631). Nickel-agarose resin coupled with either $His_6$-tagged NPH I-(residues 457–631) or $His_6$-tagged H4L-(1–195) was prepared and mixed with the respective *in vitro* translation product. 50 mM imidazole was included in the buffer during the wash step. The *top panel* shows the association of $His_6$-NPH I-(457–631) with the $^{35}$S-labeled $NH_2$-terminal region of H4L (residues 1–195). The *bottom panel* shows the binding of $His_6$-H4L-(1–195) to the $^{35}$S-labeled COOH-terminal region of NPH I (residues 457–631). The percentage of binding (indicated *below* the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager. *I*, input radioactivity; *R*, nickel-agarose resin.

*NPH I Binds H4L between Amino Acids 1 and 195*—In an attempt to map the site of interaction of NPH I on H4L, a battery of $NH_2$- and COOH-terminal truncation mutations of H4L was constructed in pCITE-4a (Fig. 2*A*). Each of these truncations was synthesized *in vitro* as $^{35}$S-labeled protein. For each of the COOH-terminal deletion mutations, a prominent degradation product co-exists with the full-length product and co-migrates roughly with the $NH_2$-terminal 195-amino acid fragment (Fig. 2*B*). This degradation product represents the $NH_2$-terminal region, since it is absent from the $NH_2$-terminal deletion mutation (residues 235–795). Each of the $NH_2$- and COOH-terminal truncation mutations of H4L was mixed with GST-NPH I coupled to glutathione-Sepharose. Compared with a GST-negative control, all of the COOH-terminal deletion mutations of H4L, including the smallest $NH_2$-terminal region of H4L (residues 1–195), bound to GST-NPH I (Fig. 2*B*). Furthermore, GST-NPH I was unable to pull down the $NH_2$-terminal deletion mutation of H4L (residues 235–795), limiting the site of interaction of NPH I to the $NH_2$-terminal 195 amino acids of H4L. In accordance with this conclusion, the prominent degradation product in each of the H4L COOH-terminal deletions bound to GST-NPH I. Despite the use of roughly equal amounts of radioactivity tested in each case, it is interesting to note that the COOH-terminal deletions of H4L exhibit a stronger binding to GST-NPH I compared with the longer H4L fragments. This may be due, in part, to the greater accessibility or exposure of the $NH_2$-terminal binding site to NPH I in the $NH_2$-terminal fragment.

*$NH_2$-terminal Region of H4L (Residues 1–195) Binds the COOH-terminal Region of NPH I (Residues 457–631)*—Previous evaluation of NPH I carboxyl-terminal deletion mutations indicated that the carboxyl-terminal end of NPH I may be involved in an interaction with one or more other components of the termination system (16). Based on the above-mentioned results, we tested whether the COOH-terminal region of NPH I interacts with the $NH_2$-terminal 195 amino acids of H4L. To this end, a nickel-agarose resin coupled with $His_6$-tagged NPH-I-(457–631) was mixed with $^{35}$S-H4L-(1–195). Compared with a nickel-agarose-negative control, $His_6$-tagged NPH I-(457–631) was capable of pulling down $^{35}$S-H4L-(1–195) (Fig. 3, *top*). In a confirmation of this result, a $His_6$-tagged H4L-(1–195) was also shown to bind to $^{35}$S-NPH I-(457–631) (Fig. 3, *bottom*). These results demonstrate that the $NH_2$-terminal 195 amino acids of H4L interact with the COOH-terminal region of NPH I (residues 457–631).

*Carboxyl-terminal Deletions of NPH I Fail to Bind H4L*—The ability of each NPH I COOH-terminal deletion mutation to interact with H4L was assessed directly. Each of these truncation mutants, 3′Δ1, 3′Δ2, and 3′Δ3, representing amino acids 1–603, 1–563, and 1–524, respectively, was expressed as GST fusion, coupled to glutathione-Sepharose resin, and mixed with equal amounts of $^{35}$S-H4L-(1–288). GST fusion of wild type NPH I was used as a positive control (Fig. 4, *lane 3*). Compared with a GST-negative control (Fig. 4, *lane 2*), each of the NPH I COOH-terminal deletions failed to bind to the $NH_2$-terminal region of H4L (Fig. 4, *lanes 4–6*). This observation shows that deletion of as few as 28 amino acids from the COOH-terminal end of NPH I abolishes its ability to interact with H4L.

*Carboxyl-terminal Deletions of NPH I Fail to Mediate Transcript Release from an Arrested Ternary Complex*—The ability of NPH I COOH-terminal deletions 3′Δ1 (residues 1–603) and 3′Δ2 (residues 1–563) as well as NPH I's Walker Box B motif specific mutation, M2 (16, 31), to mediate transcript release from bead-bound ternary complexes prepared in C50-infected cell extracts was next evaluated. The prototype $G^{21}$(TER29)$A^{78}$ transcription unit (24) 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 21–23. 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 78–81. A termination signal, TTTTTTTTT, was placed within the A-less cassette, spanning positions 29–37 (Fig. 5*A*). The use of bead-bound DNA template provided a convenient method to assay transcript release by magnetic separation of template-engaged $^{32}$P-labeled RNA products (bead-bound) from released transcripts in the supernatant. The labeled RNAs that had

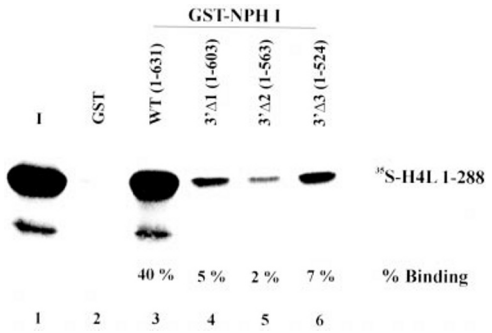


FIG. 4. **COOH-terminal deletion mutations of NPH I fail to bind H4L.** A coupled transcription/translation system was employed to make $^{35}$S-H4L $NH_2$-terminal fragment (residues 1–288). Induction of GST fusion of wild type as well as truncation mutations of NPH I was carried out at 20 °C as described previously (28). *Lanes 2* and *3* represent negative and positive controls, respectively. *Lanes 4–6* represent GST fusion of NPH I COOH-terminal deletions, 3′Δ1, 3′Δ2, and 3′Δ3, possessing amino acids 1–603, 1–563, and 1–524, respectively. The percentage of binding (indicated *below* the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager. *I*, 50% of the input radioactivity.

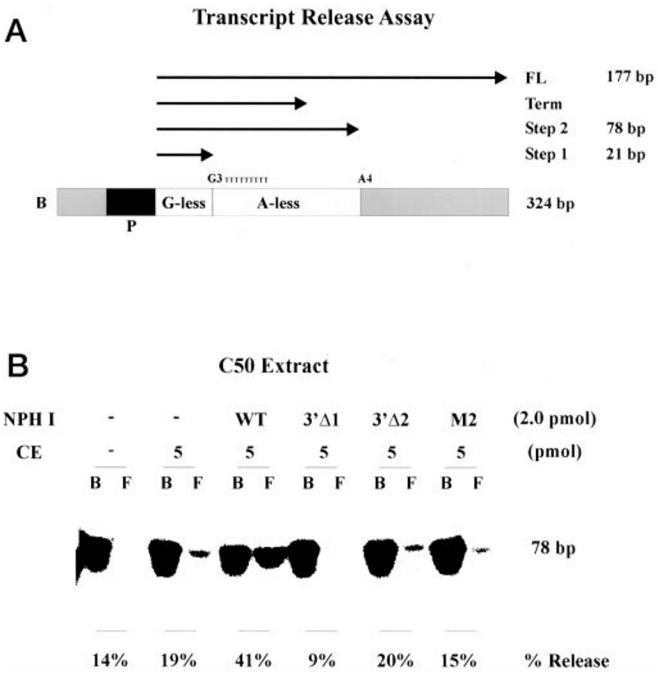


FIG. 5. **COOH-terminal deletion mutations of NPH I fail to mediate transcript release from an arrested ternary complex.** *A*, a map of the bead-bound $G^{21}$(TER29)$A^{78}$ DNA template is shown (24). The DNA template is uniquely biotinylated at the 3′-end of the template strand, which anchors the DNA to streptavidin-coated magnetic beads. The transcription unit 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 21–23. A 57-nucleotide A-less cassette was inserted downstream of the G-less cassette and flanked at its 3′ end by four A residues at positions 78–81. A termination signal, TTTTTTTTT, was placed within the A-less cassette, spanning positions 29–37. The *arrows* represent the products produced by the various reaction conditions. *FL*, full-length; *P*, promoter; *Term*, termination product. *B*, ternary complexes containing the $G^{21}$ transcript were synthesized in a C50 virus-infected cell extract (lacking NPH I), ATP, CTP, UTP, and 3′-OMeGTP. The ternary complexes were then isolated, and the nascent transcript was extended through the A-less cassette, in the presence of UTP, GTP, CTP, and cordycepin triphosphate, to yield a bead-bound ternary complex containing the $A^{78}$ transcript. Transcript release from the paused ternary complex was then assessed in the presence or absence of VTF, WT NPH I, NPH I COOH-terminal deletion mutations (3′Δ1 and 3′Δ2), or the Walker Box B motif-specific mutation of NPH I, M2. The bead-bound $A^{78}$ RNA (*lane B*, *Bound*) was separated from released $A^{78}$ RNA (*lane F*, *Free*) by centrifugation. The transcription products were analyzed by electrophoresis through a 12% polyacrylamide gel containing 8 M urea. The labeled $A^{78}$ transcript was visualized by autoradiography. The percentage of RNA released (indicated *below* the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager.

extended to $A^{78}$ in the C50-infected cell extracts lacking NPH I (16) were recovered in the template-bound fraction and then incubated with 1 mM dATP, VTF/CE, and either wild type or mutant NPH I. Both in the absence of added factors and in the presence of VTF/CE alone, the level of transcript release was minimal (14–19%). The addition of wild type NPH I along with VTF/CE was capable of mediating a significant level of transcript release (41%) from the arrested ternary complex (15) (Fig. 5*B*). Both COOH-terminal deletion mutations 3′Δ1 (residues 1–603) and 3′Δ2 (residues 1–563) as well as NPH I Walker Box B mutation M2 failed to release transcript (Fig. 5*B*), demonstrating that the COOH-terminal region of NPH I is required for the transcript release activity.

*Carboxyl-terminal End of NPH I Is Required for Mutants to Act as Dominant Negatives*—Both NPH I COOH-terminal deletion 3′Δ1 (residues 1–603) and Walker Box B mutant M2 were tested for their ability to inhibit transcript release mediated by wild type NPH I. M2 inhibited wild type NPH I-mediated transcript release activity in a concentration-dependent manner (Fig. 6*A*). In contrast to M2, up to 10 pmol of 3′Δ1 exhibited minimal inhibition of wild type NPH I-mediated transcript release activity (Fig. 6*A*), showing that the dominant negative inhibition of wild type NPH I activity by mutant NPH I requires an intact carboxyl-terminal end.

*NPH I Is Reversibly Bound to the Ternary Complex*—In order to test whether NPH I is an integral or an exchangeable component of the ternary complex, Walker B motif mutation M2 was tested for its ability to act as a dominant negative inhibitor of transcript release mediated by wild type NPH I. The addition of NPH I-M2 inhibits transcript release activity from ternary complexes possessing wild type NPH I in a concentration-dependent manner (Fig. 6*B*). These results indicate that NPH I's association with the ternary complex, via H4L, is reversible and that an intact COOH-terminal end is necessary for such an association. This provides a further support for the notion that the interaction between the COOH-terminal end of NPH I and the $NH_2$-terminal end of H4L is responsible for the recruitment of NPH I to the ternary complex.

## DISCUSSION

Early poxvirus genes are unique in that transcription terminates in a signal- and factor-dependent manner (10–12). Effective termination of early gene transcription requires the productive interplay of at least four factors: the virion RNA polymerase (17); the signal UUUUUNU in the nascent mRNA (13, 14); VTF, a multifunctional transcription factor and mRNA-processing enzyme (11); and the ATP-hydrolyzing enzyme NPH I (15, 16). Christen *et al*. (16) reported that while deletion of up to 68 amino acids from the COOH-terminal end of NPH I exhibited only a modest decrease in ATP hydrolysis and retained the ability to bind DNA, these COOH-terminal deletions failed to support early gene transcription termination *in vitro*. They also showed that deletion of up to 68 amino acids from the COOH-terminal end of NPH I eliminates the mutant's ability to inhibit wild type NPH I-mediated transcription termination activity. This suggests that the COOH-terminal deletions remove a site in NPH I, required for a function in termination other than DNA binding or ATP hydrolysis. One appealing model proposes that the COOH-terminal region of NPH I binds to one or more additional factors required for transcription termination.

Prior results support a direct interaction between NPH I and the virion RNA polymerase. Broyles and Moss (18) showed that

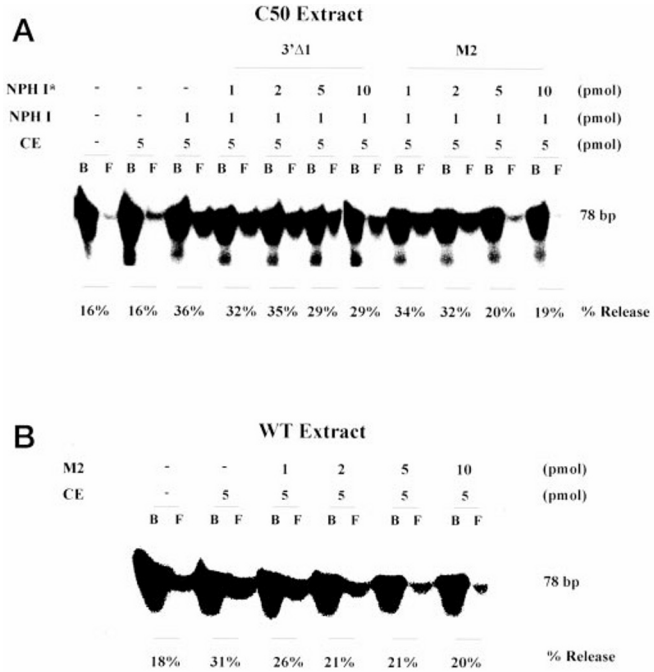


FIG. 6. **COOH-terminal end is required for NPH I mutants to act as dominant negative inhibitors of transcript release.** Transcription extracts were prepared from cells infected with either wild type virus or ts C50 virus possessing a mutation in NPH I. *A*, ternary complexes containing the $A^{78}$ transcript were synthesized using the C50 virus-infected cell extract. Where indicated, the mixtures were supplemented with 5 pmol of recombinant VTF/CE and 1 pmol of recombinant NPH I. Transcript release from the paused ternary complex was then assessed in the presence or absence of increasing concentrations of either NPH I COOH-terminal deletion mutation 3′Δ1 (residues 1–603), or NPH I Walker Box B mutant, M2. The bead-bound $A^{78}$ RNA (*lane B*, *Bound*) was separated from released $A^{78}$ RNA (*lane F*, *Free*) by centrifugation. The transcription products were analyzed by electrophoresis through a 12% polyacrylamide gel containing 8 M urea. The labeled $A^{78}$ transcript was visualized by autoradiography. The percentage of RNA released (indicated *below* the autoradiograph) was quantified by scanning the autoradiogram with a PhosphorImager. *B*, ternary complexes containing the $A^{78}$ transcript were synthesized using the WT virus-infected cell extract. Where indicated, the mixtures were supplemented with 5 pmol of recombinant VTF/CE. Transcript release from the paused ternary complex was then assessed in the presence or absence of increasing concentrations of NPH I Walker Box B mutant, M2. The percentage of RNA released is indicated *below* the autoradiograph.

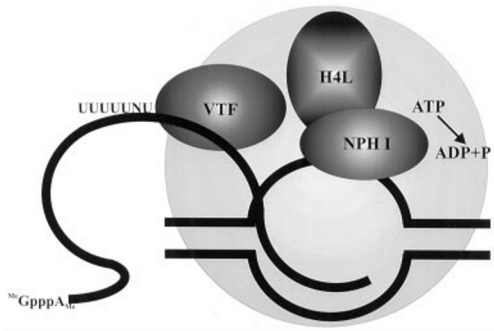


FIG. 7. **A model of the vaccinia virus early gene transcription termination complex.** Termination requires the presence of the sequence UUUUUNU in the nascent mRNA (13, 14). NPH I, a single-stranded DNA-dependent ATPase activity, is employed as an energy-transducing factor (15, 16). NPH I is depicted as binding to the nontemplate strand in the transcription bubble. Only the H4L-containing RNA polymerase is able to terminate (17), where H4L acts as a termination cofactor, recruiting NPH I to the ternary complex. VTF (11), the viral mRNA capping enzyme, is an essential factor whose role in termination is undefined.

activities corresponding to two enzymes, mRNA guanylyltransferase (capping enzyme) and nucleoside triphosphate phosphohydrolase I (DNA-dependent ATPase), partially sedimented with vaccinia virion RNA polymerase complex. Zhang *et al*. (19) demonstrated that targeting of a multicomponent transcription apparatus, including viral RNA polymerase, capping enzyme, NPH I, poly (A) polymerase, topoisomerase, and RNA helicase, into assembling vaccinia virus particles requires RAP94, the H4L subunit of the virion RNA polymerase. Furthermore, Deng and Shuman (15) demonstrated the presence of NPH I in a paused ternary complex.

Several potential NPH I interacting partners were tested, including the virion RNA polymerase subunit H4L (RAP94); the D6R subunit of the early gene transcription initiation factor, VETF; and the two subunits of the known termination factor VTF (capping enzyme), D1R and D12L. Among the proteins tested, the virion RNA polymerase subunit H4L was shown to bind to GST-NPH I. Also, H4L specifically co-purified with GST-tagged NPH I in virus-infected cells, consistent with their physical interaction. Using a series of $NH_2$-terminal and COOH-terminal truncation mutations of H4L, we were able to map the site of interaction of NPH I to the $NH_2$-terminal 195 amino acids of H4L. In addition, we showed that the COOH-terminal region of NPH I (residues 457–631) was able to bind to the $NH_2$-terminal region of H4L (residues 1–195). Moreover, carboxyl-terminal deletions of NPH I, 3′Δ1 (residues 1–603) and 3′Δ2 (residues 1–563), failed to interact with the $NH_2$-terminal region of H4L. The interaction between the $NH_2$-terminal region of H4L and the COOH-terminal region of NPH I defines H4L as a termination cofactor. However, it is not clear if H4L plays an active role in transcription termination or serves simply as a docking site for NPH I. The failure of NPH I COOH-terminal deletions to bind to H4L provides an explanation for the previous observation that NPH I-(1–603) (3′Δ1) and NPH I-(1–563) (3′Δ2) retain both ATPase and single-stranded DNA binding activities, yet they fail to support transcription termination in C50-infected cell extracts (16).

Analysis of transcript release activity of ternary complexes prepared from virus-infected cell extracts lacking NPH I demonstrated that NPH I is a required factor. The addition of GST-NPH I, along with VTF, restored transcript release activity, while the addition of either COOH-terminal deletion mutations 3′Δ1 and 3′Δ2 or Walker Box B motif-specific mutation M2, along with VTF, failed to do so, demonstrating that the intact NPH I COOH-terminal region is required. The requirement for an intact COOH-terminal end of NPH I indicates that a functional interaction between NPH I and H4L is necessary for the final step in the termination pathway. In contrast to M2, the COOH-terminal deletion 3′Δ1 failed to inhibit wild type NPH I-mediated transcript release activity in ternary complexes prepared from both C50 and wild type virus-infected cell extracts. This inhibition must be due to competition between wild type and mutant NPH I proteins for the binding to H4L. Since the M2 mutant GST-NPH I also inhibits transcript release from ternary complexes prepared with wild type virus-infected cell extract, M2 must be able to replace wild type NPH I in the ternary complex. This demonstrates 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 via NPH I's association with H4L.

Studies have indicated that early gene transcription termination occurs about 50 nucleotides downstream of the termination signal UUUUUNU (13). In marked contrast to early genes, the TTTTTNT consensus sequence is frequently found in the coding region of adjacent late genes (32–35). However, at intermediate and late times of infection, the early termination signal is disregarded by the intermediate and late transcription

machinery. It is clear that only the form of RNA polymerase that recognizes an early promoter is sensitive to signal-dependent termination (17). The H4L protein is an integral RNA polymerase subunit found only in the virion form of RNA polymerase that recognizes and initiates at early gene promoters (7, 8, 36). It is also known that NPH I provides the ATPase activity required for termination (15, 16). Therefore, the essential interaction of NPH I and H4L provides an explanation for the observed restriction of transcription termination to early genes, where only the H4L-containing RNA polymerase would be able to terminate.

It is important to point out prior results reported by Deng and Shuman (37) indicating that H4L is not required for NPH I-mediated transcription termination *in vitro*. They employed heparin to strip components of the ternary complex. The stripped complexes exhibited increased mobility in gel electrophoresis, lost the ability to terminate *in vitro*, and failed to bind H4L polyclonal antibody. Upon the addition of NPH I to the heparin-treated complexes, termination was restored. They interpreted the inability of the H4L antibody to supershift the ternary complex as a demonstration that the H4L subunit was removed from the complex. Thus, they concluded that H4L was unnecessary for NPH I-mediated termination. However, there was no direct measurement of H4L either in the heparin wash or in the stripped ternary complex. Since heparin was present during the supershift analysis, heparin could have prevented antibody binding to H4L explaining the loss of a supershift. Alternatively, heparin treatment might render the stripped complex H4L-independent by providing a means of NPH I association with the ternary complex.

A model can now be proposed in which H4L acts as a termination cofactor, recruiting NPH I to the ternary complex (Fig. 7). It is known that NPH I must bind single-stranded DNA to stimulate ATPase activity (38, 39) and that ATP hydrolysis is required for termination (15, 16, 25). Therefore, in the termination complex, NPH I must have access to ssDNA. Since much of the template strand is annealed to nascent RNA, the most likely source for single-stranded DNA is the free nontemplate strand in the paused ternary complex. The possibility that H4L is responsible for recruiting NPH I to the ternary complex permits NPH I to associate with the complex yet have access to the nontemplate strand when termination occurs. The fact that NPH I binds H4L in the absence of any other factors implies that NPH I can be recruited to the ternary complex and participate in transcription termination at any time. However, termination occurs only after the termination complex encounters the UUUUUNU signal in the nascent RNA. This suggests that other factor(s) must modulate NPH I activity in a way that will only allow for ATPase activation to occur at the time of termination. Perhaps, in the elongating transcription complex, NPH I's access to ssDNA is blocked. One possible modulator is RNA, which binds strongly to the ssDNA binding site on NPH I but fails to stimulate ATPase activity (40). Perhaps bound RNA prevents NPH I activation until it is dissociated from NPH I at the termination site. Under any circumstances, at the termination site something must be removed or altered to give NPH I access to ssDNA. A role for VTF/CE has not yet been revealed, but VTF/CE is clearly required for termination and transcript release (11, 24). One appealing scenario proposes that sensing of the termination signal, UUUUUNU, in the nascent mRNA, perhaps via VTF or an other factor, triggers conformational changes providing ssDNA to activate NPH I, resulting in termination and transcript release. According to the proposed model, an interaction between VTF and H4L, NPH I, or UUUUUNU might be expected. In this case, VTF will perhaps act as a modulator or an on/off switch for NPH I activity. Further genetic and biochemical studies are under way to evaluate aspects of this general model and define the role of VTF in termination.

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