

Nucleoside Triphosphate Phosphohydrolase I (NPH I) Functions as a 5' to 3' Translocase in Transcription Termination of Vaccinia Early Genes*

Received for publication, March 30, 2016, and in revised form, May 5, 2016. Published, JBC Papers in Press, May 6, 2016, DOI 10.1074/jbc.M116.730135

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Vaccinia virus early genes are transcribed immediately upon infection. Nucleoside triphosphate phosphohydrolase I (NPH I) is an essential component of the early gene transcription complex. NPH I hydrolyzes ATP to release transcripts during transcription termination. The ATPase activity of NPH I requires single-stranded (ss) DNA as a cofactor; however, the source of this cofactor within the transcription complex is not known. Based on available structures of transcription complexes it has been hypothesized that the ssDNA cofactor is obtained from the unpaired non-template strand within the transcription bubble. *In vitro* transcription on templates that lack portions of the non-template strand within the transcription bubble showed that the upstream portion of the transcription bubble is required for efficient NPH I-mediated transcript release. Complementarity between the template and non-template strands in this region is also required for NPH I-mediated transcript release. This observation complicates locating the source of the ssDNA cofactor within the transcription complex because removal of the non-template strand also disrupts transcription bubble reannealing. Prior studies have shown that ssRNA binds to NPH I, but it does not activate ATPase activity. Chimeric transcription templates with RNA in the non-template strand confirm that the source of the ssDNA cofactor for NPH I is the upstream portion of the non-template strand in the transcription bubble. Consistent with this conclusion we also show that isolated NPH I acts as a 5' to 3' translocase on single-stranded DNA.

Vaccinia virus is a member of the poxvirus family, which are large double-stranded DNA viruses that conduct their entire infective cycle within the cytoplasm of the infected cell. As such, vaccinia relies upon its own machinery for transcription and DNA replication to produce new viruses (1). Vaccinia expresses its genes in three temporal classes termed early, intermediate, and late (2). Early gene transcription occurs within the viral core immediately after infection and is conducted by the early gene-specific form of viral RNA polymerase (vRNAP).²

* This work was supported by National Science Foundation Grant MCB 1019969. The authors have no conflicts of interest with the contents of this article.

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² The abbreviations used are: vRNAP, viral RNA polymerase; RNAP, RNA polymerase; NPH I, nucleoside triphosphate phosphohydrolase I; ss, single-stranded; N-MR, NPH I-mediated transcript release; O-MR, oligonucleotide-mediated transcript release; CE, capping enzyme; VTF, vaccinia

vRNAP contains one subunit called Rap94 (the product of the *H4L* gene) in addition to the eight subunits it shares with the intermediate/late form of the polymerase (1, 3, 4).

Proper early gene transcription termination is essential as demonstrated by temperature-sensitive viruses with defects in components of this process (5–7). In addition to the virion form of vaccinia RNA polymerase, early gene transcription termination requires two viral factors, the mRNA capping enzyme (CE) (8) and nucleoside triphosphate phosphohydrolase I (NPH I) (1, 2, 9–11), as well as a cis-acting termination signal (12) in the nascent RNA. This termination signal consists of U₅NU (where N is any nucleotide) found 25–50 bases upstream of the 3' end of early transcripts (12). Capping enzyme, which is also known as vaccinia termination factor (VTF), has been shown to interact with the termination signal in the nascent RNA (13). NPH I is a single-stranded (ss) DNA-dependent ATPase that couples ATP hydrolysis with release of nascent RNA from the transcription elongation complex (TEC) (11, 14, 15). Both of these viral proteins are associated with vRNAP isolated from virions (7, 15–17). CE is loosely associated and can be removed by washing with low salt (7, 11). NPH I is more tightly bound to vRNAP and requires treatment with heparin to be removed (9, 10, 15, 18). vRNAP lacking VTF and NPH I is active for transcription of early genes *in vitro* but fails to terminate properly unless both factors are added back (1, 11, 15, 19, 20).

Although NPH I is a member of the DEXH box helicase family, duplex unwinding has not been observed with DNA or RNA (9, 10). However, the ATPase activity of NPH I is essential for vaccinia early gene transcription termination (10, 14). Previous work suggests that NPH I ATPase activity is coupled to release of nascent RNA from the transcription complex through a mechanism involving forward translocation of vRNAP (11). Although studies of purified NPH I have shown that it requires ssDNA as a cofactor for ATPase activity (14, 21), it is not clear where this cofactor is obtained within the TEC. It has been proposed previously that the ssDNA cofactor of NPH I originates in the non-template strand within the transcription bubble of the DNA being transcribed (21). In structures representing elongation complexes of both bacterial RNA polymerase and yeast RNA polymerase II, the non-template strand of DNA within the transcription bubble appears to be on the surface of the structures and potentially available to interact with termination factors (22, 23). In contrast, the template strand in the

termination factor; NT, non-template; T, template; SF2, superfamily 2; TEC, transcription elongation complex; US, upstream; DS, downstream.

bubble is buried within the protein structure, including in the DNA-RNA hybrid (22), which suggests it is not available to interact with termination factors.

In this study, we investigated the role of the non-template strand in transcript release. We found that release, both NPH I-dependent and -independent, requires the presence of the non-template strand in the upstream portion of the transcription bubble. Moreover, complementarity between the two strands in this region of the bubble is also required for transcript release. By using chimeric transcription templates containing segments of ribonucleotides in the non-template strand, we show that the ssDNA cofactor for NPH I is obtained from the upstream region of the non-template strand. We also show that isolated NPH I acts as a 5' to 3' translocase on single-stranded DNA.

Experimental Procedures

Ter29 in Vitro Transcription Template—All templates for *in vitro* transcription used in these studies were based on the dsDNA Ter29 template, which was designed by Dr. Stewart Shuman and co-worker (18) (Fig. 1A). Ter29 contains a strong early promoter joined to a 20-base G-less cassette followed by 4 G residues from positions +21 to +24. The G-less cassette is followed by a 57-base A-less cassette. Within the A-less cassette, a series of 9 T residues in the non-template strand serve as the termination signal when transcribed into 9 Us in the nascent RNA. The A-less cassette is followed by 4 A residues from positions +78 to +81 and then an additional 108 bp of DNA. Ter29 transcription templates were prepared by PCR as described previously and bound to streptavidin-coated magnetic beads (Promega) via a biotin on the 5' end of the template strand (8, 11, 18).

In Vitro Transcription Templates Constructed from Oligonucleotides—The oligonucleotide-based templates for *in vitro* transcription were each composed of a template (T) and a non-template (NT) strand. Each strand (T or NT) was created by ligating two oligonucleotides together including an upstream (US) and downstream (DS) oligonucleotide (Fig. 1). For both the T and NT strands, the US oligo was composed of residues corresponding to -30 to +30 based on transcription starting at +1. The DS oligo for the T strand spanned from +31 to +90. The DS oligos for the NT strand contained variations for each transcription template. For the deletion series of transcription templates, the DS oligos comprised +31 to either +90 or to various truncated 3' ends (Fig. 1B, NT90 to NT57). In some cases, the DS oligos for the NT strand contained altered sequences to generate mismatches with the template strand (Fig. 1B, NC66–68 to NC73–75). In other cases, the DS oligos contained segments of ribonucleotides (Fig. 1B). The 5' end of the US oligo for the NT strand was biotinylated to allow binding of the completed dsDNA transcription templates to streptavidin-coated paramagnetic beads. For oligo-based transcription templates that lacked non-template DNA or contained mismatches, the sequence of the completed template strand was identical to Ter29 (18) except that the termination signal was moved downstream to +46, and the sequence following the end of the A-less cassette was shortened.

For each pair of oligos that were ligated to generate a T or NT strand, the 5' end of one oligo was phosphorylated using T4 polynucleotide kinase (New England Biolabs), 20 μM ATP, and 1 μCi of [γ -³²P]ATP (PerkinElmer Life Sciences) as indicated in Fig. 1. Radiolabeling facilitated visualizing the ligated products in polyacrylamide gels during the construction process as well as quantification of each strand. Each pair of US or DS oligos were annealed to a 30-mer bridging oligo complementary to 15 nucleotides at the 3' end of the US oligo and 15 nucleotides at the 5' end of the DS oligo (*i.e.* the bridging oligo is complementary to +15 to +45). One nanomole of each oligo (US, DS, and bridge) was mixed in 60 μl of H₂O, heated to 95 °C for 15 min, and then slowly cooled over 60 min to room temperature (~25 °C). T4 DNA ligase (New England Biolabs) was then added to the annealed oligos in T4 DNA ligase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM dithiothreitol, and 1 mM ATP) and incubated for 3 h at 20 °C. The products of the ligation reactions were separated on 8% (19:1 bis) polyacrylamide gels containing 8 M urea in 1× Tris borate-EDTA. The gels were exposed to a phosphor storage screen (GE Healthcare) developed with a Storm phosphorimaging system using ImageQuant software (GE Healthcare). Bands corresponding to full-length T and NT strands were excised from the gels, and the DNA was extracted by a crush and soak procedure (24). The purified NT and T strands were resuspended in diethylpyrocarbonate-treated H₂O and quantified by liquid scintillation (Beckman). 30 picomoles of each strand were mixed and annealed by heating and slow cooling as described above. The double-stranded oligo-based transcription templates were then bound to streptavidin-coated paramagnetic particles as directed by the manufacturer.

Transcription templates that contained mismatches between the T and NT strands were based on the sequence of NT90. Mismatches were generated by changing C to G, G to C, T to A, or A to T in the NT strand within the regions indicated (Fig. 1B).

Templates with ribonucleotides in the non-template strand were based on the NT112 dsDNA template, which is identical to Ter29 except that it spans from -30 to +112. Ribonucleotides containing T and NT strands were assembled from two oligonucleotides spanning -30 to +58 and +59 to +112 (Midland Certified Reagent Co.). The US NT oligonucleotide was biotinylated on the 5' end. The DS NT oligonucleotide contained ribonucleotides corresponding to the positions in the name of each transcription template (Fig. 1). The bridging oligonucleotides for ligating both pairs of US and DS oligonucleotides for the T and NT strands spanned from +44 to +74.

Periodate Deoxy-ATP (dATP) Treatment—Contaminating ATP was removed from dATP stocks by periodate treatment as described previously (25). 5 micromoles of dATP (12.5 mM) were incubated at 37 °C for 30 min in 40 mM potassium acetate (pH 5.5) with 50 mM NaIO in a total volume of 400 μl. Following incubation, 200 μmol of glucose (500 mM) were added to inactivate the excess periodate.

Virion Extract Preparation—Vaccinia virions were purified from infected HeLa cells using sucrose density gradients (11). Viral cores were prepared from 5–10 mg of virions by incubation in 50 mM Tris-Cl (pH 8.5), 10 mM DTT, and 0.05%

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Nonidet P-40 at 37 °C for 10 min. Viral cores were collected by centrifugation at 13,000 × g for 15 min at 4 °C, and transcription extracts were then prepared as described previously (11, 26).

NPH I-mediated Transcript Release (N-MR) Assays—N-MR assays were conducted in two steps. In the first step, 1 μ l of virion extract was incubated with 100 fmol of bead-bound dsDNA template (5 nm), 1 mM ATP, 1 mM CTP, 0.25 mM 3'-O-methyl-GTP, and 5 μ Ci of [α -³²P]UTP for 15 min at 30 °C in 20 μ l of transcription buffer (20 mM Tris-HCl (pH 8.0), 6 mM MgCl₂, 2 mM DTT, and 8% glycerol). This step produces stable TECs paused at G21. Bead-bound TECs were pulled down using a magnet and washed three times in 100 μ l of transcription buffer to remove capping enzyme and unincorporated nucleotides. In the second step of the assay, transcription was resumed in 20 μ l of transcription buffer containing 1 mM CTP, 1 mM UTP, and 1 mM GTP, which yields transcription complexes stalled at C77 at the end of the A-less cassette. Where indicated, 1 mM periodate-treated dATP was added to stimulate NPH I function while preventing transcription elongation past the end of the A-less cassette. The 77-nucleotide transcripts that were released from the TEC were separated from those remaining bound to the TEC by pulling down the DNA template using a magnet. 20 μ l of 95% formamide were added to each sample, which were then boiled for 5 min and electrophoresed through 12% (19:1) polyacrylamide gels containing 8 M urea and 1× Tris borate-EDTA. Gels were exposed to phosphor storage screens and scanned using a Storm phosphorimaging system. The radiolabeled RNA products were quantified using ImageQuant software. Percent release was calculated by dividing the quantity of RNA released into the supernatant by the sum of RNA retained in the ternary complex and released RNA.

Oligonucleotide-mediated Transcript Release (O-MR) Assays—O-MR assays were conducted similarly to NPH I-mediated transcript release assays except that dATP was excluded from the second step of transcription and 2 pmol (10 μ M) of an oligonucleotide complementary to the nascent RNA from +51 to +77 were added where indicated.

Transcription Termination Assay—Transcription termination assays were conducted in two steps as described previously (11, 18). Briefly, the first step transcription was initiated in the same way as for the transcript release assays described above to produce TECs paused at G21. Bead-bound TECs were washed with transcription buffer as described for transcript release assays above. The second step of the termination assays contained all four NTPs and 10 μ M CE (VTF). The second step of transcription produces transcripts that either read through to the end of the template or terminate at approximately +75. Samples were boiled with an equal volume 95% formamide and electrophoresed through 12% (19:1) polyacrylamide gels containing 8 M urea and 1× Tris borate-EDTA. Gels were exposed to phosphor storage screens and scanned using a Storm phosphorimaging system. The radiolabeled RNA products were quantified using ImageQuant software. Percent termination was calculated by dividing the quantity of the terminated transcripts by the sum of the terminated and readthrough RNA.

Streptavidin Displacement Assay DNA Preparation—The ability of NPH I to disrupt the streptavidin-biotin interaction was tested on ssDNAs that were biotinylated at either the 5' or 3' end. To construct radiolabeled strands of DNA biotinylated at the 5' end, we needed to overcome the inability to phosphorylate 5'-biotinylated oligonucleotides with T4 polynucleotide kinase. Therefore, we constructed these ssDNAs by ligating a 5'-biotinylated oligonucleotide to a 5' end-labeled oligonucleotide comprising the 3' portion of the strand. The sequence of ssDNA utilized in these experiments was identical to that used in a previous study, 5'-GAATTCGAGCTCGGTACCCGGGG-ATCCTCTAGAGTC-3' (21). We generated a 36-mer and a 24-mer based on the sequence shown. The 24-mer was a 3' truncated version of the 36-mer. The complete strands were formed by ligating oligonucleotides spanning positions 1–12 and from 13 to the 3' terminus of 24 or 36. The 5' end of the downstream oligo beginning at position 13 was phosphorylated using T4 polynucleotide kinase in all cases, 10 μ M ATP, and 10 μ Ci of [γ -³²P]ATP. To construct either ssDNA in 60 μ l of H₂O, 5 nmol of each set of oligos (83 μ M) were annealed to 4 nmol of 15-base bridge oligo (67 μ M), 5'-GGGTACCGAGCTCGA-3', by heating and slow cooling conducted as described above. Ligation and gel purification were conducted as described above.

Analysis of NPH I Streptavidin Displacement Assays by Gel Electrophoresis—The ability of NPH I to disrupt the streptavidin-biotin interaction was also tested on the single-stranded DNAs described above that were biotinylated at either the 5' or 3' end. The streptavidin-DNA complex was generated from 500 fmol of biotinylated DNA (25 nm) mixed with 5 pmol of streptavidin (250 nm) (New England Biolabs) and allowed to bind for 1 h at 4 °C. The streptavidin-bound DNA in 20- μ l total volume containing 20 mM Tris-HCl (pH 8.0), 6 mM MgCl₂, 2 mM DTT, and 8% glycerol was mixed with 100 pmol of biotin (5 μ M) and 1 mM ATP. 10 picomoles of NPH I (500 nm) were added to the reactions and incubated at 30 °C, and samples were removed at various times. 20 μ l of quench solution (0.6% SDS, 200 mM EDTA, 0.1% bromphenol blue, 5% glycerol, and 1 mg/ml salmon sperm DNA) were added to stop the reaction. Reactions were then electrophoresed ~10 cm through a native 8% (79:1) polyacrylamide gel in 1× Tris borate-EDTA. Gels were dried and exposed to phosphor storage screens and scanned and analyzed as described for transcript release assays above.

Bead-bound NPH I Streptavidin Displacement Assays—Assays were conducted similarly to gel-based assays except DNAs were mixed with 1 μ g of streptavidin-coated paramagnetic particles instead of purified streptavidin. Reactions were conducted identically to gel-based assays. 20 μ l of quench solution (0.6% SDS, 200 mM EDTA, 0.1% bromphenol blue, 5% glycerol, and 1 mg/ml salmon sperm DNA) were added at the time points indicated to stop the reaction. Beads were separated from the supernatant using a magnet. Bound and released DNA was quantified using a scintillation counting (Beckman). Percent streptavidin displacement was calculated by dividing the quantity of released DNA by the sum of total DNA in the reaction.

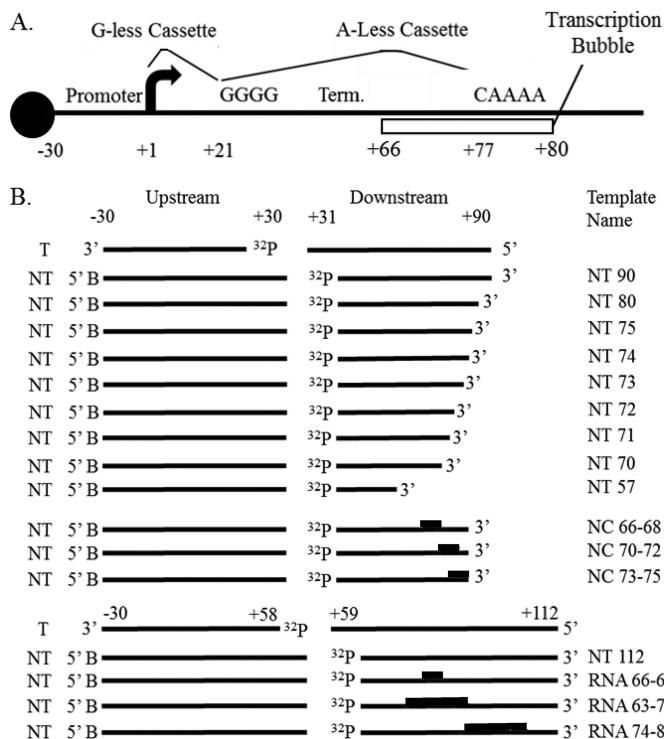


FIGURE 1. Construction of oligonucleotide-based transcription templates. *A*, schematic diagram of the features common to the *in vitro* transcription templates used in this study. The sequence of these templates was based on that of Ter29, a previously characterized synthetic transcription template (11). Each template contains a strong early promoter fused to a 20-bp G-less cassette followed by 4 G residues beginning at +21 relative to the start of transcription. This is followed by a 57-bp A-less cassette, which when transcribed contains a run of 9 Us (*Term.*) that provides the transcription termination signal. There are 4 A residues following the C residue at the end of the A-less cassette (+77). This arrangement allows the TEC to be halted at C77 when transcription elongation is performed in the absence of ATP. The unpaired bases within the transcription bubble stretch from +66 to +80 when the TEC is paused at C77 are indicated by the box below the template diagram. Templates were bound to streptavidin-coated magnetic beads through a biotin on the 5' end of the non-template strand. *B*, diagram depicting the oligonucleotides used to construct transcription templates used in this study. T and NT strands are labeled along the left side of the diagram. Template strands are listed first and are oriented 3' to 5' as they would pair to the corresponding non-template strands. The 5' end of the upstream template strand oligo and the 5' end of the downstream non-template strand oligo were end-labeled using [γ -³²P]ATP. The transcription templates with truncated downstream NTDNA (NT90, NT80, NT75, NT74, NT73, NT72, NT71, NT70, and NT57) contained the same T strand as the full-length NT90. Templates that contained mismatches introduced into the non-template strand are shown with gray boxes indicating their positions within the template (NC66–68, NC70–72, and NC73–75). Mismatches were introduced by changing the sequence of the non-template DNA from cytosine to guanine, guanine to cytosine, thymine to adenine, and adenine to thymine. The mismatch-containing templates used the same template and upstream non-template oligonucleotides as the NT90 transcription template. The oligonucleotide-based template (NT112) and ribonucleotide-containing templates (RNA66–68, RNA63–74, and RNA74–85) all shared upstream non-template oligonucleotides and complete template strands. The relative positions of ribonucleotides within the RNA-containing templates are shown by gray boxes (RNA66–68, RNA63–74, and RNA74–85).

Results

Role of the Non-template Strand in the Transcription Bubble in NPH I-mediated Transcript Release—To examine the role of the NTDNA strand within the transcription bubble in vaccinia early gene transcript release, we tested N-MR activity *in vitro* on transcription templates lacking various segments of the NT strand (Fig. 1B). These transcription templates were con-

structed by ligating pairs of oligonucleotides to form both the T and NT strands, which were then annealed. Each dsDNA transcription template was bound to streptavidin-coated magnetic beads through a biotin at the 5' end of the NT strand. The sequence of these oligo-based transcription templates was based on a previously characterized template for vaccinia early transcription called Ter29 (9, 11, 18, 20). Ter29 contains a strong early promoter followed by a 20-bp G-less cassette and then a 57-bp A-less cassette (Fig. 1A). A stretch of 9 Ts in the NT strand starting at +29 functions as the termination signal when transcribed into U9 in the nascent RNA. The only difference between the oligo-based templates and Ter29 is that the termination signal was moved to +46 in the oligo-based templates. This signal is required for termination of ongoing transcription (11); however, it is not required for NPH I-mediated transcript release from a stalled TEC (11, 15).

A schematic depiction of the TEC stalled at C77, based on prior characterization of the early vaccinia transcription complex (27), is shown in Fig. 2A. The transcription bubble is composed of 15 unpaired nucleotides starting 12 residues upstream of the active site (residue +66) and extending 3 residues downstream of the active site to residue +80. The DNA-RNA hybrid comprises 9–10 base pairs upstream of the active site at C77. The numbers listed above the schematic correspond to the 3' end of various NT strands of the oligo-based templates examined here.

Transcript release assays were conducted in two steps. In the first step, transcription was initiated using partially purified viral extract, ATP, CTP, [α -³²P]UTP, and 3'-O-methyl-GTP (11). On all of the templates used in this study, this step yielded TECs stalled at +21, the position of the first template G residue (Fig. 2B, lanes 1 and 6). These TECs with a 21-nucleotide radiolabeled nascent transcript were stable and were then washed with transcription buffer to remove unincorporated nucleotides and viral capping enzyme (VTF). In the second step, transcription was resumed in the presence of CTP, UTP, and GTP (without ATP), which resulted in elongation to the end of the A-less cassette at C77 (Fig. 2B, lanes 2 and 7). The quantity of transcripts produced from oligo-based templates was similar to that observed previously with the PCR-generated template Ter29.

The ability of NPH I to release transcripts from these stalled TECs was assessed by adding dATP to the second step of the assay. Although both ATP and dATP can be hydrolyzed by NPH I to provide the energy to release transcripts (15), dATP does not serve as a substrate for RNAP and can thus be used to test NPH I function independent of transcription elongation (10, 11, 18). Released transcripts were separated from those remaining associated with the bead-bound TECs using a magnet and visualized by denaturing PAGE. In the absence of dATP, ~10% of the transcripts were released from all transcription templates tested (Fig. 2B, lanes 2 and 3 and lanes 7 and 8, and C, Bkg). In the presence of dATP, transcript release increased to ~80% on NT90 (Fig. 2B, lanes 4 and 5). Similar results have been obtained previously with Ter29 both by us and others (9–11, 15, 20).

To examine the role of the NT strand in the transcription bubble on transcript release activity, we tested N-MR activity

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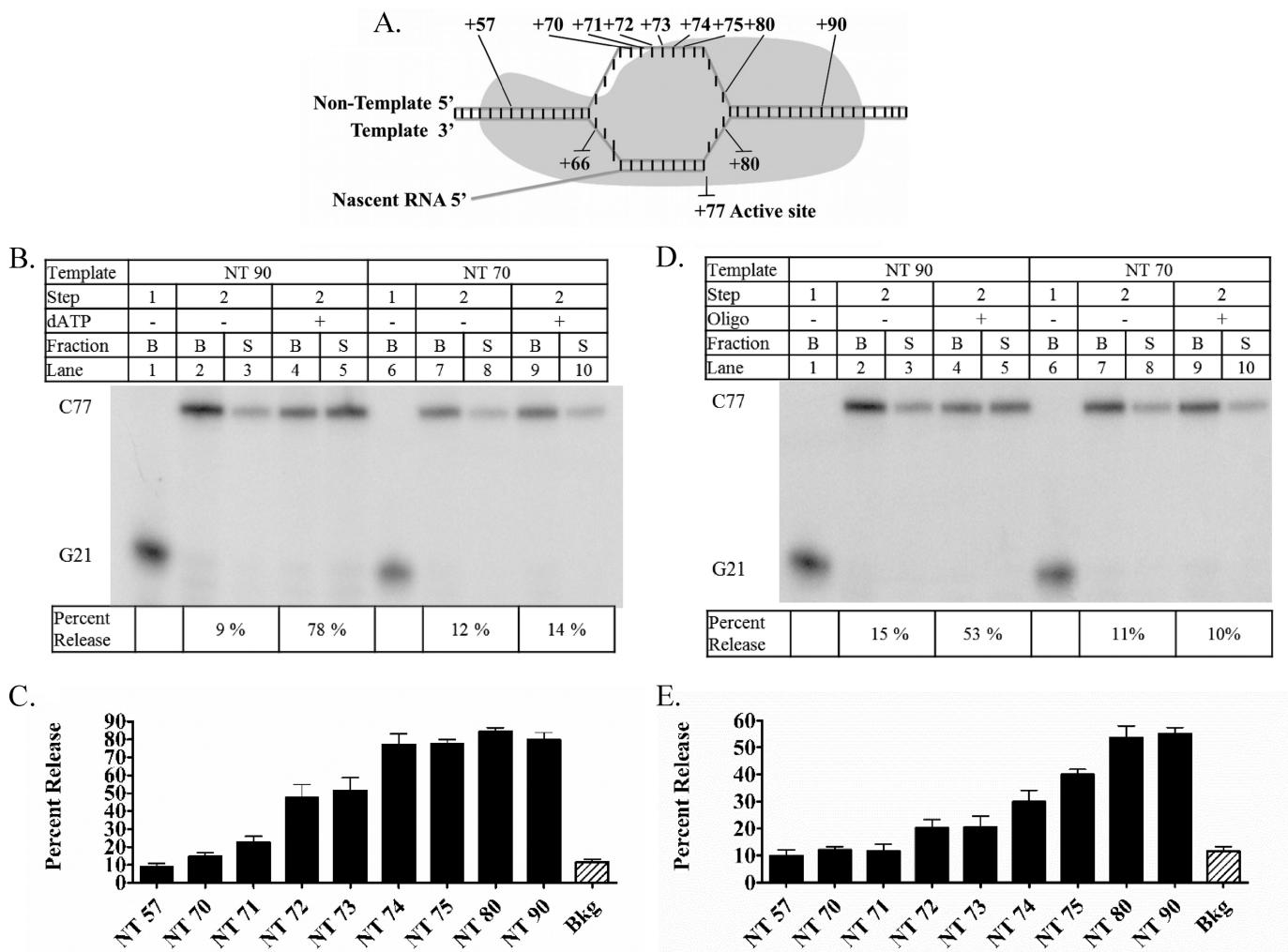


FIGURE 2. Transcript release is inhibited when the non-template strand of DNA is removed from the transcription bubble. *A*, schematic diagram of the vaccinia early transcription elongation complex paused at C77 based on previous studies (27). The transcription bubble consists of 15 unpaired nucleotides beginning 12 nucleotides upstream of the transcription active site and extending 3 nucleotides downstream of the active site. The DNA-RNA hybrid is ~10 nucleotides long. The 3' end of the NT strand for each of the *in vitro* transcription templates that were tested is indicated at the top of the diagram. *B*, phosphorimage of a 12% denaturing polyacrylamide gel with two examples of N-MR assays. Transcriptions were conducted on templates that contained either the full-length NT strand (NT90) or the NT strand truncated at residue +70 (NT70) (see *A*). The first step of transcription was conducted in the presence of [α^{32} P]UTP, CTP, ATP, and 3'-O-methyl-GTP, resulting in production of elongation complexes stalled at residue +21 denoted as G21 (lanes 1 and 6). Following step 1, transcription complexes were washed with transcription buffer. The second step of transcription involved elongation in the presence of CTP, GTP, UTP, and where indicated dATP (lanes 2–5 and 7–10). The reaction products were then separated using magnetic beads into bound (B) and supernatant (S) fractions and analyzed by PAGE. Percent transcript release was calculated by dividing the amount of transcripts released into the supernatant by the sum of both the bound and supernatant and is listed below the B and S pair of lanes for each reaction. *C*, bar graph representation of the percent NPH I-mediated transcript release on each template. Values are the averages of at least three independent trials with standard deviations shown as error bars. Background (Bkg) release activity is the average percent transcript release in the absence of added dATP from all templates tested. *D*, phosphorimage of a 12% denaturing polyacrylamide gel with two O-MR assays. Transcriptions were conducted as described in *B* except that instead of dATP an antisense oligonucleotide complementary to 26 nucleotides of the 3'-most portion of the nascent RNA was added in step 2 of all reactions (lanes 2–5 and 7–10). The reaction products were then separated and analyzed in the same manner as described above. Percent transcript release is listed below the B and S pair of lanes for each reaction. *E*, bar graph representation of the percent oligonucleotide-mediated transcript release on templates that lack portions of the non-template DNA within the transcription bubble. Values are the average of at least three independent trials with standard deviations shown as error bars. Background release activity is the average percent transcript release in the absence of added oligonucleotide from NT90 templates.

on transcription templates that lack segments of this strand (Fig. 2*A*). With transcription templates that harbor truncations of the NT strand starting at +74, +75, or +80 and continue to the 3' end of the strand (Fig. 2*A*, NT74, NT75, and NT80), NPH I activity remained similar (~80%) to that seen on the fully dsDNA NT90 (Fig. 2*C*). Hence, the 6 unpaired residues (+74 to +80) of the NT strand in the downstream portion of the transcription bubble do not appear to be required for efficient NPH I-mediated transcript release. Shortening the NT strand by 1 or 2 additional residues toward the upstream direction (NT73 or

NT72, respectively) reduced N-MR activity from ~80 to ~50% (Fig. 2*C*). Further shortening of the NT strand by 1 or 2 residues reduced transcript release to 20% or less (Fig. 2*C*, NT71 and NT70). Finally N-MR activity was reduced to background levels (~10%) when the NT strand was shortened such that it was entirely absent from the transcription bubble (Fig. 2*C*, NT57). All of these transcription templates yielded approximately equal amounts of RNA transcripts from the same quantity of DNA template. Together these results indicate that residues of the non-template DNA within the upstream region of the tran-

scription bubble are required for NPH I to efficiently (>50%) release nascent RNA from the vaccinia early gene ternary complex.

The Requirement for the Non-template Strand in the Transcription Bubble for Transcript Release Is Not NPH I-specific— Deletions of the non-template strand could impair transcript release by specifically affecting NPH I function, or these effects may be independent of NPH I. We therefore tested transcript release from these templates using an approach that does not involve NPH I activity. O-MR uses an antisense oligonucleotide complementary to the 3' end of the nascent RNA to induce transcript release. O-MR was first developed to mimic bacterial intrinsic transcription termination *in vitro* (28) and has also been shown to release transcripts from stalled vaccinia early elongation complexes (11). OM-R assays were conducted similarly to the two-step N-MR assays described above except the second step of the assay was performed in the absence of dATP and included an oligonucleotide complementary to the 3'-most 26 residues of the nascent transcript.

As seen above, in the absence of added oligonucleotide, TECs stalled at C77 at the end of the A-less cassette were stable with only 10–15% of the transcripts released (Fig. 2D, *lanes 2 and 3* and *lanes 7 and 8*). Similar results were obtained with all of the templates tested in the absence of added oligo (Fig. 2E, *Bkg*). Adding an antisense oligonucleotide complementary to 26 residues at the 3' end of the nascent transcript in the second step of the assay resulted in release of ~50% of the transcripts from the fully double-stranded template NT90 (Fig. 2, D, *Lanes 4 and 5*, and E, NT90). The efficiency of oligonucleotide-mediated transcript release (~50%) was less than that seen with NPH I-mediated transcript release (~80%), which is similar to prior observations with the Ter29 template (11). Sense oligonucleotides with the same sequence as the RNA transcript did not induce significant transcript release (data not shown).

O-MR was tested on the same templates used to test N-MR (Fig. 2A). As the NT strand was truncated toward the upstream region of the transcription bubble, the efficiency of transcript release diminished in a pattern that is very similar to that seen with NPH I-mediated release (Fig. 2, compare E and C). Hence, transcript release from stalled early gene TECs requires the presence of the non-template strand in the upstream half of the transcription bubble independently of NPH I activity.

Complementarity between the Template and Non-template Strands within the Transcription Bubble Is Required for Transcript Release— Reannealing of the template and non-template strands of the transcription bubble has been shown to be important for transcription termination and transcript release in bacteria (28, 29). This effect has been termed bubble collapse. The requirement for the NT strand in the upstream portion of the transcription bubble for both N-MR and O-MR suggests that bubble collapse may also play a role in transcript release for vaccinia early genes. To test this possibility, we examined the effects of mismatches between the T and NT strands at various locations on transcript release by NPH I and oligonucleotides. In all cases, the NT strand was altered, which maintained the sequence of the template and the RNA transcript. All the transcription templates with mismatches used in this study were based on NT75 (Fig. 2A), which contains sufficient NT strand

to yield N-MR and O-MR efficiencies similar to those of the full-length double-stranded template (Fig. 2, C and E). We examined the effects of mismatches within the upstream half of the transcription bubble between +66 and the 3' end of the NT strand (Fig. 3A).

NPH I-mediated transcript release was reduced to nearly background levels (~10%) on the template with three mismatches at the 5' end of the transcription bubble (Fig. 3C, NC66–68). When the trinucleotide mismatches were further downstream within the transcription bubble, transcript release was partially restored (Fig. 3C, NC70–72 and NC73–75). Similar results were also obtained for O-MR on these templates (Fig. 3, compare C and E). Thus, reannealing of the transcription bubble, particularly in the upstream portion, is required for early gene transcript release regardless of whether NPH I is involved.

Ribonucleotides within the Non-template Strand of Transcription Bubble Specifically Inhibits NPH I-mediated Transcript Release— One goal of our studies was to identify the source of ssDNA cofactor for NPH I in the transcription elongation complex. However, the requirement for complementarity between the template and non-template strands in the upstream region of the transcription bubble for efficient transcript release (either N-MR or O-MR) complicates interpreting the results with transcription templates lacking portions of the NT strand with regard to this goal. To better address this question, we took advantage of the observation that ssRNA competes with ssDNA for binding to NPH I, suggesting that they occupy the same binding site on the protein, but RNA does not activate ATPase activity (21). Thus, we tested N-MR and O-MR on chimeric DNA-RNA templates that contain segments of ribonucleotides within the NT strand in the transcription bubble (Fig. 4A). These transcription templates maintain complementarity between the template and non-template strands to fulfill the requirement of reannealing the transcription bubble for transcript release.

When N-MR was measured from transcription templates that contained ribonucleotides in the upstream half of the NT strand of the bubble, only background levels of release activity were observed (~10%) (Fig. 4B, compare *lanes 2 and 3* and *lanes 7–10*). These templates include RNA66–68 with only 3 ribonucleotides in the 5'-most residues of the bubble (Fig. 7A). In contrast, when the downstream portion of the NT strand of the transcription bubble was replaced with ribonucleotides (Fig. 7A, RNA74–85), N-MR was reduced only slightly (~50%) as compared with the same template with all DNA (NT112) (~75%) (Fig. 4C).

To test whether the inhibition of transcript release by the presence of ribonucleotides in the 5' region of the transcription bubble is specific to NPH I, we examined O-MR on these templates. In contrast to the results with N-MR, O-MR activity on all of the DNA-RNA chimeric templates was only slightly less than on the fully dsDNA template (Fig. 4, E and D, compare *lanes 4 and 5* with *lanes 9 and 10*). Moreover, O-MR was similar on all these templates regardless of the location of the RNA segments within the transcription bubble (Fig. 4E). These observations show that the effects of RNA on N-MR are specific to NPH I.

NPH I ssDNA 5' to 3' Translocation

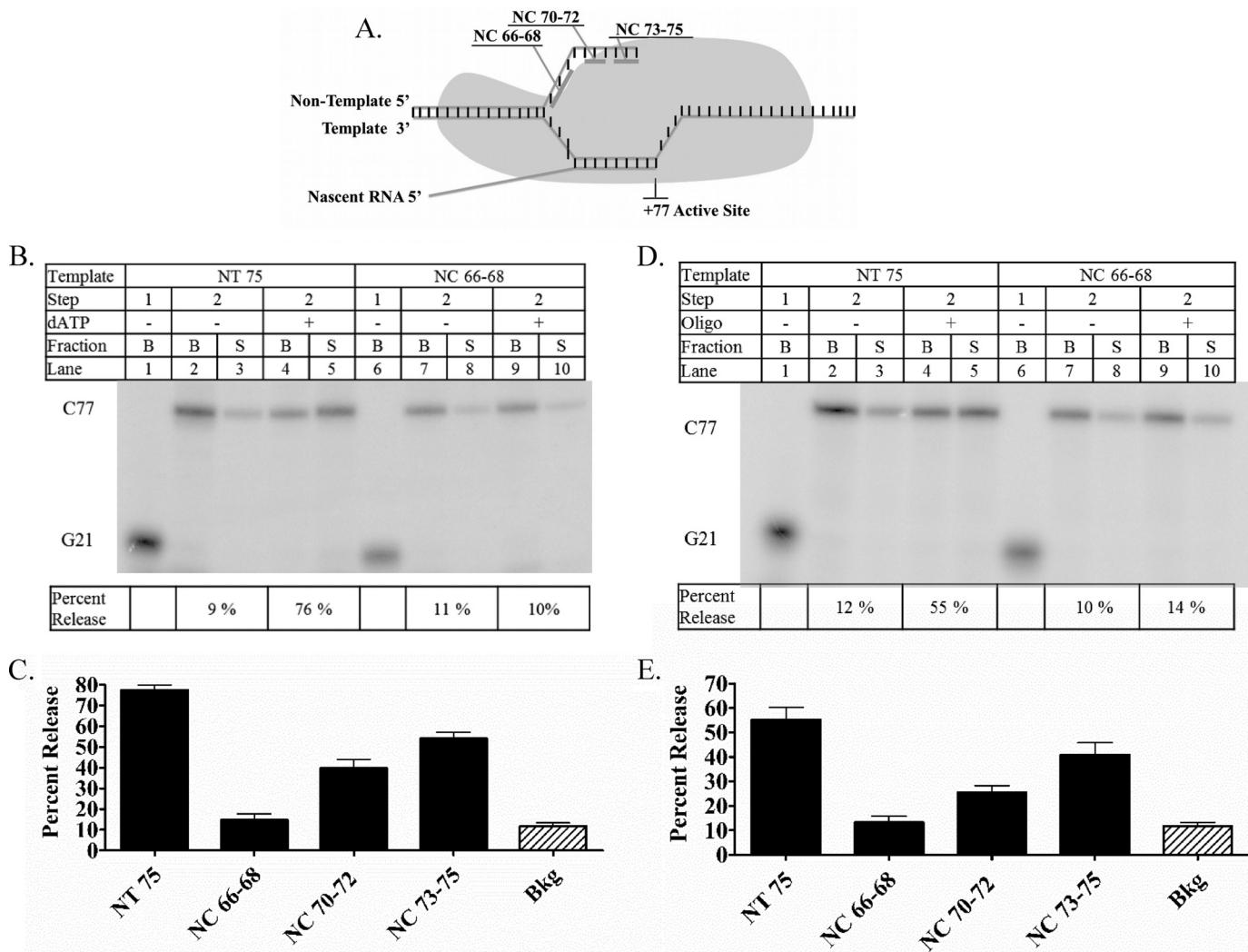


FIGURE 3. Transcript release requires complementarity between the T and NT strands within the upstream region of the transcription bubble. *A*, diagram depicting the vaccinia transcription elongation complex paused at C77 (27). The NT strand for all of these transcription templates extended to +75, which resulted in NPH I-mediated transcript release similar to the full-length (+90) NT strand (see Fig. 1*D*). The positions of substitutions that were introduced into the non-template strand to disrupt base pairing with the template strand are indicated by lines below the non-template strand. The names of the transcription templates corresponding to the positions of the mismatches are indicated at the top of the diagram. *B*, phosphorimage of a 12% denaturing polyacrylamide gel with examples of two NPH I-mediated transcript release assays conducted and analyzed as described in Fig. 2*B*. *C*, bar graph representation of the percent NPH I-mediated transcript release on templates that contain disruptions in base pairing at different positions within the transcription bubble. Values are average of at least three independent trials with standard deviations shown as error bars. Background (*Bkg*) release activity is the average percent transcript release in the absence of added dATP from NT90 template. *D*, phosphorimage of a 12% denaturing polyacrylamide gel with examples of two oligonucleotide-mediated transcript release assays on templates described in *B*. O-MR assays were conducted and analyzed as described in Fig. 2*D*. Percent transcript release is listed below the *B* and *S* pair of lanes for each reaction. *E*, bar graph representation of the percent oligonucleotide-mediated transcript release on templates that contain disruptions in base pairing at different positions within the transcription bubble. Values are average of at least three independent trials with standard deviations shown as error bars. Background release activity is the average percent transcript release in the absence of added oligonucleotide from NT90 templates.

The ssDNA Cofactor Required for NPH I-mediated Transcript Release Cannot be Provided by Adding ssDNA in Trans—Prior studies have shown that ssDNA oligonucleotides can activate ATPase activity of isolated NPH I (21). The observation that NPH I is unable to release transcripts from the RNA66–68 template allows us to examine whether the ssDNA cofactor of NPH I required for transcript release from a stalled transcription elongation complex can be provided in *trans* (21). To do so, we added a 24-mer ssDNA oligonucleotide capable of stimulating ATPase activity with isolated NPH I (data not shown) to N-MR assays on either the NT112 DNA template or on the

DNA-RNA chimeric template RNA66–68. NPH I induced efficient ($\geq 75\%$) transcript release from the fully DNA template NT112, which was not affected by the oligonucleotide (Fig. 5, lanes 2–5). As seen above (Fig. 4, *B* and *C*, RNA66–68), NPH I was unable to induce transcript release from the template with ribonucleotides in the first 3 unpaired bases of the transcription bubble (Fig. 5, lanes 7 and 8). Adding as much as 1000-fold molar excess of the DNA oligonucleotide during the second step of the assay did not affect transcript release by NPH I on this template (Fig. 5, lanes 9–12). Similar results were obtained using the NT57 template, which lacks the non-template DNA

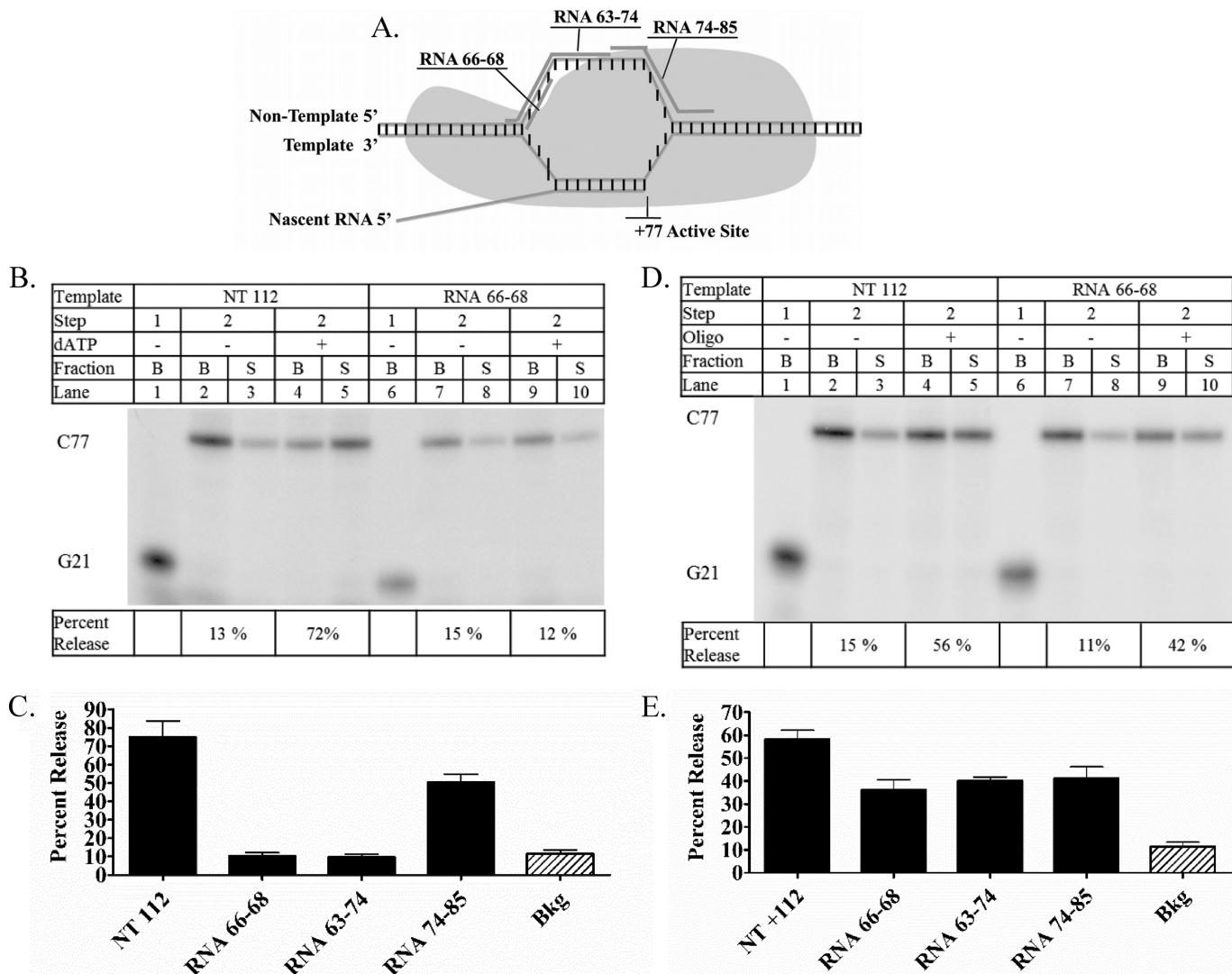


FIGURE 4. NPH-I-mediated transcript release is specifically inhibited by the presence of ribonucleotides in the upstream region of the transcription bubble. *A*, diagram depicting the vaccinia transcription complex paused at C77 (27). Transcription templates contained various lengths of ribonucleotides in the non-template strand indicated by the lines above the non-template strand. Names of the transcription templates corresponding to the positions of the ribonucleotides are indicated at the top of the diagram. *B*, phosphorimage of a representative 12% denaturing polyacrylamide gel with two NPH I-mediated transcript release assays on transcription templates with full-length dsDNA (NT112) or that contained ribonucleotides in positions +66 to +68 of the non-template strand (RNA66–68). N-MR assays were conducted and analyzed as described in Fig. 2B. Percent transcript release is listed below the bound (B) and supernatant (S) pair of lanes for each reaction. *C*, bar graph representation of the percent NPH I-mediated transcript release on templates that contain ribonucleotides at different positions within the transcription bubble. The averages of at least three independent trials, and standard deviations are shown as error bars. Background (Bkg) release activity is the average percent transcript release in the absence of added dATP from NT112 templates. *D*, phosphorimage of an example of a 12% denaturing polyacrylamide gel with two O-MR assays on the same templates described in *B*. O-MR assays were conducted and analyzed as described in Fig. 2D. Percent transcript release is listed below the B and S pair of lanes for each reaction. *E*, bar graph representation of the percent oligonucleotide-mediated transcript release on templates that contain ribonucleotides at different positions within the transcription bubble. Values are the average of at least three independent trials, and standard deviations are shown as error bars. Background release activity is the average percent transcript release in the absence of added oligonucleotide from all templates tested.

within the transcription bubble (data not shown). Together these results demonstrate that the ssDNA cofactor for NPH I-mediated transcript release cannot be provided in *trans*.

Transcription Termination Is Inhibited by Ribonucleotides within the Non-template Strand of Transcription Bubble—The N-MR transcript release assay from stalled transcription elongation complexes has the advantage of isolating NPH I function in the termination process. However, as such, this assay does not assess the entire termination mechanism. To further examine the role of the ssDNA cofactor of NPH I in transcription termination, we compared termination from actively transcribing elongation complexes *in vitro* using a DNA-RNA template

that was defective for N-MR with a full-length dsDNA template (Fig. 6). The transcription termination assay is similar to the transcript release assay described above except all four NTPs are included in the second step. Under these conditions, termination requires the presence of a U₅NU signal in the nascent RNA and the viral CE, also known as VTF, in addition to NPH I (1, 11, 13, 19, 30).

We first compared transcription termination on the NT112 transcription template with the previously characterized Ter29 transcription template (10, 11) (Fig. 6*B*). The RNAs produced with this assay include readthrough transcripts starting at +1 and continuing to the end of either template (189 nucleotides

NPH I ssDNA 5' to 3' Translocation

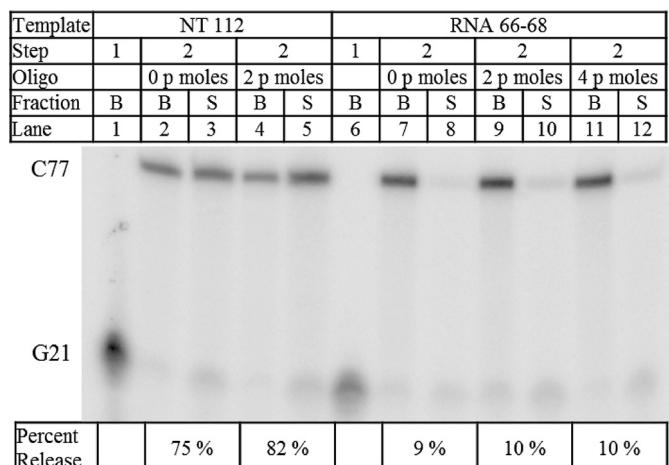


FIGURE 5. The ssDNA cofactor for NPH I-mediated transcript release cannot be provided in trans. A phosphorimage of a 12% denaturing polyacrylamide gel analysis of NPH I-mediated transcript release assays on templates that either were composed of full-length double-stranded DNA (NT112) or contained ribonucleotides in the upstream region of the transcription bubble (RNA66–68). N-MR assays were conducted as described in Fig. 2B except increasing amounts of a 24-base oligonucleotide capable of activating ATPase activity of isolated NPH I were added to the second step of transcription where indicated (lanes 2–5 and 7–12). Percent transcript release is listed below the bound (B) and supernatant (S) pair of lanes for each reaction.

for Ter29 and 112 nucleotides for NT112) and terminated transcripts, which are ~75 nucleotides for either template. As seen previously (11), in the presence of VTF and NPH I, transcription of Ter29 resulted in 50–60% of the transcripts terminating at approximately +75, whereas the remaining continued to the end of the template (Fig. 6*B*, lane 1). Transcription of NT112 in the absence of added CE yielded only readthrough transcripts (Fig. 6*B*, lane 2) (note that the 112-nucleotide transcript from NT112 is shorter than the corresponding 189-nucleotide readthrough transcript from Ter29). In the presence of CE, transcription termination increased to ~50% on NT112 (Fig. 6*B*, lane 3).

We then examined the effects of ribonucleotides in the upstream region of the NT strand within the transcription bubble when the TEC approaches the site of termination on RNA63–74 (Fig. 6*A*). Transcription of the RNA63–74 template yielded low levels (~10–15%) of termination in the absence or presence of CE (Fig. 6*B*, lanes 4 and 5). Hence, as was seen in the transcript release assays (Fig. 4), the presence of ribonucleotides on the non-template strand in the upstream region of the transcription bubble inhibits transcription termination, likely by preventing activation of NPH I.

NPH I Translocates in a 5' to 3' Direction on ssDNA—NPH I contains sequence motifs characteristic of the DEXH box superfamily 2 (SF2) helicases (10, 14). Although these motifs have been shown to be essential for ATPase activity (14), helicase activity has not been observed with NPH I on dsDNA or RNA (9, 31, 32). Prior work demonstrated that NPH I-mediated transcript release involves forward movement of vRNAP in the absence of nucleotide addition (11). Our results presented here indicate that NPH I-mediated transcript release activity relies on the non-template strand in the upstream region of the transcription bubble. Together these observations suggest that

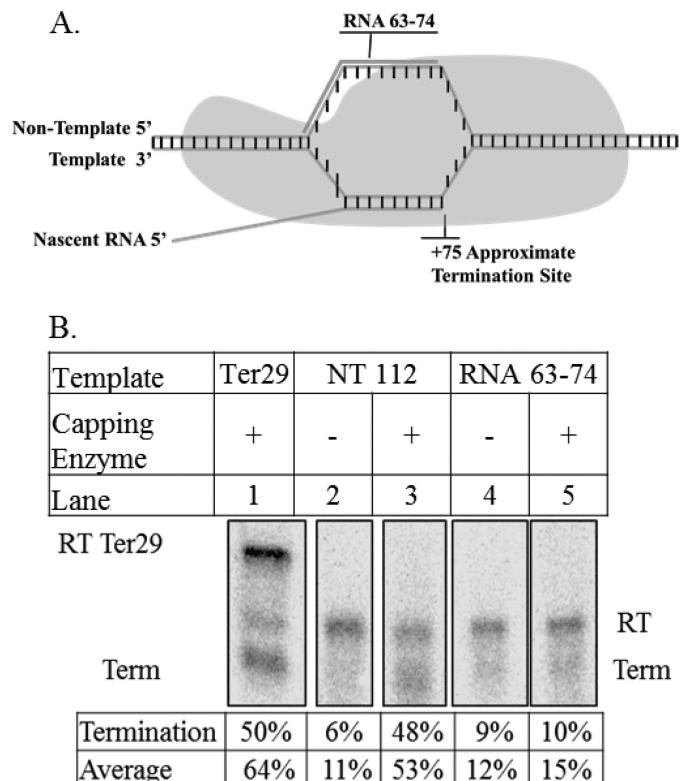
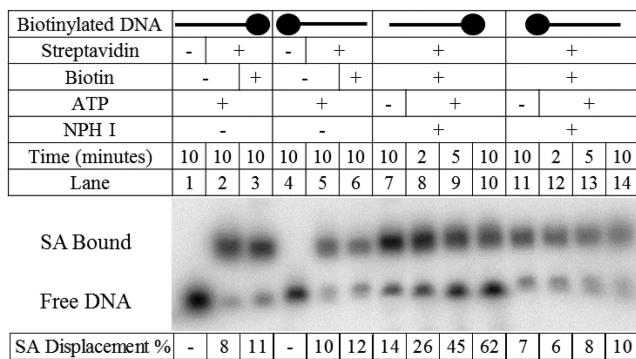


FIGURE 6. Ribonucleotides in the upstream portion of the transcription bubble inhibit vaccinia early gene transcription termination. *A*, diagram depicting the position of ribonucleotides within the ternary complex when the active site of vaccinia RNA polymerase is at the approximate site of termination, +75. The ribonucleotide positions are shown by the line above the non-template strand within the transcription bubble. The name of the template is written at the top of the diagram. *B*, several lanes from a phosphorimage of a transcription termination experiment separated on a 12% denaturing polyacrylamide gel. Transcription termination assays were conducted in two steps. The first step involved initiating transcription in the presence of [α^{32} P]UTP, CTP, ATP, and 3'-O-methyl-GTP to produce 21-nucleotide transcripts. After the first step, transcription reactions were washed with transcription buffer to remove capping enzyme and unincorporated nucleotides. The second step of transcription involved incubation in the presence of CTP, GTP, UTP, ATP, and where indicated CE (VTF). The Ter29 template produces readthrough transcripts of 189 (RT Ter29) nucleotides and terminated transcripts of ~75 nucleotides (Term). The oligo-based templates produce readthrough transcripts of 112 nucleotides (RT) and terminated transcripts of ~75 nucleotides (Term). Percent termination was analyzed using ImageQuant, and the average percent termination was calculated from three independent trials. Percent termination was calculated by dividing the radiolabeled transcripts that accumulated at the site of termination by the sum of readthrough transcripts and terminated transcripts.

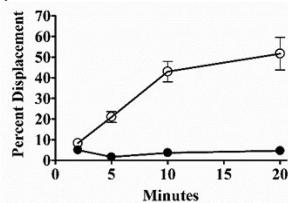
NPH I may translocate in a 5' to 3' direction on this strand as part of the transcript release mechanism.

We tested the ability of NPH I to translocate on ssDNA by examining its ability to displace streptavidin from biotinylated oligonucleotides using a previously described assay (33–38). Streptavidin displacement was tested using 24-mer or 36-mer oligonucleotides that were biotinylated at either the 5' or 3' end and visualized by native gel electrophoresis (Fig. 7*A*). Streptavidin-bound DNA migrates slower than free DNA (Fig. 7*A*, compare lanes 1 and 2 and lanes 4 and 5). To prevent rebinding of the displaced streptavidin to biotinylated DNA, free biotin was included in the reactions. Free biotin did not displace the streptavidin interaction from either oligonucleotide (Fig. 7*A*, lanes 3 and 6). When NPH I was incubated with either the 5'-biotinylated or 3'-biotinylated 36-mer oligonucleotide in the

A.



B.



C.

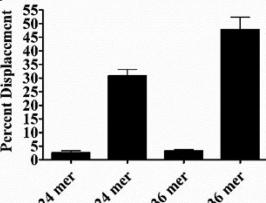


FIGURE 7. NPH I translocates 5' to 3' on single-stranded DNA. A, phosphorimage of a 10% native polyacrylamide gel of a streptavidin displacement assay. Assays were conducted on 36-mer oligonucleotides that were biotinylated at either the 3' end or 5' end as depicted at the top of the figure. Streptavidin was allowed to bind to the oligonucleotides for 1 h. Then biotin, ATP, reaction buffer, and NPH I were added to the streptavidin (SA)-bound oligonucleotides. Reactions were incubated at 30 °C for the times indicated and then stopped with quench buffer. The location of the free DNA and streptavidin-bound DNA are indicated to the left. Percent streptavidin displacement was calculated by dividing the amount of free DNA by the total DNA in the reaction. **B,** line graph representing the percent streptavidin displacement from 36-mer oligonucleotides versus incubation time from magnetic bead-bound streptavidin displacement assays. Open circles represent streptavidin displacement from 36-mer DNA biotinylated at the 3' end, and closed circles are for the same DNA biotinylated at the 5' end. Background streptavidin displacement in the absence of ATP was subtracted from the values in the presence of ATP. Values are the average of at least three independent trials with standard deviations shown as error bars. **C,** bar graph representing the percent streptavidin displacement at 10 min from bead-bound oligonucleotides biotinylated at either the 5' or 3' ends. The oligonucleotides were either 24 nucleotides long or 36 nucleotides long and are indicated in the names along the x axis.

absence of ATP, streptavidin displacement was not observed (Fig. 7A, lanes 7 and 11). However, when ATP was included in the reactions, NPH I was capable of disrupting the biotin-streptavidin interaction only when the biotin was at the 3' end of the oligonucleotide (Fig. 7A, compare lanes 8–10 and lanes 12–14).

We also examined the ability of NPH I to displace biotinylated DNA from streptavidin-coated magnetic beads. This assay has the advantages of being rapid and quantitative (Fig. 7, B and C). Using this assay, we found that streptavidin displacement from the 3' end of either the 24-mer or 36-mer increased with time up to 10 min and then plateaued (Fig. 7B). NPH I was slightly more efficient at displacing streptavidin from the 3' end of a 36-mer than from a 24-mer oligonucleotide (Fig. 7C). Streptavidin displacement was not observed when the biotinylated oligonucleotides were annealed to complementary oligonucleotides, consistent with the prior observation that NPH I is incapable of helicase activity (10, 14, 21) (data not shown).

Discussion

Transcription termination involves the cessation of RNA polymerization and release of nascent RNA from the transcription complex. Vaccinia virus early gene transcript release is mediated by NPH I in an ATP-dependent manner (10, 11, 15). In addition, NPH I acts as a positive elongation factor to assist transcription through problematic sequences (10). Although the amino acid sequence of NPH I contains DEXH SF2 helicase motifs, unwinding of duplex nucleic acid has not been observed (10, 14, 21). Isolated NPH I requires a ssDNA cofactor for ATPase activity (21); however, this cofactor does not need to be added to *in vitro* transcription reactions to observe NPH I-mediated transcript release activity (11, 15), indicating that it is provided by the transcription template.

In this study, we demonstrate that NPH I acquires its single-stranded DNA cofactor from the non-template strand in the upstream portion of the transcription bubble. When the non-template DNA was removed from the transcription bubble, transcript release decreased. Additionally, we found that complementarity between the non-template and template DNA within the transcription bubble is required for vaccinia early gene transcript release. When ribonucleotides were introduced into the non-template DNA within the upstream portions of the transcription bubble, NPH I-mediated transcript release activity was specifically inhibited (Fig. 4).

A prior study demonstrated that NPH I-mediated transcript release is mediated by a forward translocation mechanism (11). In other systems, forward translocation of RNA polymerase has been shown to displace the active site from the 3' end of the nascent transcript, shorten and destabilize the DNA-RNA hybrid, and lead to the release of the nascent transcript from the transcription complex (11, 28, 39). Together the observations that NPH I utilizes the upstream portion of the non-template strand as its ssDNA cofactor and that it induces forward movement of RNAP (11) suggest that NPH I uses the energy of ATP hydrolysis to translocate along ssDNA in the 5' to 3' direction. Consistent with this suggestion, we observed that isolated NPH I can displace streptavidin from the 3' end of biotinylated oligonucleotides but not from the 5' end (Fig. 7). The same 5' to 3' translocation mechanism utilized in transcript release could also be utilized by NPH I to act as a positive elongation factor and assist transcription elongation through difficult sequences.

Translocation in a 5' to 3' direction is unusual among members of the SF2 DEXH box helicases, most of which translocate in the 3' to 5' direction (32, 40–44). The xeroderma pigmentosum group D proteins are the only characterized SF2 helicases that translocate 5' to 3' direction along DNA (32, 33, 45). The iron-sulfur cluster responsible for coupling ATP hydrolysis to 5' to 3' DNA translocation in the archaeal xeroderma pigmentosum group D protein family member Rad3 (33, 46) is not present in the sequence of NPH I. Thus, another feature of NPH I determines 5' to 3' translocation directionality.

Several helicases and translocases from other organisms function in transcription termination similarly to NPH I. In bacteria, factor-dependent transcript release is mediated by two well characterized factors, Rho (47, 48) and Mfd (29, 39). Rho is an RNA helicase that translocates 5' to 3' along the

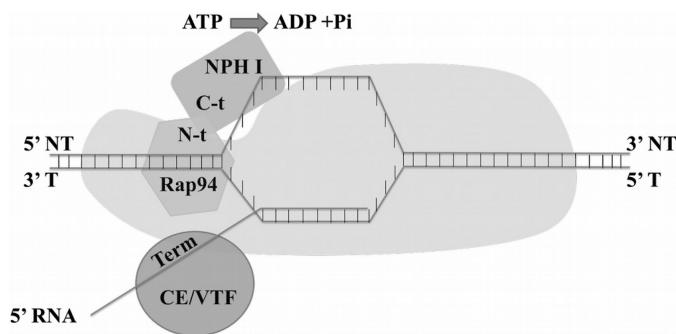


FIGURE 8. Updated model of early gene transcription termination. A depiction of the vaccinia early gene transcription elongation complex based on prior structural studies (27) is shown. NPH I is bound to the transcription complex through interaction between the C-terminal (*C-t*) region of NPH I and the N-terminal region of Rap94 (*N-t*) (15, 19). NPH I is located in the upstream region of the transcription bubble where it acquires its ssDNA cofactor from the NT strand. CE/VTF interacts with the termination signal in the nascent RNA (*Term*), pausing RNA polymerase near the site of termination (7). Hydrolysis of ATP by NPH I induces forward translocation of the elongation complex in the 5' to 3' direction relative to the non-template DNA strand and leads to release of the nascent RNA and transcription termination (11).

nascent RNA as part of the transcription termination mechanism (49, 50). Mfd binds to dsDNA upstream of stalled transcription complexes and translocates downstream to release transcription complexes at sites of DNA damage as part of the transcription-coupled repair mechanism (39). Examples of eukaryotic factors shown to dissociate the ternary complex *in vitro* include TTF2 (51, 52) and Sen1 (53, 54). TTF2 is a dsDNA-dependent ATPase that contains helicase motifs and couples ATP hydrolysis with transcript release (51). Sen1 is an RNA helicase from budding yeast that functions similarly to Rho (54). Additionally, *Arabidopsis thaliana* contains a Rho-like protein called Rhon1, which is involved in transcription termination in plastids (55). Understanding the mechanism of NPH I transcript release may also provide insight into a more widely used mechanism of transcript release.

In vaccinia, another DEXH box SF2 helicase family protein called A18 functions in transcription termination of intermediate and late genes (56, 57). Viruses harboring a temperature-sensitive mutation in A18 produce abnormally long intermediate gene transcripts during infection at the non-permissive temperature (58). A18 is also a ssDNA-dependent ATPase that has been shown to unwind short DNA duplexes in the more commonly observed 3' to 5' direction (32, 57, 59, 60). If A18 also induces transcript release through a forward translocation mechanism similarly to NPH I, then it likely utilizes the template strand to translocate in a 3' to 5' direction to move vRNAP forward. Prior attempts to replace NPH I with A18 for early gene transcription termination were unsuccessful.³ The intermediate/late form of vaccinia RNA polymerase lacks the early gene-specific subunit Rap94, which interacts with NPH I (19). Instead A18 has been shown to interact with two viral proteins, G2 and H5, involved in transcription regulation of intermediate and late genes (61, 62).

An updated model of the arrangement of the ternary complex and mechanism of vaccinia early gene transcription termination can be proposed from the results presented in this study

and prior studies (Fig. 8). Based on cross-linking studies, Rap94, CE/VTF, and three subunits of vaccinia RNA polymerase (J6, A24, and D7) are arranged in the ternary complex such that they interact with the emerging nascent RNA (13). NPH I is bound to the transcription complex through an interaction between the C-terminal region of NPH I and the N-terminal region of Rap94 (19). NPH I is located in a position close to the upstream region of the transcription bubble where it acquires its ssDNA cofactor. When CE/VTF induces pausing of transcription near the site of termination (7), NPH I hydrolyzes ATP to translocate in the 5' to 3' direction on the non-template DNA in the absence of nucleotide addition. The movement of NPH I results in the forward movement of vRNAP, destabilizes the DNA-RNA hybrid, and leads to the release of the nascent RNA from the transcription complex (11).

Author Contributions—R. H. conducted the experiments, analyzed the results, and wrote the paper. P. G. conceived the idea for the project, acquired funding for the project, and wrote the manuscript with R. H.

Acknowledgments—We thank Dr. Rich Condit, Dr. Ed Niles, Dr. Stewart Shuman, and Dr. Nissin Moussatche for critical review of the manuscript and conducting the experiments. We especially want to thank Nissin Moussatche and Nicole Kay for help preparing the vaccinia virus used in these studies. We also thank Andrea Patterson and Sally Enriquez for help in purifying NPH I. We also thank Dr. Jessica Tate for suggestions and technical advice in performing the experiments.

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