# Interaction of the Vaccinia Virus Nucleoside Triphosphate Phosphohydrolase I with Linear Oligonucleotides†

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ABSTRACT: Vaccinia virus nucleoside triphosphate phosphohydrolase I (NPH I) serves as the ATPase activity employed in early gene transcription termination [Deng, L., and Shuman, S. (1998) *Genes Dev. 12*, 538−546; Christen, L. M., et al. (1998) *Virology 245*, 360−371]. Since ATPase activity requires binding of single-stranded DNA, a full understanding of the mechanism of oligonucleotide activation is essential for the elucidation of its role in transcription termination. To initiate detailed structure−function studies of NPH I, we undertook combined kinetic and binding analyses of the interaction of linear oligonucleotides with NPH I. In the presence of single-stranded DNA, ATP exhibits complex saturation kinetics. The apparent $K_m$ for ATP is independent of DNA concentration, demonstrating that ssDNA binding alters the $k_{cat}$ for the reaction. Linear ssDNA oligonucleotides from 18 to 48 nucleotides in length stimulated activity in a saturatable fashion. As the oligonucleotide length increases, the $K_{act}$ decreases and the $V_{max}$ increases. The increase in affinity is paralleled by an increase in the level of binding as measured by EMSA. The kinetic activation observed for 36-nucleotide ssDNA is dependent upon ATP concentration. At low ATP levels, sigmoidal saturation kinetics are observed, while at saturating ATP levels, near-hyperbolic kinetics are seen, suggesting that NPH I may adopt two conformational states. Linear oligonucleotides 18, 24, and 36 bases in length bind one, two, and three molecules of NPH I maximally, respectively, indicating that the NPH I binding site is no more than 12 bases in length. In contrast, single-stranded RNA does not stimulate ATPase activity, yet RNA binds as well as DNA of a similar length. Both RNA and DNA can be photo-cross-linked to NPH I by UV light. ssDNA and ssRNA cross-compete in UV photo-cross-linking to NPH I, indicating that both oligonucleotides share a common binding site. ssRNA prevents ssDNA activation of ATPase activity, confirming that both oligonucleotides bind to the kinetically important oligonucleotide activation site on NPH I. ssDNA inhibits transcription termination in vitro. Inhibition is overcome by adding NPH I, demonstrating that oligonucleotide inhibition is mediated through NPH I.

The poxvirus life cycle is unique in that although poxviruses possess a double-stranded DNA genome, replication takes place in the cytoplasm of infected cells. Vaccinia, the most extensively studied poxvirus, encodes most of the proteins employed in gene transcription and mRNA processing (*1*). Viral gene expression is complex. Vaccinia virus genes are divided into three classes differentiated by the conformation of the template used and the time of transcription (*2*). Early genes are transcribed in the virus core immediately upon infection, by a virion-specific form of RNA polymerase (*3*). After 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.

Signal-dependent transcription termination is restricted to early genes (*4*). Termination requires the sequence TTTTTNT on the nontemplate strand (*5*, *6*) which is transcribed into the signal UUUUUNU in the nascent transcript (*7*). The virion form of RNA polymerase capable of initiating at early promoters is responsive to signal-dependent termination (*8*). A virion-encoded factor VTF (vaccinia termination factor) is required (*9*). VTF is a multifunctional protein corresponding to the viral mRNA capping enzyme. Energy provided by the hydrolysis of ATP or dATP is coupled to the termination event (*9*, *10*). Termination-specific ATP hydrolysis is catalyzed by nucleoside triphosphate phosphohydrolase I (NPH I) (*11*, *12*), the product of gene D11L (*13*, *14*). NPH I was purified from virions and shown to be a monomer with a molecular mass of 72 kDa (*11*, *16*) and to catalyze single-stranded DNA-dependent hydrolysis of ATP (*15*, *16*). Initial kinetic characterization demonstrated that NPH I exhibited a requirement for single-stranded DNA that could not be fulfilled with either RNA or duplex structures. NPH I also exhibited a marked preference for adenine nucleoside triphosphates and could employ Mg, Mn, or Ca as divalent cations (*16*).

The location of temperature-sensitive mutations in gene D11L demonstrates that NPH I is essential for virus

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replication (*17*−*19*). Phenotypic characterization of the ts mutations indicates that at the nonpermissive temperature virus intermediate and late gene transcription is impaired (*20*). Notable decreases in the levels of both early gene transcription and DNA replication can be observed in some cell lines (*21*, *22*). These pleiotropic effects observed in the NPH I ts mutations may indicate that NPH I serves multiple functions in the viral life cycle. In vitro transcription analyses show that NPH I facilitates elongation through a T-rich sequence, at low UTP levels (*11*). It is not known if ATP hydrolysis is required for this elongation enhancement activity of NPH I.

To elucidate the mechanism of early gene transcription termination, the role of NPH I in the process must be defined. Since ATPase activity is essential for transcription termination, and binding to single-stranded DNA is required for ATPase activity, a detailed understanding of the mechanism of ATPase activation by DNA is desirable. In this report, we extend prior studies (*15*, *16*) and describe the activation potential of oligonucleotides differing in length. In addition, evidence is presented for the existence of multiple states of NPH I, a resting inactive form and single-stranded DNA-dependent active conformations. Although RNA is capable of binding to the DNA activation site, it is incapable of inducing the active conformation. Finally, we demonstrate that single-stranded DNA is capable of serving as a potent inhibitor of termination in vitro through its interaction with NPH I.

## EXPERIMENTAL PROCEDURES

*Expression and Isolation of NPH I.* The NPH I coding sequence was cloned into pGEX-4T1, and after expression in *Escherichia coli* TB-1, GST−NPH I was isolated as described by affinity chromatography on glutathione Sepharose, followed by heparin agarose (*12*). The NPH I coding sequence was also inserted into pET30a, and a $His_6$-tagged version was isolated by chromatography on Ni agarose, phosphocellulose, and Sephacryl S100 HR as described by Deng and Shuman (*11*). Electrophoretically homogeneous enzyme was obtained. The enzyme is stable indefinitely while stored at −80 °C. The absorbency at 280 nm of a 1 mg/mL solution of GST−NPH I or $His_6$−NPH I was estimated to be approximately 0.8, on the basis of the amino acid composition of the protein.

*ATPase Activity Measurements.* ATPase activity was measured by a colorimetric assay as described by Myette and Niles (*23*). Standard assays were carried out in duplicate in 100 $\mu$L reaction mixtures containing 1 mM ATP, 1 mM $MgCl_2$, 40 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, and 5 $\mu$g of single-stranded calf thymus DNA. The rate of product formation is linear with time for at least 10 min at enzyme concentrations of up to 0.5 pmol per reaction. For analysis of the activation capacity of a series of defined oligonucleotides, calf thymus DNA was replaced with varying concentrations of individual oligonucleotides. In competition studies, activity was measured at a single concentration of a 36-base single-stranded DNA, DNA36, in the presence of increasing levels of RNA30. Assays were conducted for 10 min at 37 °C unless otherwise indicated.

*Preparation of Oligonucleotides.* The kinetic activity of a family of oligonucleotides of mixed sequence, varying in length from 15 to 48 bases, was tested (Figure 1A). Oligonucleotides were prepared commercially, and purified by preparative gel electrophoresis in 10% polyacrylamide gels containing 8 M urea. The positions of the oligonucleotide bands were determined by shadowing, and the oligonucleotides were eluted from the crushed gel fragments overnight at 37 °C essentially as described previously (*23*). The oligonucleotide-containing solutions were extracted with phenol/chloroform (1:1) and precipitated with 3 volumes of 95% ethanol. After resuspension, the oligonucleotides were dialyzed overnight to remove salt. The purity of the oligonucleotides was determined by gel electrophoresis after end labeling with polynucleotide kinase and [$\gamma$-$^{32}$P]ATP (Figure 1B). $VGF_{30}$ RNA was synthesized commercially and purified as described above. $VGF_{30}$ corresponds in sequence to the 3′-end of the vaccinia growth factor mRNA and is often employed as a primer for poly A polymerase assays (*24*). In this study, $VGF_{30}$ serves simply as a ribooligonucleotide of mixed sequence and defined length.

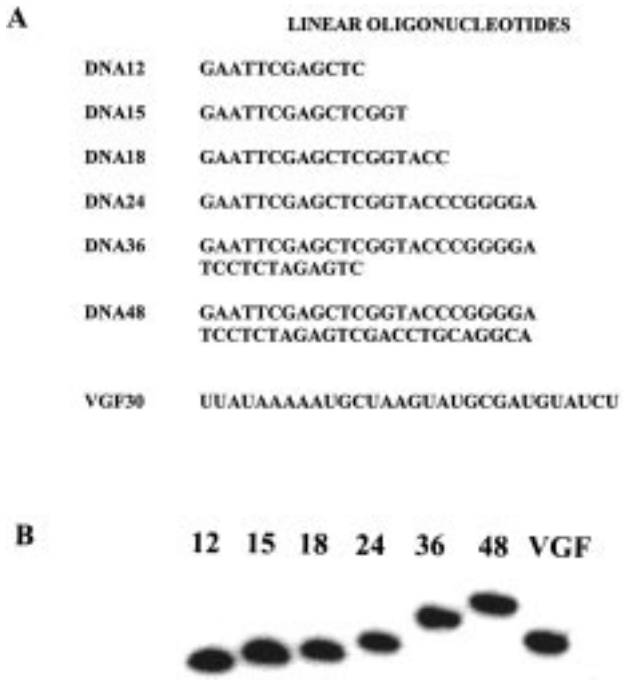



FIGURE 1: Sequences of oligonucleotides employed in these studies. (A) A series of oligonucleotides of mixed sequence, capable of limited secondary structure and derived from the polylinker region of standard cloning plasmids, were synthesized. VGF30 corresponds to the 3′-terminal sequence of a viral mRNA (*23*). (B) The 5′-end-labeled oligonucleotides were separated by urea−polyacrylamide gel electrophoresis, and their migration positions were determined by autoradiography.

*Oligonucleotide Binding to NPH I.* The extent of binding of 5′-end-labeled oligonucleotides to $His_6$−NPH I was determined by electrophoretic mobility shift analysis as described previously (*12*). The 5′-end-labeled oligonucleotides were heated and quick cooled just prior to use. Protein was incubated in a 20 $\mu$L total volume containing 50 mM Tris (pH 8.0), 10 mM mercaptoethanol, 10% glycerol, and 10 or 20 fmol of oligonucleotide on ice for 5 min prior to separation at 4 °C. The positions of the bound and free oligonucleotides were determined by phosphorimage analysis. The half-saturation value, $S_{0.5}$, for each oligonucleotide was determined from a plot of the amount of free oligonucleotide remaining at each protein concentration.

*Transcription Termination Analysis.* Early gene transcription termination was assayed in 20 $\mu$L reaction mixtures containing 1 mM ATP, 0.1 mM UTP, 20 $\mu$M CTP, 4 $\mu$Ci of [$^{32}$P]CTP, 100 $\mu$M 3′-*O*-methyl-GTP, 0.4 $\mu$g of supercoiled DNA template, 6 mM $MgCl_2$, 2 mM dithiothreitol, 20 mM Tris-HCl (pH 7.4), 8% glycerol, and 5−10 $\mu$L of wild-type virus-infected cell extracts (*12*). The template pSBterm contains a G-less cassette harboring tandem termination signals downstream from a strong synthetic early promoter. Read-through transcription yields a 540-base RNA product. Termination produces a 450-nucleotide RNA species (*8*). Termination in vitro requires the addition of 0.05 pmol of GST−NPH I or $His_6$−NPH I and 2 pmol of VTF per reaction. VTF was expressed in and purified from recombinant *E. coli* (*25*).

*UV Photo-Cross-Linking.* The 5′-end-labeled single-stranded DNA and RNA were cross-linked to GST−NPH I using a GE 15 W G15T8 germicidal UV lamp. The lamp was positioned 10 cm above a 96-well dish floating on an ice/water bath. The cross-linking reaction mixture contained in a 50 $\mu$L total volume 25−50 pmol of GST−NPH I, 0.1−2 pmol of [$^{32}$P]DNA or -RNA, 25 mM Tris (pH 8.0), 10 mM mercaptoethanol, 1 mM EDTA, and 10% glycerol. The time of irradiation was varied from 5 to 45 min with the maximum level of photolinkage observed at 30 min. The extent of cross-linking was dependent upon the concentration of both protein and oligonucleotide. Cross-linking to GST-NPH I was equivalent to $his_6$−NPH I. Competition was evaluated by adding increasing concentrations of unlabeled RNA or DNA to the cross-linking reaction. After separation by SDS gel electrophoresis and staining with Coomassie brilliant blue, the bound radioactivity in the dried gel was observed by phosphorimage analysis. The extent of competition was calculated from the phosphorimage results and plotted as a percentage of radioactivity bound at different competitor concentrations. Little UV light-induced damage to the protein was observed after Coomassie blue staining under these conditions.

## RESULTS

*Kinetic Activation of NPH I.* Initial characterization of NPH I demonstrated a requirement for single-stranded DNA for ATPase activity (*15*). Neither single- nor double-stranded RNA, nor double-stranded DNA, was effective. Homopolymers served as poor activators, yet hybrids formed from homopolymers exhibited substantial activity, leading to the proposal that duplexes possessing single-stranded ends were efficacious activators. In these initial studies, oligonucleotides heterogeneous in length were employed. To more precisely define the oligonucleotide requirements for ATPase activation, we conducted a series of kinetic studies. Linear single-stranded oligonucleotides of mixed sequence from 12 to 48 bases in length were commercially synthesized, gel purified (Figure 1A,B), and employed in these experiments.

ATP exhibits complex saturation kinetics with the $V_{max}$ being independent of $Mg^{2+}$ concentration but the apparent $K_m$ being determined by the $Mg^{2+}$ level (Figure 2A). At ATP concentrations greater than the $Mg^{2+}$ concentrations, decreased activity was observed, indicating that free ATP is inhibitory. The apparent $K_m$ for ATP is 0.3 mM at 1 mM $Mg^{2+}$, similar to the value of 0.14 mM reported by Paoletti et al. (*16*). The turnover number (moles of phosphate released per minute per mole of enzyme) is about 8000−10000 with denatured calf thymus DNA as the activator, somewhat less than the value of 16000 reported by Deng and Shuman (*11*). As shown in Figure 2B, the observed $V_{max}$ is dependent upon the concentration of ssDNA but the ATP concentration dependence is not. At each DNA concentration that was tested, activity reached a maximum followed by a decrease at higher ATP levels.

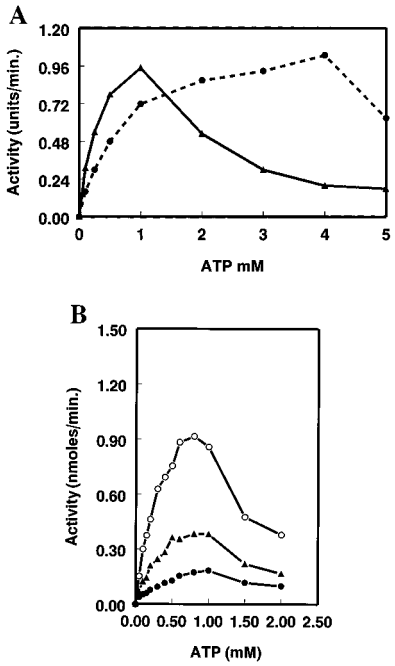



FIGURE 2: ATP saturation kinetics. (A) The initial observed velocity was plotted against the initial concentration of ATP. Reactions were conducted in either 1 mM (▲) or 5 mM (●) $MgCl_2$, 0.3 pmol of enzyme, and 5 ng of 36-base single-stranded DNA. (B) The ATP concentration dependence was measured at three single stranded DNA concentrations: 0.1 (●), 0.25 (▲), and 2.5 ng (○) per assay.

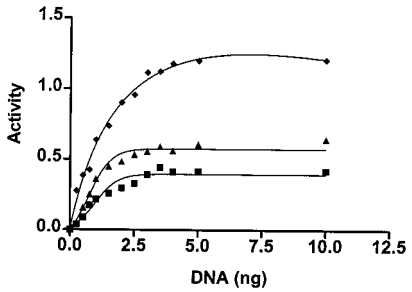



FIGURE 3: Level of activation of NPH I by a 36-base single-stranded DNA measured with 0.3 pmol of NPH I and 1 mM $MgCl_2$ at different initial ATP concentrations: (■) 0.1, (▲) 0.25, and (◆) 1.0 mM. The 0.1 and 0.25 mM ATP data are fitted to a sigmoid curve and the 1.0 mM data to a bireactant enzyme using Prism 2.01. Activity is expressed in nanomoles per minute. The results presented are the average of two independent experiments, each carried out in triplicate.

ATPase activity requires ssDNA. The observed kinetics of ssDNA activation is dependent upon the initial concentration of ATP, at 1 mM $Mg^{2+}$ (Figure 3). At low ATP concentrations, $^1/_2K_m$, or $K_m$, a sigmoidal activation pattern is seen, exhibiting a Hill coefficient of 1.6−1.7. At the optimal ATP concentration (1 mM), however, sigmoidicity is reduced and the Hill coefficient lowered to 1.3. Similar values are obtained at 1 mM ATP and $Mg^{2+}$ for single-

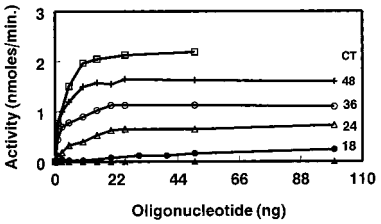



FIGURE 4: Oligonucleotide length dependence for NPH I activation was measured at 0.25 pmol of NPH I, 1 mM ATP, and 1 mM $MgCl_2$. Data obtained for VGF30 are also presented in this figure (▲). Lengths of activating oligonucleotides are listed aside each curve. CT is single-stranded calf thymus DNA.

stranded DNA activators 24 or 48 bases in length. There are two possible explanations for these observations. One, a classic allosteric model, proposes that NPH I exists in multiple forms, a resting inactive form and a ssDNA-dependent active form. ATP alone cannot induce the formation of the active conformation. The transition from an inactive to active form requires ssDNA binding. However, the activation of NPH I by ssDNA is influenced by the ATP level even though ATP alone cannot replace the oligonucleotide activator. A second model posits that there is no preexisting conformational equilibrium. In this model, the observed kinetic behavior is the result of the existence of two kinetic routes for forming the ternary complex (enzyme, ATP, and DNA). In such a random steady-state system, the observed velocity will be dependent upon the pathway employed which in turn will be dependent upon the concentrations of ATP and DNA in the reaction mixture.

The dependence of ATPase activity upon DNA concentration was measured for linear DNAs differing in length from 15 to 48 nucleotides, at 1 mM ATP and $Mg^{2+}$ (Figure 4). A 15-base oligonucleotide was inactive, and little activity is seen for 18-base DNA. For oligonucleotides from 24 bases to 48 bases in length, the observed $V_{max}$ is dependent upon DNA length. The observed $K_{act}$, the concentration of DNA required for half-maximal activation, exhibits a small dependence upon DNA length from 6 ng for 24-base DNA to 1.6 ng for 48-base DNA. Since $V_{max}$ is dependent upon oligonucleotide length, the intrinsic activity of the DNA-bound enzyme must be different for each oligonucleotide. This observation indicates that NPH I is capable of adopting multiple active conformations dependent upon oligonucleotide length, each exhibiting a different $k_{cat}$. However, single-stranded RNA does not activate NPH I at any concentration tested, consistent with prior observations (*15*).

*EMSA Analysis of Oligonucleotide Binding to NPH I.* The relative affinity of each oligonucleotide for NPH I was estimated by EMSA analyses. Binding of 5′-end-labeled oligonucleotides to $His_6$−NPH I was carried out on ice for 15 min, and the bound complexes were separated from free by gel electrophoresis, at 4 °C. The binding observed by this method exhibited dramatic length dependence for linear oligonucleotides (Figure 5). Meaningful binding was not observed for oligonucleotides of less than 15 bases. The apparent affinity increased 20-fold between 15 and 48 oligonucleotides (Table 1). The general pattern of length dependence for ATPase activation mediated by linear oligonucleotides was reflected in the binding analysis (Figure 4). For molecules 15 and 18 bases in length, a single binding species was observed throughout the titration of $His_6$−NPH I, indicating that a single protein binds to each oligonucleotide. For a 24-base oligonucleotide, two species are seen, one at low concentrations, which is replaced by a slower migrating complex at high protein levels. This behavior is consistent with two protein molecules binding maximally at high protein concentrations. For linear molecules 36 or 48 bases in length, at least three binding complexes can be observed (Figure 5), indicating that three or more proteins can bind to each oligonucleotide. The sum of these data suggests that NPH I binds to a site maximally 12 bases in length.

Surprisingly, single-stranded RNA bound with the highest observed affinity (Figure 5 and Table 1). NPH I-bound RNA also exhibits two components indicating that two proteins can bind at high protein levels, consistent with the model of a 12-base binding site. However, RNA is incapable of activating NPH I (Figure 4). Clearly, kinetic activation of NPH I is not simply a reflection of oligonucleotide binding.

*Oligonucleotide Binding Sites.* A comparison of the kinetic activation results to the EMSA binding observations demonstrates that ribooligonucleotides can bind with high affinity to NPH I yet not activate the ATPase active site. One model posits that there are multiple oligonucleotide binding sites on NPH I: one of unknown function binds RNA and the other responsible for kinetic activation binds DNA. An alternative model proposes that both RNA and DNA bind to the same site but only DNA binding is capable of activating the enzyme. To determine whether RNA and DNA share a common binding site, two experiments were conducted.

In the first, UV cross-linking to GST−NPH I of 5′-end-labeled single-stranded 24-base DNA and 30-base RNA was carried out (Figure 6). The extent of cross-linking was dependent upon the time of irradiation and the concentration of NPH I and oligonucleotide. The kinetics of cross-linking was equivalent for GST−NPH I and for $His_6$−NPH I, demonstrating that the GST domain does not influence the cross-linking reaction (data not shown). This is consistent with the lack of an effect of the GST domain on EMSA or catalytic activity (*12*). In a cross-linking reaction mixture containing 20 pmol of enzyme and 2 pmol of 24-base DNA, about 1% of the enzyme molecules were linked to DNA (data not shown). To determine whether both single-stranded RNA and DNA bind to the same site, the ability of each oligonucleotide to reduce the level of UV-induced incorporation of radioactivity was evaluated (Figure 6A,B). The levels of both DNA and RNA cross-linking to NPH I were reduced by both RNA and DNA, respectively, indicating that the site of UV cross-linking is shared by both DNA and RNA. RNA competes with DNA cross-linking better than DNA competes with RNA cross-linking (Figure 6B), which is consistent with the affinity of each determined by EMSA (Figure 5 and Table 1). The simplest interpretation is that there is a single oligonucleotide binding site capable of binding both RNA and DNA. These data do not rule out the possibility that there are multiple sites that bind both RNA and DNA. Furthermore, there is the remote possibility that there are two sites, one that binds only RNA and the other DNA, and that binding to one site prevents binding to the second site.

In the second experiment, the ability of 30-base RNA to inhibit 36-base DNA activation of NPH I was measured. If NPH I possesses a single oligonucleotide binding site and

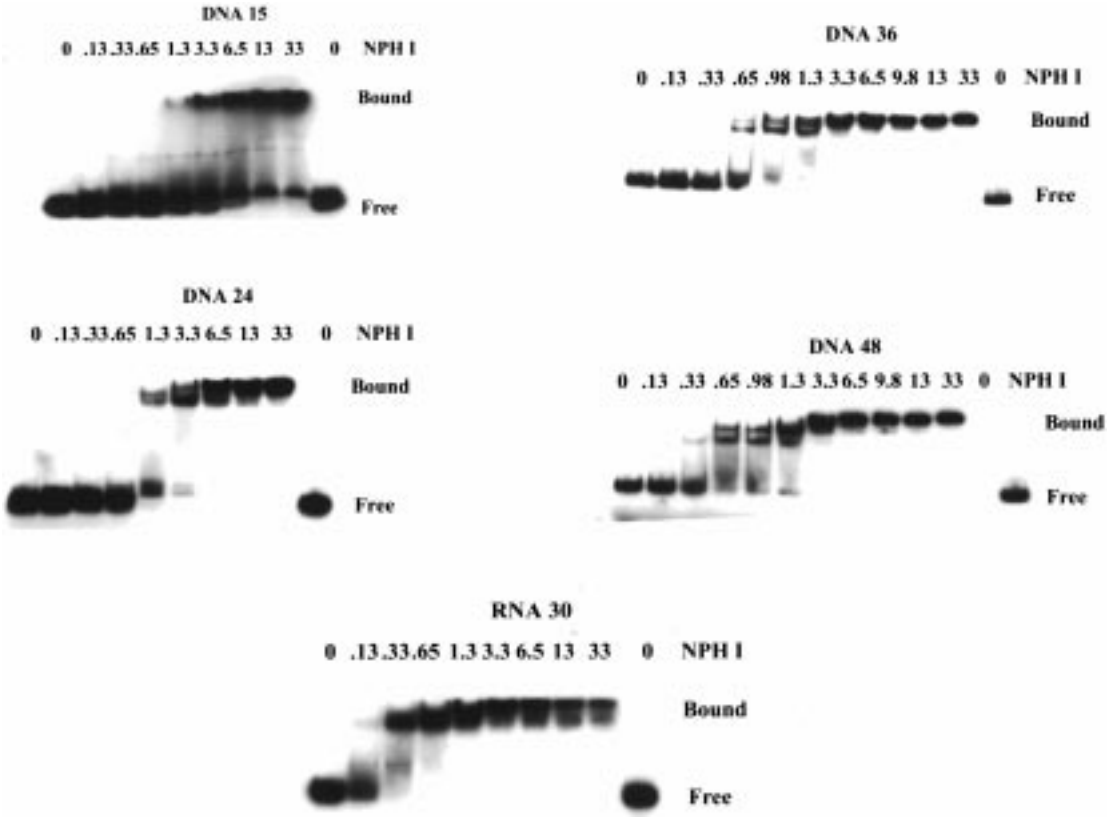



FIGURE 5: EMSA analysis of oligonucleotide binding to NPH I. Reaction mixtures containing 20 fmol of 5′-end-labeled DNA and varying concentrations of NPH I (picomoles) were set up at 0 °C and separated at 4 °C.

Table 1: Summary of Kinetic and Binding Analyses[a]

| oligonucleotide length (bases) | kinetic constants | | | EMSA |
|---|---|---|---|---|
| | $K_{act}$ (ng) | $K_{act}$ (nM) | $V_{max}$ | $S_{0.5}$(NPH I) (nM) |
| 15 | – | | – | 400 |
| 18 | – | | – | 50 |
| 24 | 6 | 18 | 0.7 | 75 |
| 36 | 2 | 6 | 1.1 | 30 |
| 48 | 2 | 6 | 1.6 | 20 |
| CT | 2 | 6 | 2.2 | – |
| VGF30 | – | – | – | 10 |

[a] CT is heat-denatured, quick-cooled calf thymus DNA. VGF30 is 30-nucleotide single-stranded RNA. $K_{act}$ is the concentration of DNA that gives half-maximal activation presented either in nanograms or in base molarity. $S_{0.5}$ is the concentration of $His_6$−NPH I that gives half-maximal oligonucleotide binding. $S_{0.5}$ was measured as total binding and does not refer to binding to a specific DNA species. $V_{max}$ is in nanomoles per minute per 0.25 pmol of enzyme.

RNA binds to the activation site yet does not activate ATPase, RNA should serve as a competitive inhibitor of ssDNA activation. The effect of addition of increasing concentrations of 30-base RNA on 36-base DNA activation was measured at three 36-base DNA concentrations. At each 36-base DNA level, 30-base RNA acted as an inhibitor (Figure 7), achieving complete inhibition at high 30-base RNA levels. These results demonstrate that both RNA and DNA bind to the same site and this site is the kinetically relevant activation site.

*Single-Stranded DNA Inhibits Transcription Termination in Vitro.* NPH I provides the ATPase activity required for transcription termination (*11*, *12*). If binding to single-stranded DNA is required for NPH I to act in termination, the addition of free single-stranded DNA might affect the transcription termination efficiency, in vitro. To assess the

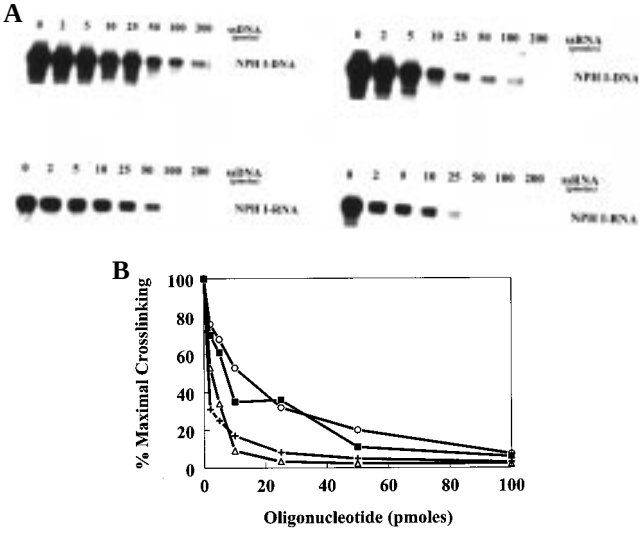



FIGURE 6: Photo-cross-linking of oligonucleotides to NPH I. (A) UV cross-linking of 5′ end labeled 30 base RNA or 24 base DNA was conducted in the absence or the presence of competitor oligonucleotides. (B) The level of incorporation of radioactivity was quantified by phosphorimage analysis. The percentage of maximal incorporation was plotted against the concentration of competitor: (■) 5′-end-labeled DNA and DNA competitor, (△) 5′-end-labeled DNA and RNA competitor, (○) 5′-end-labeled RNA and DNA competitor, and (+) 5′-end-labeled RNA and RNA competitor.

affect of oligonucleotide addition upon transcription termination, in vitro, two assay systems were tested (*8*, *12*). One contained a transcription competent extract generated from wild-type virus-infected cells. This extract contains the wild-type NPH I, presumably associated with the transcription machinery. The other system was derived from the C50 ts

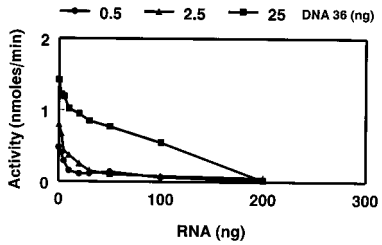



FIGURE 7: Inhibition of single-stranded DNA activation of NPH I by single-stranded RNA. ATPase activity was measured at three concentrations of 36-base single-stranded DNA, (●) 0.5, (▲) 2.5, and (■) 25 ng, in the absence or presence of varying concentrations of competitor single-stranded RNA.

mutant virus harboring a defective NPH I gene. Extracts prepared from cells infected at the nonpermissive temperature with the NPH I ts virus support transcription but lack measurable NPH I-dependent transcription termination activity (*12*). To stimulate transcription termination in these latter extracts, GST−NPH I prepared from induced *E. coli* was added. Both extracts require the addition of the vaccinia termination factor (VTF) for termination activity (*8*, *12*). In each assay, addition of single-stranded DNA preferentially inhibits transcription termination while exhibiting a modest reduction in the overall level of transcription (Figure 8A,B). To determine whether this loss of termination activity is due to inhibition of NPH I, we tested if addition of NPH I would restore termination. In Figure 9A,B, it can be seen that addition of 0.5 pmol of GST−NPH I increases the level of transcription termination to more than 50% of that observed in the absence of added ssDNA. Inhibition of termination by free single-stranded DNA is significantly more pronounced in the NPH I-supplemented ts virus-infected cell extract than in the wild-type virus-infected cell extract. This observation suggests that the ssDNA binding site on NPH I is relatively inaccessible to free DNA in the wild-type virus-infected cell extract. Single-stranded 30-base RNA also inhibits transcription termination in vitro but requires a 2-fold higher concentration (data not shown).

## DISCUSSION

NPH I plays an essential role in vaccinia virus early gene transcription termination (*11*, *12*). Through hydrolysis of ATP, NPH I provides the energy required to mediate release of transcript from the paused ternary complex (*11*). Prior in vitro analyses demonstrated that ATP hydrolysis requires single-stranded DNA, and this requirement cannot be fulfilled by single-stranded RNA (*15*). One pleasing model proposes that the single-stranded DNA requirement for NPH I ATPase activity is fulfilled by interaction with the free nontemplate strand in the paused ternary complex (*12*). To provide an elementary understanding of the mechanism of oligonucleotide activation of NPH I, essential to defining its role in transcript release, we undertook these studies.

Kinetic analyses of substrate saturation demonstrate that like other ATP-utilizing enzymes, NPH I binds to an ATP−$Mg^{2+}$ complex. Free ATP competes with substrate binding, resulting in the observed inhibition at high ATP concentrations. The pattern of ATP saturation is not influenced by the concentration of activator DNA. However, the maximal activity is directly dependent upon the concentration of DNA, demonstrating that $k_{cat}$ and not $K_m$ for ATP is influenced by DNA binding.

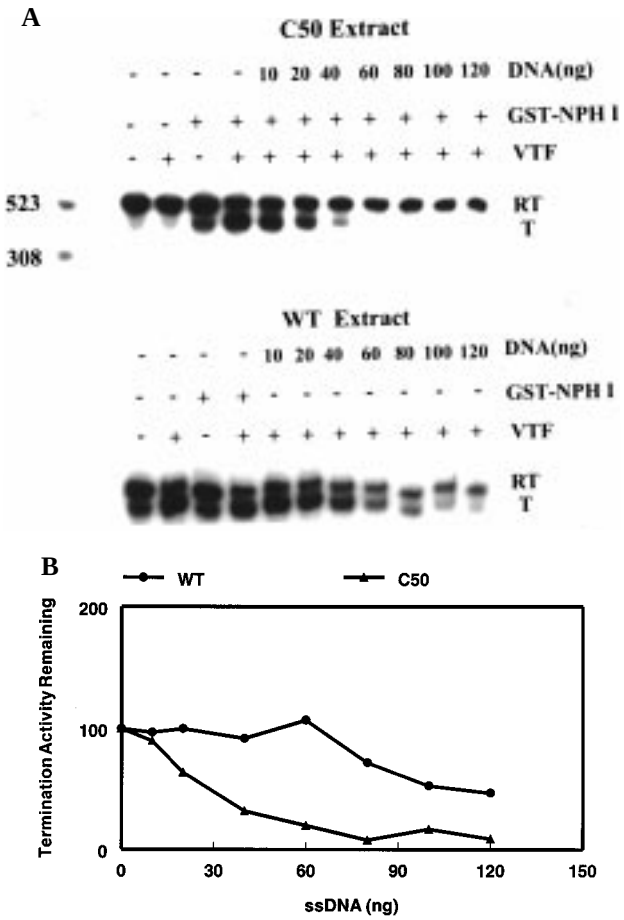



FIGURE 8: Inhibition of transcription termination in vitro by single-stranded DNA. (A) RNA was synthesized in vitro in extracts prepared either from wild-type virus-infected cells supplemented with 2 pmol of VTF or in extracts prepared from ts C50 virus that harbors a ts mutation in the D11L gene. The C50 extract lacks endogenous NPH I activity and must be supplemented with both 2 pmol of VTF and 0.05 pmol of GST−NPH I to observe transcription termination. RNA synthesis was observed by acrylamide gel electrophoresis and quantified by phosphorimage analysis. R is the read-through transcript; T is the termination product. (B) Single-stranded DNA inhibition of transcription termination in wild-type virus-infected cell extract and ts mutant virus-infected cell extract was quantified. The percent termination activity was plotted against the concentration of single-stranded DNA.

Activation by linear DNA molecules is complex. The kinetic pattern of DNA activation is influenced by the concentration of substrate. At low ATP concentrations, activation follows a nonhyperbolic pattern. The sigmoidicity of the plot is ameliorated by addition of maximal ATP. One interpretation of these data proposes that NPH I exists in multiple forms. A resting inactive form predominates in the absence of DNA. DNA binding favors an active conformation. ATP alone does not induce the formation of the active form, since DNA is required for activity. However, ATP clearly influences the ability of single-stranded DNA to stabilize the active conformation. This model of allosteric activation is usually applied to multisubunit enzymes. NPH I is clearly a monomeric enzyme (*11*, *16*). A second model proposes that the saturation patterns observed in Figure 3 could be a kinetic phenomenon observed for steady-state bireactant enzymes (*26*) and not due to a DNA-induced conformational change in NPH I. In a random sequential bireactant enzyme, two possible pathways can be followed

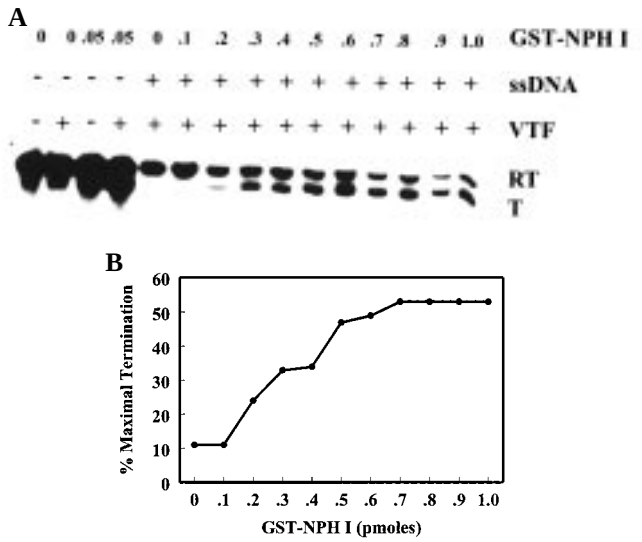
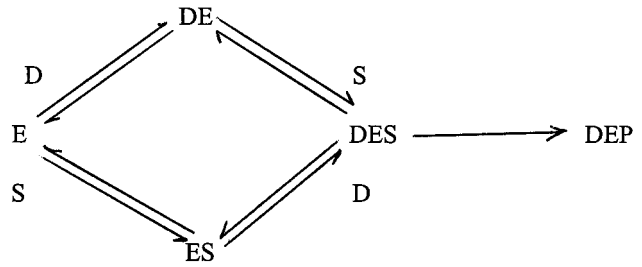



FIGURE 9: Single-stranded DNA inhibition of transcription termination in vitro is reversed by the addition of GST−NPH I. (A) RNA synthesis was assessed as described in the legend of Figure 8, in the presence of 60 ng of 24-base DNA, 2 pmol of VTF, and varying amounts of GST−NPH I (picomoles). Read-through (RT) and terminated (T) RNA are separated by acrylamide gel electrophoresis and quantified by phosphorimage analysis. (B) Transcription termination activity was calculated at different concentrations of added GST−NPH I and plotted vs the concentration of GST−NPH I.

to achieve the active ternary enzyme (E), substrate (S), and DNA (D) complex:




In this model, D is DNA, S is ATP, P is product, and E is enzyme, and the rate of the top path is greater than that of the lower path. The observed velocity is dependent upon which path is employed in forming DES. Path selection is determined by the concentrations of S and D and the relative rate constants for DE, ES, and DES formation. At a low fixed concentration of S, titration of D will exhibit sigmoidal kinetics. This is due to the fact that the slower path will be favored at a low D concentration. As you increase the concentration of D in the reaction mixture, the faster pathway will be preferred, yielding a steep rise in the saturation curve. Alternatively, at a low fixed concentration of D, the faster pathway will be preferred at low S levels. As the concentration of S is increased, the slower path will predominate. This will generate complex saturation kinetics in which the observed velocity rises initially at low S levels. However, at high S levels, the slower pathway predominates so the observed velocities are depressed. This model is consistent with the kinetic behavior presented in Figures 2 and 3. At this point, our data do not discriminate between the two-state conformational change model or the kinetic model. To elucidate the role of NPH I in termination, it is important to ascertain whether DNA binding results in a conformational change in NPH I. Furthermore, since RNA binds to NPH I but does not activate it, it is important to determine whether RNA binding induces the same conformational change. Experiments are underway to determine whether single-stranded DNA induces a conformational change in NPH I and whether single-stranded RNA lacks this capacity.

The ability of linear oligonucleotides to activate NPH I is dependent upon their length. Little activation is observed with purified oligonucleotides with no more than 18 bases. Between 18 and 48 bases, the activation capacity increases dramatically. Oligonucleotides between 24 and 48 bases in length exhibit similar half-activation concentrations, $K_{act}$ (Table 1). However, the maximal level of activation is directly dependent upon length. One explanation for this behavior is that NPH I adopts multiple closely related active forms determined by the length of the oligonucleotide employed as the activator.

Direct binding measurements demonstrate that the affinity of NPH I for single-stranded DNA is dependent upon the length of the oligonucleotide between 15 and 36 bases. Gel analysis of bound complexes permits the identification of multiple binding species. The number of species observed is dependent on both the length of the oligonucleotide and the concentration of NPH I in the binding reaction mixture. For short oligonucleotides, 15 or 18 bases in length, a single binding complex is seen, indicating that a single molecule of NPH I binds. We can conclude that oligonucleotides of this length are too short to bind two molecules of NPH I. For a 24-base DNA, two binding species are seen, indicating that up to two proteins can bind to a single DNA molecule. This is increased to three species observed with a 36-base DNA, consistent with three NPH I molecules bound. Similar results were observed for a 48-base DNA, but complexes containing three or four NPH I molecules would not be resolved in this analysis. These studies indicate that NPH I binds to a site that is about 12 bases in length, which is shorter than the free nontemplate strand found in the ternary complex (*29*).

Although single-stranded RNA does not activate NPH I, it binds to NPH I with a greater affinity than single-stranded DNA of a similar length. Since RNA and DNA cross-compete in an UV photolinkage analysis, both RNA and DNA must bind to the same site(s). Since addition of single-stranded RNA prevents single-stranded DNA activation of NPH I, RNA must bind to the kinetically relevant activation site on NPH I. However, it is clear that simple oligonucleotide binding to NPH I is not sufficient for activation. It is not at all clear whether RNA binding to NPH I may be important in vivo. One might imagine that NPH I is present at a site in the ternary complex that does not permit access to RNA so binding to RNA is not relevant. Consistent with this model, single-stranded RNA is a less effective inhibitor of transcription termination, in vitro (not shown).

To test whether oligonucleotides of known length and composition would be useful reagents for evaluating the termination pathway, the ability of single-stranded DNA to influence termination in vitro was evaluated. Two transcription systems were tested. In one, NPH I was added exogenously along with VTF to a ts mutant virus-infected

cell extract that lacks endogenous NPH I. In the second, a wild-type virus-infected cell extract containing NPH I was employed. In the first extract, added NPH I must bind to a transcription complex and assume its active conformation. In the latter extract, NPH I would be expected to be present in its active position in the transcription complex. When both NPH I and DNA were added together to an NPH I deficient transcription reaction mixture, termination was preferentially inhibited. The observed inhibition was overcome by addition of NPH I, demonstrating that inhibition is mediated through DNA binding to NPH I. When a wild-type virus-infected cell extract was analyzed, it was clear that DNA was a much less efficient inhibitor. This result indicates that the oligonucleotide binding site on NPH I in a preformed ternary complex is relatively inaccessible to free DNA. Perhaps the oligonuclotide binding site on NPH I is occupied in the wild-type-infected cell extract. The mechanism of DNA inhibition is unclear. One possibility is that DNA binding to free NPH I prevents its association with the ternary complex, similar to the affect of heparin on transcription termination (*27*). Alternatively, for NPH I in a transcription complex, binding to a free DNA rather than to the DNA in the transcription bubble might cause NPH I to assume an unfavorable conformation, resulting in a decreased level of termination.

Analysis of a series of mutations in NPH I demonstrates that ATPase activity is essential for transcription termination activity, in vitro (*11*, *12*, *28*). Carboxyl terminal mutations in NPH I which retain ATPase activity fail to terminate, indicating that this region plays a role in termination other than catalysis of ATP hydrolysis. Recent data demonstrate that NPH I interacts with at least one additional component of the ternary complex, the H4L subunit of the virion RNA polymerase (M. Ragaa and E. G. Niles, unpublished). However, since termination activity can be restored to an NPH I deficient virus-infected cell extract (*12*) or to a heparin-stripped ternary complex (*11*), NPH I is not required to be an integral component of the transcription complex. These data support the following model of NPH I activity in transcription termination. NPH I is a nonintegral component of the ternary complex. Binding to the ternary complex is mediated in part through the H4L subunit of the virion RNA polymerase. In the absence of template DNA binding, the ATPase activity of NPH I is silent. Upon binding single-stranded DNA, the nontemplate strand of the transcription bubble, NPH I adopts an alternative conformation that is active in ATP hydrolysis. Through the interaction of NPH I with the H4L subunit of RNA polymerase, or perhaps through an interaction with one or more additional proteins, the energy released from ATP hydrolysis is coupled to transcript release and the dissociation of the RNA polymerase. This model, although undoubtedly oversimplified, lends itself to detailed experimentation.

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