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Effect of selected mutations in the C-terminal region of the vaccinia virus nucleoside triphosphate phosphohydrolase I on binding to the H4L subunit of the viral RNA polymerase and early gene transcription termination in vitro

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Abstract

Vaccinia virus nucleoside triphosphate phosphohydrolase I (NPH I) is an essential early gene transcription termination factor. The C-terminal end of NPH I binds to the N-terminal end of the H4L subunit (RAP94) of the virion RNA polymerase. This interaction is required for transcription termination and transcript release. To refine our understanding of the specific amino acids in the C-terminal end of NPH I involved in binding to H4L, and to develop a collection of mutations exhibiting various degrees of activity to be employed in in vivo studies, we prepared a set of short deletions, and clustered substitutions of charged amino acids to alanine, or bulky hydrophobic amino acids to alanine mutations. These NPH I mutant proteins were expressed, purified, and tested for ATPase activity, binding to H4L, and transcription termination activity. Most mutations in amino acids 609 to 631 exhibited reduced activity. Deletion of the terminal five amino acids (627–631), or substitution of Y_{629} with alanine or glutamic acid, dramatically reduced NPH I mediated transcription termination. Deletion of the terminal F_{631}, or substitution of F_{631} with alanine, reduced binding to H4L and eliminated termination activity. These observations demonstrate that the terminal five amino acids directly participate in binding to RNA polymerase and in early gene transcription termination.

Introduction

Vaccinia virus gene expression is divided into three temporal classes (reviewed in Moss, 1990, 2001). Early genes are transcribed in the virion core by a virion encoded multi-subunit RNA polymerase, which is modulated by viral early gene-specific transcription initiation and termination factors. Intermediate mRNA synthesis begins after the onset of DNA replication under the direction of both viral and nuclear proteins. Late gene transcription follows, requiring a different class of viral gene products and nuclear proteins. Only early gene transcription is responsive to signal-dependent transcription termination machinery.

Early gene transcription termination is a complex process, which requires multiple factors. Early viral genes contain the sequence TTTTTNT in the 3′-end area, which is required for termination. TTTTTNT is transcribed yielding the sequence UUUUUNU embedded within 50 bases of the mRNA 3′ end. This sequence serves as an essential signal, which is required for termination (Rohrmann et al., 1986; Yuen and Moss, 1986, 1987). The vaccinia termination factor (VTF) was isolated from virions and shown to be required for UUUUUNU-dependent termination in vitro (Shuman et al., 1987). VTF contains two virus-encoded subunits, the products of genes D1R and D12L (Morgan et

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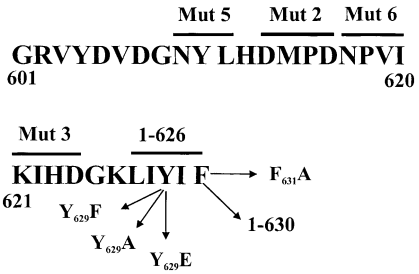

Fig. 1. Location of the mutations employed in this study. In mutations 2, 3, 5, and 6 charged or bulky hydrophobic amino acids were substituted with alanine. 1-626 and 1-630 are deletions of the terminal five amino acids or the terminal F, respectively, Other mutations are single amino acid substitutions, as indicated.

al., 1984; Niles et al., 1989). VTF is a multifunctional protein that also catalyzes the first three steps in mRNA cap formation (Ensinger et al., 1975; Wei and Moss, 1974) and serves as an intermediate gene transcription initiation factor (Vos et al., 1991). Transcription termination also requires energy provided by the hydrolysis of ATP to ADP and Pi (Hagler et al., 1994; Shuman and Moss, 1987). ATPase activity is catalyzed by the viral nucleoside triphosphate phosphohydrolase I (NPH I) (Christen et al., 1998; Deng and Shuman, 1998), the product of gene D11L (Broyles and Moss, 1987; Rodriguez et al., 1986). Single-stranded DNA induces a conformational change in NPH I (Christen et al., 1999), which reveals a cryptic ATPase activity (Paoletti and Moss, 1974). This requirement implies that single-stranded DNA must be available to bind to NPH I at the time of termination. Virions possess two multi-subunit forms of RNA polymerase. One lacks the H4L subunit and is incapable of transcribing early genes (Ahn et al., 1994; Ahn and Moss, 1992; Deng and Shuman, 1994; Kane and Shuman, 1992). The H4L protein is also known as RAP94, the product of gene H4L (Ahn and Moss, 1992). The H4L-containing RNA polymerase transcribes early genes in vitro. The H4L subunit is involved in providing transcription initiation specificity. Furthermore, the N-terminal region of H4L is essential for transcription termination (Mohamed et al., 2002). Deletion of the C-terminal 28 amino acids of NPH I eliminates binding to H4L and transcription termination activity, demonstrating that this interaction is required for transcription termination in vitro (Mohamed and Niles, 2000).

To evaluate the significance of the NPH I/H4L interaction in virus-infected cells, mutant genes must be crossed into the virus and the phenotypes of the mutants must be investigated. To this end, a collection of site-specific mutations in the C-terminal 28 amino acids of NPH I were constructed with the intent of identifying a set of mutations that yielded NPH I proteins possessing a spectrum of activities. The studies herein describe the effect of amino acid substitutions on ATPase activity, H4L binding, and transcription termination efficiency in vitro. Within this collection are mutants that range from wild-type activity to total inhibition.

Results

Expression of wild-type and mutant NPH I in Escherichia coli

A set of short deletions, clustered amino acid substitutions, and single amino acid changes were incorporated into the C-terminal end of genes encoding NPH I (Fig. 1). NPH I C-terminal mutations were constructed in pET30a. Both wild-type and mutant NPH I were expressed in *E. coli* HMS174(DE3) and isolated by sequential chromatography of the S100 fraction on nickel agarose and phosphocellulose (Christen et al., 1999; Deng and Shuman, 1998). SDS denaturing gel electrophoretic analysis revealed a set of highly purified proteins. The wild-type enzyme, along with the Motif2, Motif5 (Christen et al., 1998), and Y629F mutations yielded a single full-length component (Fig. 2). Mutants 2, 3, 5, 6, 1–626, and Y629A exhibited a minor degree of degradation. Mutants Y629E, F631A, 1–630, and the double-mutant Motif 2-NPH I_{1-626} contained two components, a full-length product that ranged from 30 to 60% of the protein and a truncation product that was reduced about 10% in length. Since the shorter protein was isolated by Ni-agarose, the N-terminal his6 tag must be intact and the C-terminal end must be truncated. Prior studies demonstrated that such C-terminal truncations retain ATPase activity yet do not bind to H4L (Mohamed and Niles, 2000) or support transcription termination, indicating that their presence along with the full-length protein will not complicate the evaluation of this latter group of mutations. Further support for this contention is provided by Mutant 2, which exhibits wild-type termination activity and slightly reduced H4L binding. This pattern of truncations of the mutant NPH I proteins was found with multiple preparations of induced enzyme and remains an important caveat for these studies. Single-stranded DNA-dependent ATPase activity was determined for each purified enzyme as a measure of their purity and proper folding (Fig. 3). ATPase activity ranged from about 57 to 105% of the wild-type enzyme. A value of

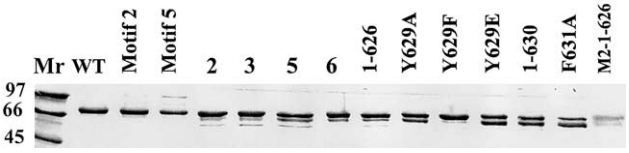

Fig. 2. SDS gel electrophoretic analysis of purified wild-type and mutant NPH I. NPH I was purified from the S100 fraction of induced bacteria by sequential chromatography on Ni-agarose and phosphocellulose. After dialysis into low salt buffer, approximately 2 μg of each sample was denatured, separated in a 12.5% polyacrylamide gel, and stained with Coomassie brilliant blue. M2-1-626 is a Motif 2 mutant that lacks the five C-terminal amino acids. This lane was taken from a different gel.

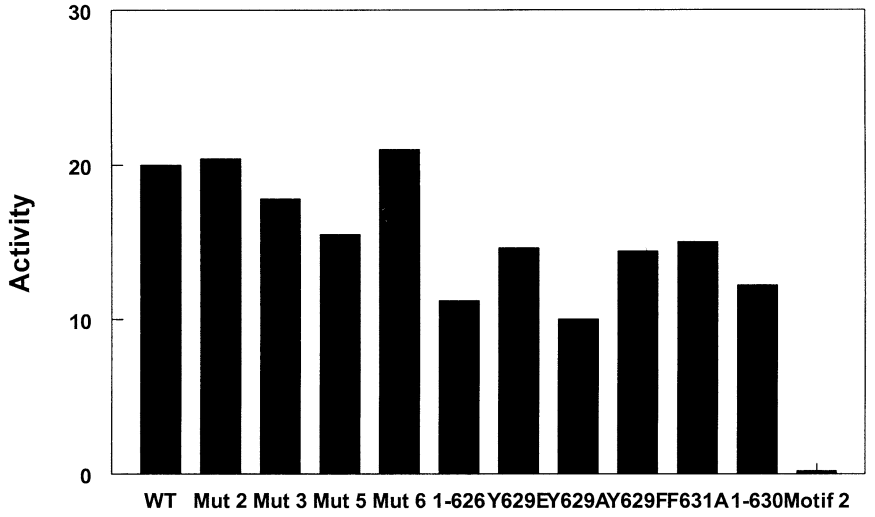

Fig. 3. NPH I ATPase activity. The single-stranded DNA-dependent ATPase activity was measured for each purified protein using the colorimetric assay for free inorganic phosphate (Christen et al., 1998). Assays were done in triplicate. Each bar represents the average of at least two sets of measurements. Activity is described as nmol of inorganic phosphate released in 1 min by 1 pmol of enzyme.

20,000 units of ATPase activity obtained for the wild-type protein agrees well with 16,000 and 10,000 (Christen et al., 1998, 1999; Deng and Shuman, 1998) previously reported.

Effect of C-terminal mutations on transcription termination in vitro

Transcription termination was measured in reactions containing the plasmid template pSBterm (Christen et al., 1998), which has a strong early promoter that drives transcription through a 540 base-pair G-less cassette containing tandem transcription termination signals embedded in the nontemplate strand. Transcription yields a 540-base read-through product that ends when the first G on the template strand is encountered at the end of the G-less cassette. Signal-dependent termination generates a shorter 450-base product. Enzymes were provided by an extract of cells infected with vaccinia virus tsC50 (Condit et al., 1996). Cells were infected under conditions that inactivate the temperature-sensitive NPH I protein, providing an extract that is transcription competent but requires the addition of NPH I to participate in early gene transcription termination (Christen et al., 1998). In addition, extracts possess varying levels of VTF, so that they must be supplemented with purified VTF to restore termination activity (Condit et al., 1996).

The results of a representative set of termination analyses are presented in Fig. 4A, and the average of multiple analyses are presented graphically in Fig. 4B and summarized in Table 1. The background level of radioactivity in the samples that lacked VTF and NPH I yielded a calculated termination efficiency of 10 to 15%, due to the smear in radioactivity, which was not subtracted from the values presented in Fig. 4B. In Table 1, the observed termination efficiency is reported at two NPH I concentrations. Mutations with reduced termination activity can be readily sorted into groups differing in the severity of the mutation based on the stimulation observed over the 10-fold range in NPH I concentration.

In the case of wild-type NPH I, in the absence of NPH I and VTF, only a read-through product is observed (Fig. 4A (top), lane 1 (WT)). Addition of VTF alone had no effect (lane 2). Addition of NPH I alone restores minimal termination activity due to the presence of a low level of VTF activity in the infected cell extract (lane 3). However, addition of VTF and NPH I yields a concentration-dependent restoration of transcription-termination activity (Fig. 4A, lanes 4–7; Fig. 4B). Motif 2 and Motif 5 mutations were shown previously (Christen et al., 1998) to exhibit dramatically reduced transcription termination activity (Fig. 4A, bottom; Fig. 4B). Each mutation lies in a sequence motif that is conserved in Superfamily II helicases and is essential for ATPase activity (Fig. 3). Mutant 2 (Fig. 4A, left) exhibits wild-type termination activity demonstrating that these poorly conserved charged amino acids (Fig. 7) do not play an essential role in this process. Mutants 3 and 5 display reduced termination activity (Fig. 4A and B) at high NPH I concentrations, suggesting at least 10-fold increase in the concentration of NPH I was required for half-termination activity. Mutant 6 displays markedly reduced termination activity that is just above the background level at the highest NPH I concentration tested.

Selected mutations in the C-terminal five amino acids exhibit dramatic reductions in transcription termination in vitro (Fig. 4A and B, Table 1). Deletion of the terminal five amino acids (Fig. 4A, right) completely eliminates termination activity. Substitution of Y_{629} with glutamic acid also destroys termination activity. However, substitution of Y_{629}

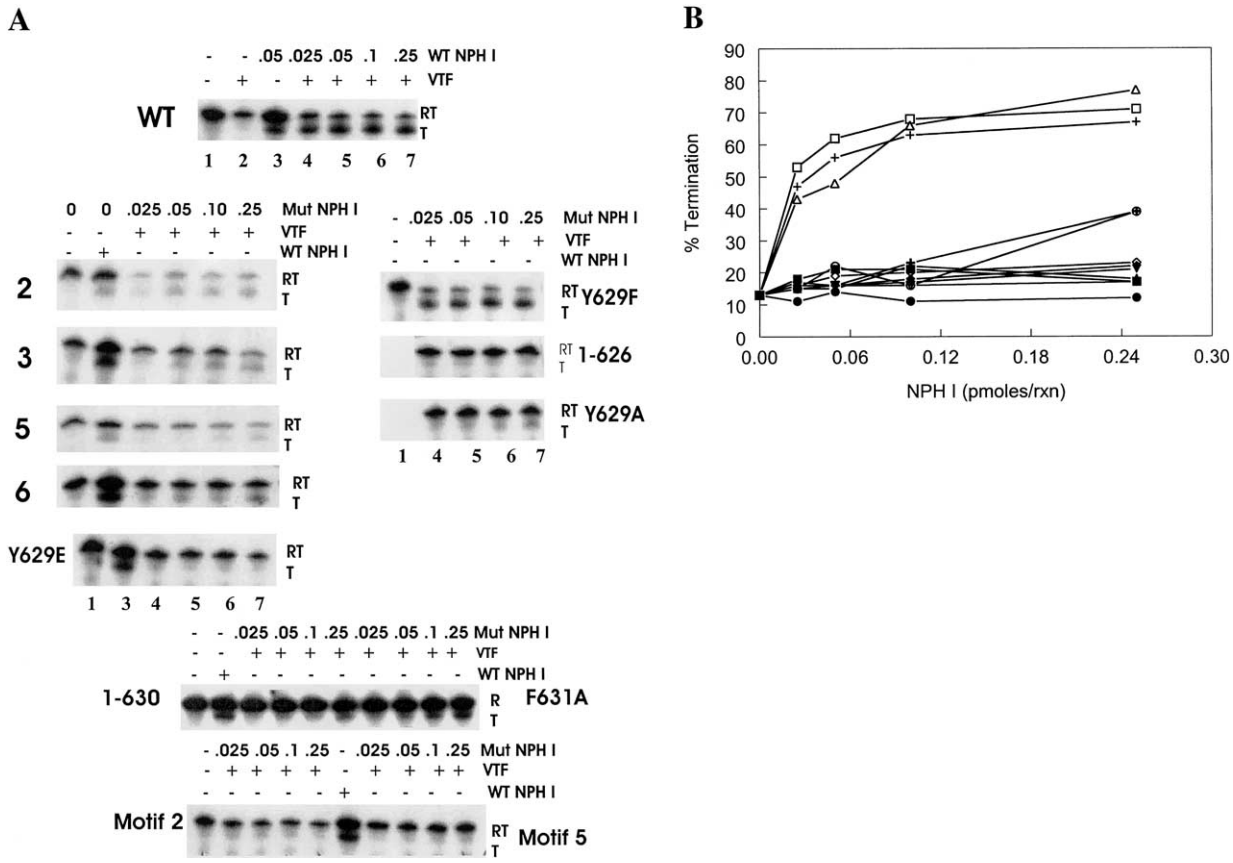

Fig. 4. Transcription termination activity of wild-type and mutant NPH I. (A) This is a composite figure containing the results of a series of transcription termination assays done at different concentrations of wild-type or mutant NPH I. Transcription-competent extract was prepared from tsC50 virus-infected cells, which lack NPH I activity (Christen et al., 1998). Transcription of the plasmid template pSBterm yields a full-length 540 nucleotide read-through (RT) product or a 450-base termination (T) product. Top, WT, wild-type NPH I was tested. Lane 1, no additions. Lane 2, 5 pmol of VTF was added in the absence of NPHI. Lane 3, 0.05 pmol of wild-type NPH I was added in the absence of VTF. The rest of the lanes contain 5 pmol of VTF plus varying levels (pmol) of wild-type NPH I. For the rest of the mutants in the composite, the mutant form of NPH I that was tested is listed alongside the results. In each case, the lane noted 0 had no additions. (B) Graphical depiction of the results obtained by averaging at least three independent termination assays. The background level of radioactivity in the samples that lacked VTF and NPH I yielded a calculated termination efficiency of 10 to 15%, which was not subtracted from the values presented in this figure. (+), WT; (△), Mut 2; (○) Mut 3; (+) Mut 5; (▲) Mut 6; (●) 1-626; (▽), Y629E; (◇) Y629A; (□) Y629F; (▼) 1-630; (♦) F631A; and (■) Motif 2.

with alanine yields a protein with substantially reduced ability to participate in transcription termination (Fig. 4A and B). These observations demonstrate that the terminal five amino acids provide an essential termination function. Importantly, substitution of Y_{629} with F generates an enzyme with full activity, indicating that the hydroxyl group in tyrosine is not involved in NPH I activity.

The terminal F_{631} is conserved in many orthopoxvirus NPH I proteins but it is not found in poxviruses that are distantly related to vaccinia virus (Fig. 7). F is replaced with either one or two amino acids or extended up to five amino acids and terminated with charged residues. Substitution of F_{631} with A (Fig. 4A, bottom) yields an enzyme with substantially reduced termination activity, similar to that observed in the Y_{629}A mutation (Fig. 4A and B). Deletion of F_{631} also reduces the termination activity level, indicating that F_{631} indeed provides an essential function in the poxviruses closely related to vaccinia virus.

Interaction of wild-type and mutant NPH I with the N-terminal end of H4L

Prior studies demonstrated that NPH I amino acids 457 to 631 were required for binding to the N-terminal end of H4L containing amino acids 1–256 (Mohamed and Niles, 2000). Furthermore, deletion of 28 C-terminal amino acids from NPH I generated a protein that lost its capacity to bind to the N-terminal end of H4L. To evaluate the effect of the C-terminal amino acid mutations on H4L binding, a series of pull-down measurements were conducted. Each assay contained amylose resin bound to a fusion protein consisting of the mal E protein fused to $H4L_{1–256}$. C-terminal mutations were introduced into NPH $I_{457–631}$ in pCite 4a and ^{35}S-labeled mutant C-terminal fragments were expressed in a reticulocyte-based transcription/translation cocktail. Subsequent to incubation, resins were washed, and bound proteins were denatured, separated by gel electrophoresis, and observed by fluorogra-

Table 1
Summary of the ATPase activity, transcription termination activity, and H4L binding for a set of NPH I C-terminal mutations

NPH I	ATPase activity[a]	Termination[b] [NPH I]		Pull-down efficiency[c]
		0.025 pmol	0.25 pmol	
Wild-type	20	47	67	26.9
Mutant 2 D613A, D616A	20.4	43	77	14.5
Mutant 3 K621A,D624A,K626A	17.8	15	39	2.3
Mutant 5 N609A,Y610A,L611A	14.4	17	39	10.1
Mutant 6 N617A,P618A,V619A,I620A	21.0	15	18	5.1
1-626 (Δ627–631)	11.2	11	12	1.9
Y629E	14.6	16	17	2.0
Y629A	10.0	16	23	3.0
Y629F	14.4	53	71	21.6
F631A	15.0	16	22	16.3
1-630 (Δ631)	13.2	15	21	7.9
Motif 2	0.4	18	6	ND

Note. ND, not determined.
[a] Nanomole of Pi formed per minute per picomole of NPH I.
[b] Mole percentage of terminated RNA formed.
[c] Percentage of input NPH I bound to malH4$L_{1–195}$ resin.

phy. Binding was compared to input NPH $I_{457–631}$, corrected for trapping by amylose resin containing mal E, and expressed as a percentage of the input protein. Data from a representative experiment are presented in Fig. 5A. The average of multiple pull-down analyses are presented in Fig. 5B and summarized in Table 1. H4$L_{1–256}$ precipitated 28% of the input wild-type ^{35}S NPH $I_{457–631}$ in this experiment, similar to prior observations (Mohamed and Niles, 2000). Mutant 2 also showed binding (54% of wild-type NPH $I_{457–631}$), consistent with its wild-type level of transcription termination (Fig. 4A and B). Mutant 5 exhibited an intermediate level of interaction (38%), which correlates well with its ability to support termination at a reduced level. Deletion of the five terminal amino acids, NPH $I_{1–626}$, or substitution of Y_{629} with glutamic acid, severely reduces binding to less than 10% of the wild-type activity. This would be expected based on the inability of these mutants to support transcription termination (Table 1). Likewise, substitution of Y_{629} with alanine, a mutation that sup-

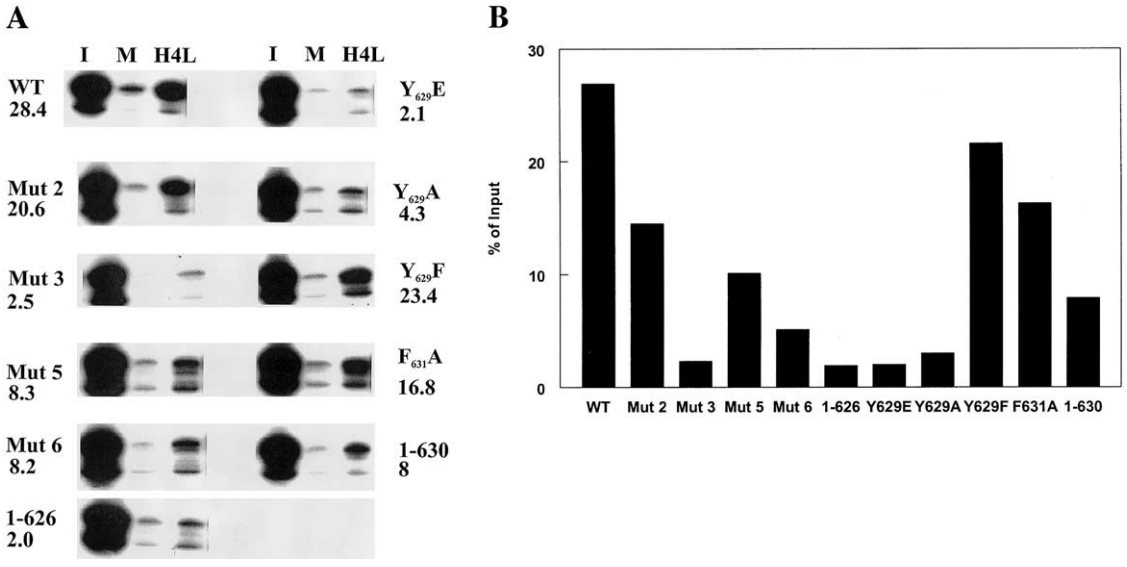

Fig. 5. Interaction of wild-type and mutant NPH $I_{457–631}$ with Mal E-H4$L_{1–256}$. (A) This composite contains the results of a single set of interaction measurements. Amylose resin was prepared that contained bound Mal E-H4$L_{1–256}$. Resin containing about 2 μg of Mal E-H4$L_{1–256}$ was incubated with 2 μl of ^{35}S NPH $I_{457–631}$, prepared by coupled transcription/translation, with rocking overnight at 4°C. After four washes, the resin-bound protein was solubilized in SDS, separated by gel electrophoresis, and observed by fluorography. Samples were quantified by densitometry and after correction for the radioactivity trapped by the Mal E resin control, the percentage of input radioactivity bound was calculated and listed below each mutation. I, 50% of input radioactivity;M, radiolabeled protein trapped by the control Mal E resin; H4L, radiolabeled protein bound to the Mal E-H4$L_{1–256}$ resin. (B) Graphical results of the binding activity for wild-type and mutant NPH $I_{457–631}$ which is the average of at least three independent measurements.

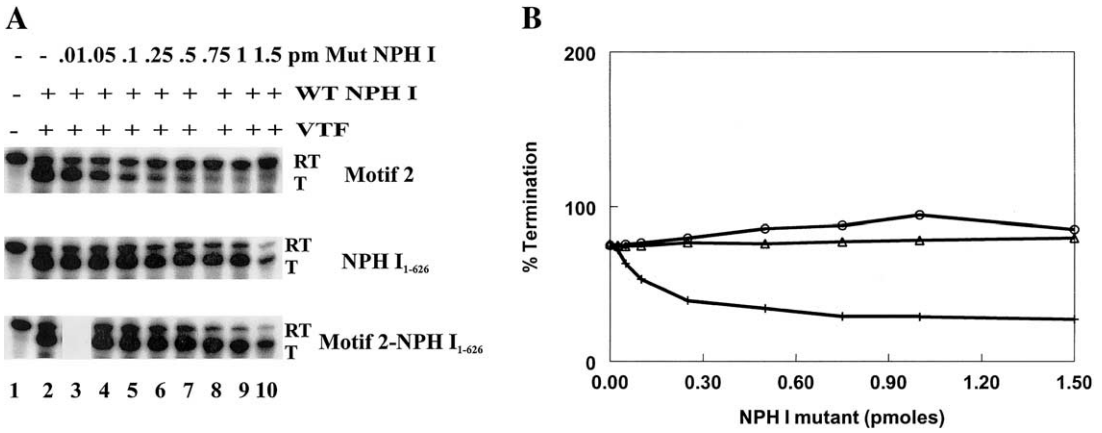

Fig. 6. NPH I_{1-626} is unable to inhibit wild-type NPH I participation in transcription termination in vitro. (A) A series of transcription termination assays was conducted which compared the ability of the Motif 2 mutation, NPH I_{1-626}, and Motif 2-NPH I_{1-626} to inhibit transcription termination in vitro. The Motif 2 mutation lacks ATPase activity and fails to function in termination (Christen et al., 1998). Reactions were setup using an extract of tsC50 virus-infected cells (NPH I-), which lacked additions (lane 1) or possessed 0.05 pmol of wild-type NPH I and 5 pmol of VTF (lane 2). Lanes 3 to 10 contained VTF, wild-type NPH I, and varying amounts of mutant NPH I. (+) Motif 2 mutant NPH I; (△) NPH I_{1-626}; (○) Motif 2-NPH I_{1-626} double mutant. Full-length read-through transcripts (RT) and terminated RNA (T) were observed by autoradiography of gel-separated samples. (B) RNA synthesis was quantified by densitometry of the film. The termination efficiency was calculated as described above and plotted against the concentration of mutant NPH I added to the reaction mixture. The background radioactivity seen in the no addition control was not subtracted in these calculations. These results are the average of two independent determinations.

ports low but measurable termination activity, yields a protein that exhibits poor binding (16%) to $H4L_{1-256}$. A phenylalanine substitution of Y_{629} shows near normal interaction (80%) with $H4L_{1-256}$. This mutation also provides a wild-type level of transcription termination activity.

Four mutations exhibit binding activity that does not correlate with their ability to mediate transcription termination in vitro. Mutant 3 exhibits low but measurable transcription termination activity yet binds poorly (8.6%) to $H4L_{1-256}$. In contrast, Mutant 6 and the deletion of F_{631} yield forms of NPH I that show intermediate binding activity (19 and 29%, respectively) but support transcription termination at only a very low level. Substitution of F_{631} with alanine generates a NPH I derivative that binds well to $H4L_{1-256}$ (61%), yet shows substantially reduced termination activity. Perhaps this latter group of mutants affects NPH I in a function that is required subsequent to $H4L_{1-256}$ binding. Although there is a direct correlation between transcription termination activity and $H4L_{1-256}$ binding for most of the mutants studied, this latter group contains variants that may provide new insights into the role of NPH I in transcription termination.

A NPH I deletion mutant lacking amino acids 627–631 is unable to act as a dominant-negative mutation

Our initial observation related to the importance of the C-terminal end of NPH I in transcription termination was based on the inability of C-terminal truncations to act as dominant-negative mutations in vitro (Christen et al., 1998). To extend this observation, we determined whether the removal of the terminal five amino acids would also yield a protein that was unable to exhibit dominant-negative characteristics. Transcription termination assays were conducted in vitro in the presence of wild-type NPH I and with varying concentrations of either the NPH I Motif 2 mutation, with the C-terminal deletion mutation NPH I_{1-626} or the Motif 2-NPH I_{1-626} double mutant. Consistent with prior studies, addition of the Motif 2 mutation, which lacks ATPase activity but retains an intact C-terminus, prevents transcription termination (Fig. 6A and B) in the presence of the wild-type NPH I. This is due to its ability to compete with wild-type NPH I for binding to H4L (Christen et al., 1998; Mohamed and Niles, 2000). NPH I_{1-626} alone or Motif 2-NPH I_{1-626} exhibits little reduction in transcription termination as expected for mutants that have lost the ability to bind to H4L. Both Motif 2 and NPH I_{1-626} fail to support measurable transcription termination in vitro at the levels employed in these studies.

Discussion

Segments of the NPH I C-terminal amino acid sequence are well-conserved throughout the poxvirus family (Fig. 7). The sequence is identical among the orthopoxvirus group members that are closely related to vaccinia virus, including variola, the causative agent for smallpox. As the comparison is expanded to include other poxviruses, the degree of conservation decreases. It is most notable that the hydrophobic sequence LIYI, amino acids 627–630 in vaccinia NPHI, is highly conserved. To the left, other well-conserved regions can be noted. This study describes the effects of site-specific mutagenesis of the C-terminal 23 amino acids on NPH I function.

Mutant 2 ($D_{613}A$, $D_{616}A$) lies in a poorly conserved

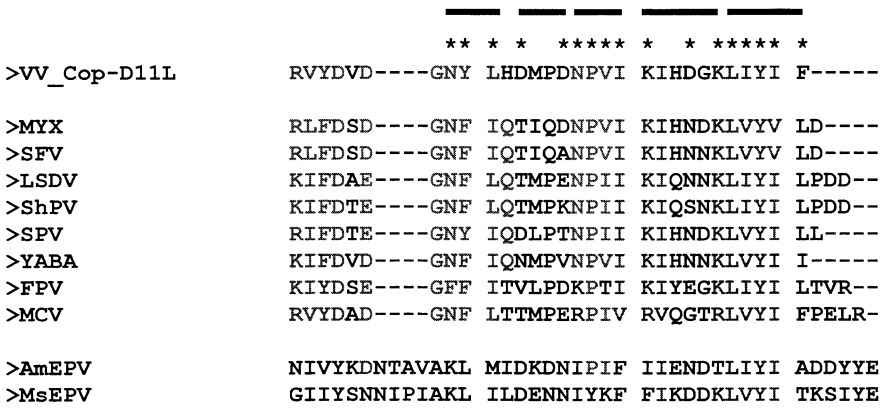

Fig. 7. Comparison of the NPH I C-terminal amino acid sequences. The base sequence of the NPH I gene is known for 21 poxvirus strains. Pileup analysis was conducted at the Poxvirus Bioinformatics Resource Center (www.poxvirus.org) and made available to the public as a Poxvirus Ortholog Cluster. This figure is based on the 30 terminal amino acids of the vaccinia virus Copenhagen strain NPH I protein. Ten orthopoxviruses closely related to vaccinia virus Copenhagen possess the identical terminal sequence. This sequence is represented by the top line. Numbers at the top indicate the position of several of the NPH I mutants described in this report. * Amino acids that were changed. —, amino acids that are present in the insect poxviruses but absent in the other strains. VV_Cop, Copenhagen strain of vaccinia virus; MYX, myxoma; SFV, shope fibroma virus; LSDV, lumpy skin disease virus; ShPV, sheepox virus; SPV, swinepox virus; YABA, yaba-like disease virus; FPV, fowlpox virus; MCV, molluscum contagiosum virus; AmEPV, *Amsacta moreii* entomopoxvirus; MsEPV, *Melanoplus sanguinipes* entomopoxvirus.

sequence. Appropriately, the exchange of two aspartic acid residues for alanine showed little effect on either H4L binding or transcription termination. Mutant 3 ($K_{621}A$, $D_{624}A$, and $K_{626}A$) contains alanine substitutions of three charged amino acids. Two mutations, $K_{621}A$, and $K_{626}A$, change well-conserved lysines. These substitutions yield a protein that shows reduced transcription termination activity and poor binding to H4L. Likewise, mutant 5 ($N_{609}A$, $Y_{610}A$, $L_{611}A$) changes three highly conserved amino acids. This mutation also exhibits reduced termination activity. However, unlike mutant 3, mutant 5 retains significant binding to H4L. Mutant 6 (N617A, P618A, V619A,I620A) substitutes a stretch of bulky hydrophobic amino acids with alanine. This clustered mutation virtually eliminates both termination and binding to H4L. It is not clear whether these amino acids directly interact with H4L amino acid side chains or provide the correct folding of the C-terminal end of NPH I, a sequence required for this interaction.

Deletion of the NPH I terminal five amino acids eliminates transcription termination activity and the interaction with H4L. Substitution of Y_{629} with alanine significantly reduces the ability of NPH I to participate in termination and interact with H4L, demonstrating that Y_{629} provides an essential side chain. Insertion of a glutamic acid residue in place of Y629 eliminates NPH I activity, indicating that the presence of a charged amino acid in this stretch of hydrophobic amino acids is most deleterious. Substitution of Y_{629} with phenylalanine, however, yields a fully functional protein, proving that the hydroxyl group on tyrosine is not needed, and indicates that the tyrosine side chain may be employed in a hydrophobic interaction with H4L.

The terminal phenylalanine is conserved among orthopoxviruses but not among NPH I's encoded by the more distantly related viruses (Fig. 7). Substitution of F_{631} with alanine dramatically reduces termination activity yet retains significant H4L binding. This shows that the F_{631} aromatic ring plays a role in NPH I function. Deletion of F_{631} also reduces NPH I participation in termination yet permits binding to H4L. These observations support two conclusions. First, despite the fact that F_{631} is not conserved through the poxvirus family, this amino acid plays an important role in NPH I function among the orthopoxviruses. One would predict that there is retention of a complimentary sequence in the orthopoxvirus H4L subunit sequence that would accommodate F631 during NPH I binding. Second, mutation of F_{631} provides a protein that lacks termination activity yet retains significant H4L binding. This suggests that these mutations may well effect a function in transcription termination that is required subsequent to binding to H4L. In the distantly related poxviruses, F631 is most often replaced by short extensions of two to five amino acids that contain charged residues. One would predict a coevolution of the NPH I binding site in H4L that would accommodate both the increase in length and the acquisition of charged residues in the C-terminal end of NPH I.

Materials and methods

Cells and viruses

Wild-type vaccinia virus WR and a temperature-sensitive mutation C50 were obtained from Dr. Richard C. Condit. Viruses were propagated at 37 and 31°C, respectively, on BSC 40 cells, as described (Condit and Motyczka, 1981; Condit et al., 1983).

Expression plasmid construction and mutagenesis

The preparation of pET30aD11L and pET30a NPH $I_{457-631}$ was described (Christen et al., 1999; Mohamed and Niles, 2000). Mutagenesis of pET30a NPH $I_{457-631}$ was conducted by a two-step PCR method using the Quickchange Site-Directed Mutagenesis kit from Stratagene. Double-stranded mutant oligonucleotides and flanking oligonucleotides were purchased from Invitrogen. The 5′-flanking oligonucleotide has a *Bam*HI site near its 5′ end and the 3′-flanking oligonucleotide had a *Sal*I site at its 5′ end. The linear double-stranded DNA products were inserted into the pCRIItopo vector from Invitrogen and used to transform *E. coli* XL-1 Blue from Stratagene. Kanamycin-resistant colonies were selected and recombinant plasmids were identified by gel electrophoresis of *Bam*HI and *Sal*I digestion of plasmid DNA. The inserts were sequenced to ensure the identity of each mutation. Full-size pET30a NPH I mutants were constructed by a three-way ligation. The major gene D11L fragment was derived from pGEM3Zf+-D11L by cleavage in a 5′-flanking *Bg*1II site and an internal *Pvu*I site. The C-terminal gene D11L fragment was derived from the pET30aD11$L_{457-631}$ mutant plasmid by cleavage at the internal *Pvu*I site and the *Sa1*I site in the vector. The two fragments were joined and inserted into pET30a that had been digested with *Bam*HI and *Sa1*I. pET30a-Motif 2-NPH I_{1-626} was constructed by swapping *Bam*HI to *Sa1*I fragments from the two parental plasmids. Plasmid structures were confirmed by restriction endonuclease digestion analysis and by determination of the plasmid sequence in the region of each mutation. pCite4a derivatives were constructed by insertion of the *Nco*I to *Sa1*I fragment of each pET30aNPH $I_{457-631}$ mutant into pCite4a cleaved with *Nco*I and *Sa1*I. The DNA sequence in the region of each mutation confirmed their identity.

Expression and purification of NPH I

Wild-type and mutant pET30a NPH I plasmids were transformed into *E. coli* HMS(DE3). NPH I expression was induced by the addition of 100 μM IPTG to mid log phase cells that had been chilled on ice to 20°C. Cells were shaken overnight at 20°C. NPH I was purified from 40 g of induced cell paste by sequential chromatography of the S100 fraction on Ni agarose and phosphocellulose, as described (Christen et al., 1999; Deng and Shuman, 1998) and the pooled fractions were dialyzed against Buffer A: 25 mM Tris–HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, and 10% glycerol. Purity was evaluated by Coomassie brilliant blue staining of proteins separated by SDS–gel electrophoresis. A value of 0.8 A_{280} equals 1 mg/ml of NPH I was used to estimate the concentration of the purified enzyme. Each mutant protein was purified from multiple independent inductions and yielded consistent results.

Determination of ATPase activity

ATPase activity was measured by a standard colorimetric assay, as described (Christen et al., 1999). An amount of 2.5 pmol of enzyme was added to a standard 0.5 ml reaction mix containing 40 mM Tris–HCl pH 8, 1 mM ATP, 1 mM $MgCl_2$, 2 mM DTT, and 200 pg denatured calf thymus DNA. At selected times, 100 μl aliquots were quenched by the addition of 12.5 mM EDTA and the amount of released inorganic phosphate was determined. Moles of Pi released were determined by comparison to a standard curve. Each assay was done in triplicate. Specific activity is expressed as nanomoles of Pi released per minute per picomole of NPH I. The results presented in this article are the average of two independent ATPase assays, each done in triplicate.

In vitro transcription termination assays

Transcription assays were conducted as described previously (Condit et al., 1996). Briefly, assays were carried out in 20 μl reactions containing the following: 20 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM UTP, 1 mM ATP, 20 μM CTP, 4 μCi [α^{32}P]CTP, 0.1 mM 3′ *O*-methyl-GTP, and 0.4 μg pBS_{term} plasmid DNA. RNA polymerase and the required transcription initiation factor VETF were provided by adding 6 μl of a cytoplasmic extract of virus-infected cells. VTF, the vaccinia virus transcription factor (Shuman et al., 1987), was purified as described (Higman et al., 1992) and 5 pmol was added to each reaction. The virus-infected cell extract was prepared from cells infected with tsC50 virus under conditions that inactivated the temperature-sensitive NPH I protein (Christen et al., 1998). Transcription termination requires the addition of NPH I to the reaction mix. To observe transcription termination, 0 to 0.25 pmol of either wild-type or mutant NPH I was added to each reaction. RNA synthesis was conducted at 30°C for 30 min. RNA was isolated, separated by denaturing gel electrophoresis, and evaluated by autoradiography. The intensity of the RNA bands produced was determined by densitometry and the percentage termination was calculated as the mole ratio of the terminated RNA product over the sum of the termination product and the read-through transcript. The assay of each mutant was done from two to four times and the results presented are an average of the total determinations. The background level of radioactivity in the samples that lacked VTF and NPH I yielded a calculated termination efficiency of 10 to 15%.

The ability of termination incompetent mutant NPH I proteins to act as dominant-negative inhibitors of transcription termination was evaluated. Each reaction contained the standard ingredients described above in addition to varying levels of NPH I mutants: Motif 2, which lacks ATPase activity (Christen et al., 1998); the 1–626 mutant that lacks the C-terminal five amino acids, and the Motif 2-NPH I_{1-626} double mutant. RNA synthesis was determined as described above.

NPH I–H4L interaction assays

Specific binding of wild-type and mutant NPH I proteins to the N-terminal region of H4L was measured by pull-down assays. Each reaction was conducted employing a fusion of the *E. coli* mal E protein and $H4L_{1-256}$ bound to amylose resin (Mohamed and Niles, 2000). The amount of resin-bound protein was determined by Coomassie blue staining of SDS gel-separated amylose resin samples. Wild-type and mutant NPH $I_{457-631}$ was radiolabeled with [^{35}S]met using a coupled transcription/translation reaction driven by the pCite 4a NPH I plasmids described above. The Novagen STP3 kit was employed. Each interaction assay was conducted in 350 μl, containing 50 μl resin containing about 2 μg of either mal E or the mal E-$H4L_{1-256}$ fusion, 2 μl of the translation product, in a buffer containing 25 mM Tris–HCl, pH 6.8, 1 mM EDTA, 100 mM NaCl, and 10% glycerol. Tubes were precoated with 0.1% bovine serum albumin and dried before use. Samples were rocked overnight at 4°C, collected by centrifugation, and washed four times with the same buffer containing 0.05% Tween 20. Samples were collected by centrifugation, solubilized by boiling in 20 μl SDS sample buffer, and separated by gel electrophoresis. Radiolabeled proteins were observed by fluorography and quantified by densitometry. Pull-down efficiency was calculated as the percentage of input NPH I in the pellet corrected for protein that was trapped by the mal E control resin. Pull-down results presented are the average of three to six determinations.

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