Role of Forward Translocation in Nucleoside Triphosphate Phosphohydrolase I (NPH I)-mediated Transcription Termination of Vaccinia Virus Early Genes

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Background: The enzyme NPH I is involved in vaccinia early gene transcription termination.

Results: Transcript release occurred in the presence of NPH I and dATP only when RNA polymerase can move forward in the absence of transcription elongation.

Conclusion: NPH I causes transcript release during vaccinia early gene transcription termination using the mechanism of forward translocation.

Significance: Vaccinia offers insight into the mechanisms behind eukaryotic transcription termination.

Termination of transcription of vaccinia virus early genes requires the virion form of the viral RNA polymerase (RNAP), a termination signal (UUUUUNU) in the nascent RNA, vaccinia termination factor, nucleoside triphosphate phosphohydrolase I (NPH I), and ATP. NPH I uses ATP hydrolysis to mediate transcript release, and in vitro, ATPase activity requires single-stranded DNA. NPH I shows sequence similarity with the DEAH-box family of proteins, which includes an *Escherichia coli* ATP-dependent motor protein, Mfd. Mfd releases transcripts and rescues arrested transcription complexes by moving the transcription elongation complex downstream on the DNA template in the absence of transcription elongation. This mechanism is known as forward translocation. In this study, we demonstrate that NPH I also uses forward translocation to catalyze transcript release from viral RNAP. Moreover, we show that NPH I-mediated release can occur at a stalled RNAP in the absence of vaccinia termination factor and U5 NU when transcription elongation is prevented.

The mechanism of transcription termination in eukaryotes has been difficult to elucidate, in part due to temporally overlapping post-transcriptional modifications. Much of the current understanding of transcription termination mechanisms comes from studies of bacterial systems. In bacteria, two distinct types of transcription termination have been identified as follows: intrinsic and factor-dependent (1). Intrinsic termination relies only on cis-acting elements, including a GC-rich stem-loop followed by 7–9 U residues in the nascent transcript, whereas factor-dependent termination requires trans-acting ATPases, such as the Rho protein (1). One model, known as forward translocation, suggests that these two termination methods use the same underlying mechanism to cause termination (2, 3). This model suggests that downstream movement of RNAP in the absence of RNA chain elongation is, at least in part, responsible for terminating transcription (Fig. 1). This movement results in rewinding of the upstream portion of the transcription bubble, displaces the 3’ end of the RNA from the active site, and shortens the DNA/RNA hybrid in the transcription bubble. Together, these events destabilize the elongation complex and result in transcript release.

Eukaryotic termination may share features with prokaryotic termination given the structural similarities of RNAPs as well as the correlation between the stability of the DNA/RNA hybrid with transcript release (4, 5). Vaccinia early gene transcription termination provides a unique model system to examine eukaryotic termination possessing both cis- and trans-acting components that is uncoupled from splicing and polyadenylation (6, 7).

Vaccinia is a cytoplasmic double-stranded DNA pox virus that encodes most of the proteins needed for gene transcription and mRNA processing (7). Vaccinia genes are categorized into three temporal classes based on time of expression, regulatory sequences, and transcription factors (6). Early gene transcription occurs in the virion core immediately following infection and employs a virion-specific form of RNAP (vRNAP), which contains one subunit (called Rap94 or H4, which is the product of the *H4L* gene) in addition to the other eight subunits present in all forms of vaccinia RNAP (8, 9). Termination of early gene transcription is dependent on a UUUUUNU signal in the nascent RNA, and termination occurs within a 10-base range, 30–50 bases downstream of the signal (10–12). Two virally encoded termination factors are also required for early gene transcription termination as follows: vaccinia termination factor (VTF) (13, 14) and nucleoside triphosphate phosphohydrolase I (NPH I) (15–17). VTF is multifunctional, catalyzing the

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3 The abbreviations used are: RNAP, RNA polymerase; vRNAP, viral RNAP; VTF, vaccinia termination factor; TEC, transcription elongation complex; IC, 5′-ido-CTP; BrU, 5′-bromo-UTP; NPH I, nt, nucleotide; N-MR, NPH I-mediated transcript release; O-MR, oligonucleotide-mediated release; oligo, oligonucleotide; NPHI, nucleoside triphosphate phosphohydrolase I.
Forward Translocation in Vaccinia Transcription Termination

The mechanism by which NPH I functions in termination is not known. We assessed the function of NPH I in transcription elongation and in termination using two assays referred to as either termination or transcript release. Termination assays assess the production of released transcripts from actively elongating vRNAP, whereas transcript release assays evaluate transcript release from artificially stalled vRNAP. Prior studies have demonstrated the requirement of VTF and the termination signal for both termination and transcript release activity when NPH I is stimulated with ATP (16, 24). In this study, we show that NPH I-mediated transcript release can occur independent of VTF and U₅NU when NPH I is stimulated with dATP, as opposed to ATP. We also provide evidence that NPH I uses the mechanism of forward translocation to release transcripts. In addition to NPH I, transcript release can also be stimulated by antisense oligonucleotides complementary to the nascent transcript in the absence of NPH I ATPase activity. Similar to NPH I, these oligonucleotides also appear to stimulate transcript release using the mechanism of forward translocation. We also present data indicating that NPH I acts as a positive elongation factor by preventing vRNAP from backtracking.

MATERIALS AND METHODS

Template Preparation for in Vitro Transcription Assays—The Ter29 plasmid used in these studies was designed by Dr. Stewart Shuman (Memorial Sloan Kettering) as a template for in vitro transcription termination and transcript release assays (Fig. 2A) (25). This template consists of a strong early gene promoter fused to a 20-bp G-less cassette followed by four Gs and then a 57-bp A-less cassette, in pBlueScript (Stratagene). The A-less cassette contains a stretch of nine Ts beginning at +29 that functions as a signal for termination. The A-less cassette is followed by four A residues and then 108 bp of random sequence. Templates used in EcoRI Q111E roadblock experiments were created by site-directed mutagenesis of Ter29. The EcoRI sites flanking the promoter were first removed, and an EcoRI site was then created at the desired location on the template. Templates lacking the termination signal had the T9 termination sequence replaced with 5'-CGTGTGTTC-3' and were designed and provided by Dr. Edward Niles (24). Transcription templates were generated by PCR amplification of the appropriate plasmids using the M13 forward and reverse primers with the latter containing biotin at the 5' end (IDT). The amplified linear double-stranded templates were purified by electrophoresis on agarose gels and then extracted from the gel using a Qiagen spin-column purification kit. Purified templates were then bound to streptavidin-coated paramagnetic beads (Promega or Dynal) and stored in water at 4 °C.

Virion Extracts—Transcription extracts were prepared from purified virions as described previously (10). Vaccinia virions were a generous gift from Dr. Edward Niles. Approximately 5 mg of purified virions were incubated on ice in 500 µl of Buffer A (250 mM KCl, 100 mM Tris-Cl, pH 8.4, 100 µM EDTA, 10 mM DTT) containing 0.2% sodium deoxycholate for 1 h. The virion extract was then passed through a 25-gauge needle six times to shear DNA and reduce viscosity. Insoluble materials were pelleted by centrifugation at 13,000 x g for 20 min at 4 °C. The resulting supernatant was then applied to a DEAE-cellulose column equilibrated with Buffer A. 500-µl fractions were collected of the flow-through and assayed for transcriptional activity (10).

Purification of Q111E EcoRI—A plasmid with the gene for the cleavage-deficient EcoRI E111Q (EcoRIQ) protein was a kind gift from Dr. Paul Modrich (Duke University) (37). The coding
sequence for both EcoRI* and the associated methylase were amplified by PCR and ligated into NdeI- and BamHI-cut pET17b (Novagen). Six histidine (His) codons were added to the amino terminus of the EcoRI* gene using the QuickChange mutagenesis kit from Stratagene. In addition, the methylase gene was amplified by PCR separately and ligated into the tetracycline resistance (tet) gene of pACYC184 using BamHI and Sall such that the methylase gene is expressed from the tet gene promoter. Both plasmids were transformed into *E. coli* BL21(DE3). The resulting strain was grown in LB with ampicillin (100 µg/ml) and chloramphenicol (30 µg/ml) at 37 °C until the *A*₅₀₀ reached 0.5, at which point isopropyl-1-thio-β-D-galactopyranoside was added to 0.4 mM, and growth was continued for 4 h. Cells were harvested by centrifugation and broken with a French pressure cell, and EcoRI* was purified using nimmel-agarose as per manufacturer’s instructions (Qiagen).

**In Vitro Transcription Termination Assays**—Transcription termination assays involved a two-step pulse-chase reaction as described previously (21, 25). Step 1 of transcription was conducted with 1 mM ATP, 1 mM CTP, 0.25 mM 3′O-methyl-GTP, 5 µCi of [α-32P]UTP, 100 fmol of template, and 0.5 µl of virion extract in transcription buffer (20 mM Tris-HCl, pH 8.0, 6 mM MgCl₂, 2 mM DTT, 8% glycerol). Reactions were incubated at 30 °C for 15 min. Step 1 of transcription allows initiation and elongation to the end of the G-less cassette to yield a stable transcription elongation complex (TEC) with a 21-base radioactive nascent transcript (see Fig. 2A). Where indicated, transcription complexes were stripped of NPH I with heparin (625 ng/ml) and chloramphenicol (30 µg/ml) and incubated for 10 min at 30 °C prior to transcription.

*In Vitro Transcription Release Assays*—Transcription release assays from stalled TECs were carried out in a three-step process. The first two steps were done as described for termination assays, except that ATP was omitted from Step 2, which results in the TECs stalling at C77 at the end of the A-less cassette (see Fig. 2A). These stalled TECs were washed three times with transcription buffer and then incubated at 30 °C for 10 min (Step 3). Where indicated, 1 mM ATP or deoxy-ATP, 17 ng of oligonucleotide, and/or 1 mM VTF was added in Step 3. Transcripts were separated by electrophoresis through a 12% (19:1) polyacrylamide 8 m urea gel. The RNA was visualized by autoradiography as described for transcription termination assays, and the fraction of released transcripts was calculated by dividing the amount of released RNA by the sum of the RNA released and that remaining in the ternary complex. For experiments with EcoRI Q111E, 15 ng (484 fmol) of EcoRI* was added per 100 fmol of DNA template and incubated in transcription buffer for 10 min at 30 °C prior to transcription.

**RESULTS**

*U₅NU and VTF Are Required for Early Gene Transcription Termination but Not for NPH I-mediated Transcript Release at a Stalled vRNAP—*We analyzed early gene transcription in vitro using bead-bound dsDNA templates based on the synthetic Ter29 template (Fig. 2A) (25). Ter29 contains a strong early promoter immediately followed by a 20-bp G-less cassette, which lies upstream of a 57-bp A-less cassette containing a run of 9 Ts within the nontemplate strand. Once transcribed into RNA, these nine Us constitute the termination signal. Transcription reactions were generally performed in two steps. In Step 1, transcription initiates at the early promoter, and in the absence of GTP and the presence of 3′O-methyl-GTP, transcription continues to the end of the G-less cassette to yield stable TECs containing a 21-base radiolabeled nascent RNA (G21) (26). In some cases (where indicated), endogenous VTF was washed away with transcription buffer, and endogenous NPH I was stripped from the TECs by heparin treatment following Step 1 (26). The second step of the transcription assay consisted of a single round of elongation of the template-bound TEC by addition of all four NTPs. The intrinsic hydrolytic activity of vRNAP cleaves the 3′O-methyl-GMP from the 3′ end of the nascent RNA to regain productive elongation complexes (27). Individual reactions were supplemented with VTF and/or NPH I prior to Step 2. Transcription reactions yielded 189-base readthrough transcripts or ~75 nucleotide terminated transcripts (Fig. 2A).

As seen previously, with the template encoding the U₅NU termination signal, terminated transcripts are only observed when both VTF and NPH I are present (Fig. 2B) (24, 28). This observation also demonstrates that endogenous VTF and NPH I were efficiently removed from the TECs by the washing steps after Step 1 of the transcription assay. In the absence of the termination signal (CGUGUGUC-3′ in place of 5′-UUUUUUUUU-3′), only read-through transcripts were produced in the absence or presence of VTF and/or NPH I (Fig. 2B). As seen previously (24, 28), these results highlight the requirement for all termination factors (U₅NU, VTF, (d)ATP, and NPH I) to achieve early gene transcription termination in a standard transcription reaction.

To specifically examine the function of NPH I in the termination process, we employed an assay for NPH I-mediated transcript release (N-MR) independent of VTF and the U₅NU termination signal. For this transcript release assay, VTF and NPH I were removed from the TECs at the end of the G-less cassette. Step 2 of transcription was performed with GTP, CTP, and UTP in the absence of ATP, which results in vRNAP stalling at the end of the A-less cassette at residue C77 (Fig. 2A). These stalled TECs are stable and competent to resume transcription, with only ~10% of the transcripts being released into the supernatant after 10 min at 30 °C. Step 3 of transcription involved an additional incubation of the TECs stalled at C77 in the presence of dATP and either in the presence or absence of NPH I and/or VTF. When dATP was added in the absence of NPH I, only
10–20% of the transcripts were released regardless of the presence of VTF (Fig. 2C, lanes 1–4). This low amount of transcript release likely occurs from a small fraction of TECs from which NPH I was not removed by the heparin treatment after Step 1. These observations are consistent with prior studies showing that transcript release is dependent on NPH I ATPase activity (15, 16, 25, 26).

When WT NPH I was added in the presence of dATP during Step 3, ≥50% of the transcripts were released from the ternary complex when the TEC stalled at C77 (Fig. 2C, lanes 5 and 6). Under these conditions, transcript release was not dependent on VTF, although a slight difference is observed in this particular gel (Fig. 2C, lanes 5–8). Similarly, the U₅₆NU termination signal is not required for transcript release under these conditions (Fig. 2C, lanes 15–18). Addition of the ATPase-deficient NPH I mutant, K61A (16), resulted in background level of transcript release (Fig. 2C, lanes 9 and 10, and 19 and 20). Together these observations support the conclusion that when vRNAP is stalled on the template, NPH I can mediate transcript release through its ATPase activity independent of VTF or the U₅₆NU sequence.

Our results indicate that the requirements for transcript release from a stalled TEC (Fig. 2C) differ from those to induce transcription termination from an actively elongating TEC (Fig. 2B). Specifically, transcription termination requires the U₅₆NU signal and VTF in addition to NPH I activity, whereas transcript release requires only NPH I activity and is independent of the termination signal and VTF. Our results also differ from previously published studies of transcript release from vRNAP stalled at the end of the A-less cassette of Ter29, which demonstrated a requirement for VTF and the U₅₆NU sequence in the nascent transcript in addition to NPH I (16). However, in those experiments, ATP was used to activate NPH I rather than dATP.

We therefore compared NPH I-mediated transcript release (N-MR) in the presence of ATP to that seen above with dATP
In the absence of VTF, the presence of ATP resulted in production of some transcripts that are shorter than C77 and others that elongated past C77, presumably to A81 by addition of four A residues (Fig. 3A, lanes 7 and 8). All of these transcripts remain stably bound to the DNA template and are not released by ATP-activated NPH I. When these elongated transcripts were allowed to form in the presence of ATP followed by activation of NPH I with dATP, transcript release was still not observed (Fig. 3A, lanes 9 and 10). This result indicates that these TECs are incompetent for release.

To examine the role of the U5NU sequence in transcript release under these conditions, these experiments were repeated with a template lacking the T5NT sequence (Fig. 3B). The appearance of the shorter bands was less evident using this template, which may be the result of different template sequence at the end of the A-less cassette. The last 10 templated residues of the A-less cassette in Ter29 are 5’-GGUCGCCUGUC-3’, whereas the last 10 residues in the template lacking the termination signal are 5’-GUUCCUGUU-3’. In general, a similar pattern of transcript release was observed on the template lacking the termination signal (Fig. 3B, lanes 1–12) as on the template containing the termination signal (Fig. 3A, lanes 1–12). However, in this case ATP no longer stimulated transcript release at C77 on the –U5NU template in the presence of VTF compared with the +U5NU template (compare Fig. 3B, lanes 13 and 14, with A, lanes 13 and 14). These observations confirm that both U5NU and VTF are required for transcript release when vRNAP is stalled at the end of the A-less cassette and when ATP is used as a substrate for NPH I. Some transcript release from this template is seen beyond A81 in the presence VTF and ATP (Fig. 3B, lane 14).

One possible explanation for the different requirements for transcript release in the presence of dATP as opposed to ATP is that although both nucleotides activate NPH I, only ATP serves as a substrate for RNAP. This difference is particularly relevant when the TEC is stalled at C77 in Ter29 because the next four templated residues to extend the RNA are A residues (Fig. 2A). Examination of the results in Fig. 3A shows that in the presence of ATP a significant fraction of the TECs that were stalled at C77 become elongated in the absence of VTF or the U5NU signal (presumably to A81), and these transcripts remain bound to the ternary complex (Fig. 3, A and B, lanes 7 and 8). Additionally, in these reactions a class of TECs that are located upstream of C77 do not elongate in the presence of ATP (Fig. 3, A and B, lanes 7 and 13). The presence of VTF and the termination signal significantly increased NPH I-mediated transcript release from TECs at C77 in the presence of ATP (Fig. 3A, compare lanes 7 and 8 and 13 and 14); however, transcripts that extended beyond C77 largely remained bound to the TEC. These observations show that, as seen previously (16, 24), both VTF and the termination signal are necessary for NPH I-mediated release when vRNAP has the ability to elongate.

**NPH I Prevents TECs from Backtracking**—To examine the formation of the heterogeneous transcripts (stretching from approximately +72 to +77 nts) seen in Fig. 3A, we examined the length of the transcripts from TECs stalled at C77 before and after they were incubated for 10 min following Step 2 (Fig. 2A). As seen above, following Steps 1 and 2 of the transcription

### FIGURE 3. Transcription elongation inhibits NPH I-mediated release. In vitro transcript release assays with TECs stalled at the end of the A-less cassette in Ter29 (+U5NU). Following Step 2 of transcription, TECs were washed with transcription buffer to remove NTPs and incubated for an additional 10 min at C77 (Step 3). NPH I was stimulated with 1 mM of either dATP or ATP. The effects of VTF were examined on NPH I-mediated transcript release in the presence (A) or absence (B) of the termination signal. Released transcripts in the supernatant (S) were separated from those remaining on the bead-bound ternary complexes (B) and analyzed on a 12% polyacrylamide gels. The location of an RNA ending at C77 is indicated. The percentage of transcripts released at C77 was calculated by dividing the amount of +77 RNA in the supernatant by the total RNA at C77 in the reaction (B + S) and listed below the B and S pair of lanes for each reaction.

| Lane | % Release at C77 |
|------|------------------|
| 1    | 1                |
| 2    | 1                |
| 3    | 73               |
| 4    | 21               |
| 5    | 20               |
| 6    | 76               |
| 7    | 79               |
| 8    | 72               |
| 9    | 77               |
| 10   | 43               |
| 11   | 15               |
| 12   | 15               |
| 13   | 15               |
| 14   | 15               |

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reaction the TECs (including NPH I) were stably stalled at C77 (Fig. 4A, lane 1). Adding dATP at the start of Step 2 had little effect on the length of the C77 transcripts (Fig. 4A, lane 2), although a small fraction of the TECs showed extension beyond C77, presumably due to a trace amount of ATP contamination of dATP.

We then examined the effects of incubating these TECs stalled at C77 for an additional 10 min under various conditions, which we term Step 3 of the transcript release assay (Fig. 2A). In the presence of only transcription buffer alone, the majority of the transcripts that were at C77 after Step 2 became shorter during Step 3 (Fig. 4A, lane 3). A similar phenomenon has been observed with other RNA polymerases, including bacteria (29) and yeast (30, 31), and has been shown to be the result of a process termed backtracking. Backtracking occurs when RNAP moves backward on the DNA template, which can lead to arrest of transcription. Such arrested complexes can be reactivated by cleaving the 3' end of the nascent transcript so as to realign the terminal residue with the active site of the enzyme. Prior studies have shown that CTP activates the 3'-exonuclease activity of vRNAP (27, 28). Adding CTP to the Step 3 incubation also resulted in the presence of shorter transcripts (Fig. 4A, lane 6), suggesting that they result from the intrinsic cleavage activity of vRNAP (27). These shortened transcripts are competent for elongation as seen upon addition of all four NTPs (Fig. 4A, lane 7), indicating that when the transcripts are cleaved, the 3' end of the RNA is aligned with the active site of vRNAP (27). The presence of dATP during Step 3 prevented shortening of the C77 transcripts, presumably due to NPH I activity (Fig. 4A, lane 4).

The results presented in Fig. 4A show that activating NPH I with dATP prevents accumulation of shortened transcripts from TECs stalled at C77. This effect could be the result of NPH I preventing formation of the shortened transcripts or due to NPH I facilitating the repair/extension of these transcripts after they are formed. To distinguish between these two possibilities, we allowed the shortened transcripts to form during Step 3 as above (Fig. 4A, lane 3), washed the complexes, and then added dATP to stimulate NPH I (Step 4) (Fig. 4A, lane 8). In this case, dATP had no effect on these shortened transcripts, suggesting that activated NPH I prevents formation of these short transcripts but cannot repair shortened transcripts after they are formed. Furthermore, the cleaved transcripts are stable during Steps 3 and 4 as observed by their ability to elongate in the presence of all four NTPs (Fig. 4A, lanes 7 and 11). For reasons that remain unclear, NPH I stimulated by ATP failed to prevent formation of these cleaved transcripts (Fig. 4A, lanes 5 and 9). This difference in NPH I-mediated backtracking inhibition when ATP is employed as substrate for NPH I in lieu of dATP may again reflect that ATP is a substrate of vRNAP but dATP is not.

An alternative explanation for the difference in the effects of ATP and dATP on backtracking could be that dATP interferes with the 3'-exonuclease activity of vRNAP directly. To examine whether the effects of dATP on backtracking are mediated by NPH I, the experiment in Fig. 4A was repeated using vRNAP from which NPH I was removed by heparin following Step 1 of transcription (Fig. 4B). As before, the shortened transcripts formed in the absence of nucleotides during Step 3 (Fig. 4B, lane 1) and were competent for elongation upon addition of all four NTPs (Fig. 4B, lane 5). However, addition of dATP to TECs lacking NPH I did not prevent the formation of the shortened transcripts to the same extent as in the presence of NPH I (compare Fig. 4, B, lane 2, with A, lane 4). These observations confirm that the effects of dATP on backtracking inhibition is due to the ATPase activity of NPH I and not due to a direct interference with the vRNAP.

Shortening of the transcripts after RNA polymerase stalls at C77 might occur to realign the 3' end of the transcript with
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RNAP active site of backtracked RNAPs, rendering these TECs competent for elongation. The GreA and GreB proteins serve a similar function in E. coli (32) as does TFIIS in yeast (33). If backtracking precedes transcript cleavage during Step 3 of our transcript release assay, then strengthening the DNA/RNA hybrid should inhibit backtracking and therefore reduce the formation of the shortened transcripts. When vRNAP is stalled at C77 during Step 3 of transcription on the Ter29 template, 6 out of 9 residues of the RNA in the DNA/RNA hybrid are C or U (Fig. 2A). Incorporating nucleotide analogues such as 5′-ido-CTP (IC) and 5′-bromo-UTP (BrU) into the nascent transcript increases the stability of the DNA/RNA hybrid and has been shown to inhibit backtracking of E. coli RNApolymerase (29). We found that the presence of IC and BrU in Step 2 of the transcription reaction with vRNAP resulted in formation of fewer short transcripts than seen in the presence of CTP and UTP (Fig. 4C, compare lanes 1 and 5). NPH I had no effect on the length of these transcripts containing BrU and IC when stimulated with dATP (Fig. 4C, lane 6). These transcripts were elongated by addition of all four NTPs indicating that the presence of IC and BrU did not arrest or release the TECs (Fig. 4C, lanes 4 and 8). Furthermore, CTP no longer resulted in accumulation of short transcripts in the presence of IC and BrU (Fig. 4C, lanes 3 and 7). These results suggest that the short transcripts formed during Step 3 of transcription are the result of the TEC backtracking followed by transcript cleavage. They also suggest that NPH I prevents the formation of these short transcripts by preventing backtracking of the TEC.

NPH I could prevent accumulation of these shortened complexes in one of the following two ways: by releasing transcripts before backtracking occurs or by realigning the 3′ end of the transcript with the vRNAP active site before transcript cleavage occurs. To distinguish between these two possibilities, the homogeneous transcripts formed during Step 3 in the presence of NPH I and dATP were chased with all four NTPs (Fig. 4D). If NPH I prevents the shortened transcripts by transcript release, then there should be little to no elongation past C77. However, if NPH I repairs backtracked complexes before transcript cleavage can occur, then a significant amount of the transcripts will be elongated in the presence of all four NTPs. As seen previously (Fig. 4, A, lane 3, and B, lane 1), the majority of the transcripts were shortened in the absence of dATP (Fig. 4D, lane 1), but in the presence of dATP the transcripts remained 77 nts (Fig. 4D, lane 2). When the transcripts were incubated with dATP and NPH I during Step 3 and then chased with all four NTPs during Step 4, ~50% of the transcripts were extended to readthrough (Fig. 4D, lane 3). These results suggest that NPH I is preventing the formation of shortened transcripts at least in part by fixing backtracked TECs.

DNA/RNA Hybrid Strength Affects NPH I-mediated Transcript Release and Transcription Termination—Destabilizing the DNA/RNA hybrid is a key component of transcript termination mechanisms, including forward translocation (34). If shearing the DNA/RNA hybrid is a requirement for NPH I-mediated transcript release, then stabilizing this hybrid should inhibit release. When NPH I-mediated transcript release was measured at C77, transcripts containing IC and BrU, which stabilize the hybrid, showed ~3-fold less release than those containing C and U (Fig. 5A, compare lanes 3 and 4 with 7 and 8; summarized in Fig. 5B). Similarly, the presence of IC and BrU in the nascent transcript reduced VTF-dependent transcription termination >4-fold (Fig. 5, C and D). In addition to strengthening the DNA/RNA hybrid, the presence of BrU in the nascent transcript is also known to interfere with the interaction between VTF and the U5NU termination signal (14, 35). However, because NPH I-mediated transcript release from a stalled TEC in the presence of dATP can occur independent of VTF and U5NU with a stalled TEC, the decreased transcript release observed in the presence of IC and BrU is most likely due to their effects on the DNA/RNA hybrid. Hence, destabilizing the DNA/RNA hybrid appears to be a significant component of both NPH I-mediated transcript release (N-MR) and may also contribute to the ability of BrU to abolish transcription termination.

Forward Translocation as Mechanism of NPH I-mediated Transcript Release—NPH I-mediated transcript release only occurs in the absence of VTF and U5NU when vRNAP is stalled, and transcription elongation cannot occur (Figs. 2C and 3A). This finding together with the sequence similarity in the DEHX-box motifs between NPH I and the bacterial transcript release protein Mfd suggest that NPH I may also stimulate transcript release through the mechanism of forward translocation. To test this hypothesis, we examined the effects of blocking the forward movement of vRNAP with a protein bound to the DNA template. The cleavage-deficient EcoRI Q111E (EcoRI′) mutant protein stably binds to its recognition sequence, GAATTC, but does not cleave the DNA. EcoRI′ bound to the DNA template blocks forward progression of an actively transcribing RNAP (2, 36–38). We estimated the distance from the active site of vRNAP to the first G of the GAATTC EcoRI-binding site by comparing several different length runoff transcripts from truncated Ter29 templates to the transcript formed when the TEC was blocked by EcoRI′. The results of these studies indicated that this distance is 17 bp (see supplemental material). Moreover, prior studies have determined the distance between the leading edge of vRNAP and the active site to be 15 bp (39), and the distance between the upstream edge of EcoRI′ and the first G in GAATTC to be 2 bp (39). The sum of these two distances agrees well with our estimate of 17 bp.

Based on the information described above, EcoRI′-binding sites were placed downstream of the A-less cassette of Ter29 such that there were 0, 2, or 6 bp between the leading edge of vRNAP stalled at C77 and EcoRI′ (Fig. 6A). TECs halted at the end of the A-less cassette (C77) by omitting ATP from Step 2 of the transcription reactions predominantly (~97%) remained bound to the TEC (Fig. 6, B, lanes 1 and 2, and C). When NPH I was activated by dATP in the absence of EcoRI′, ~80% of the transcripts were released (Fig. 6, B, lanes 3 and 4, and C). Placing the EcoRI′ roadblock immediately downstream of vRNAP (+0) reduced the amount of NPH I-mediated transcript release (N-MR) 2.5-fold (Fig. 6B, lanes 5 and 6). Moving the roadblock 2 or 6 bp downstream from the leading edge of vRNAP (+2, +6) restored N-MR to nearly the same level seen in the absence of EcoRI′ (Fig. 6B, lanes 7–10). The results indicate that forward movement of vRNAP by at least 2 bp is required for efficient NPH I-mediated transcript release.
Forward Translocation in Vaccinia Transcription Termination

Antisense Oligonucleotides Stimulate Transcript Release on vRNAP by Forward Translocation—Intrinsic terminators in bacteria are characterized by two features in the RNA, including a GC-rich stem-loop structure immediately followed by a stretch of 7–9 U residues (1). One model for the mechanism of intrinsic termination suggests that formation of the terminator stem-loop structure in the nascent transcript induces forward movement of RNAP paused at the U-stretch (Fig. 1) (2). Support for this model comes from studies in which antisense DNA oligonucleotides were annealed to the nascent transcript to mimic formation of the terminator hairpin to release the transcript (2). Moreover, these oligonucleotide-mediated release (O-MR) studies showed that oligonucleotide binding induces forward translocation of RNAP on the DNA template.

We used O-MR to further assess whether vaccinia RNAP is susceptible to forward translocation as a mechanism of transcription termination or whether NPH I may provide some additional function beyond physically moving vRNAP forward. Transcript release assays were performed in a two-step manner as described above. Step 2 of the transcription reactions, which consists of elongation from G21 to the end of the A-less cas- sette, was performed in the absence of ATP (or dATP) so that NPH I was inactive. In the absence of added oligonucleotides (oligos), nearly 90% of the transcripts remained bound to the TEC stalled at C77 (Fig. 7B, lanes 1 and 2). We then tested the effects of adding oligonucleotides complementary to various regions of the nascent transcript during Step 2 of the transcription reaction (Fig. 7A). When the TEC is stalled at C77, the DNA/RNA hybrid extends from +69 to +77, and RNAP protects +60 to +77 of the nascent transcript (28). The oligos tested were complementary to the nascent RNA starting at +51 and extending to between +65 and +77. The presence of oligos that bound to +69 or farther downstream on the nascent transcript during Step 2 resulted in release of >50% of the transcripts (Fig. 7B, lanes 11–18), whereas oligos that did not extend to +69 failed to stimulate transcript release (Fig. 7B, lanes 3 and 4, and 7–10). Hence oligo-mediated transcript release (O-MR) from vRNAP stalled at C77 occurs when the oligo extends into the DNA/RNA hybrid by one or more residues. To demonstrate that base pairing to the 3′ end (+69) of the nascent RNA is required for O-MR, an oligo was designed to be complementary to +51–65 of the nascent RNA followed by five residues that are not complementary to the RNA sequence (+65 + 5MM). This oligonucleotide failed to stimulate transcript release (Fig. 7B, lanes 5 and 6), indicating that O-MR requires base pairing to the nascent RNA into the DNA/RNA hybrid. These observations are similar to those seen by Yarnell.
and Roberts (40) for O-MR with E. coli RNAP. Although the antisense oligos were added to the transcription reaction at the start of Step 2, transcript release only occurred when the TEC reached C77 (Fig. 7B). Moreover, an oligo complementary to the transcript corresponding to the upstream region of the A-less cassette (51–71) failed to stimulate transcript release upstream of C77 (Fig. 7B, lanes 3 and 4). These observations suggest that O-MR only functions when vRNAP is stalled and not when it is actively transcribing.

To examine whether forward translocation is required for O-MR, we again tested the effects of blocking the forward movement of vRNAP with EcoRI*. EcoRI* was positioned on the DNA templates as described in Fig. 4A such that the protein was 0, 2, or 6 nts downstream from the leading edge of vRNAP. O-MR assays were performed on EcoRI*-bound templates, using the /H1100151–71 antisense DNA oligonucleotide (Fig. 7C and D). When the roadblock was positioned immediately adjacent to vRNAP (+0), O-MR decreased 2-fold (Fig. 7C, lanes 5 and 6, and D). When the roadblock was moved 2 or 6 bases downstream, O-MR increased to similar levels seen in the absence of a roadblock. These observations indicate that forward movement of RNAP corresponding to ~2 residues on the DNA template is necessary for efficient O-MR. Again these results are similar to those seen with E. coli RNAP (40) and suggest that vRNAP is also susceptible to oligo-mediated forward translocation as a means of transcription termination.

To examine whether the strength of the RNA/DNA hybrid also affects O-MR, we tested the effect of stabilizing the hybrid by incorporation of IC and BrU in the nascent transcript. TECs were elongated to the end of the A-less cassette in the presence of either IC and BrU or CTP and UTP. O-MR was stimulated by addition of the +51–75 oligo. The presence of IC and BrU in the nascent transcript completely inhibited O-MR as compared with CTP and UTP (Fig. 7E).
**Forward Translocation in Vaccinia Transcription Termination**

**DISCUSSION**

Prokaryotic transcription terminators are generally classified as intrinsic, which involves only specific RNA features, or factor-dependent, which relies on trans-acting proteins to cause termination (1). Intrinsic terminators consist of a GC-rich RNA stem-loop immediately followed by a run of 7–9 U residues. Factor-dependent termination relies on trans-acting factors, such as E. coli Rho or Mfd proteins, which use energy from ATP hydrolysis to cause transcript release. Evidence has been presented that suggests a common underlying mechanism of forward translocation is employed for both types of termination (2, 3, 41, 42). Forward translocation involves an external force moving RNAP forward on the DNA template in the absence of RNA chain extension. Forward translocation of RNAP leads to three key outcomes as follows: misalignment of the 3′ end of the nascent RNA with the active site of RNAP, shortening the DNA/RNA hybrid, and collapse of the upstream region of the transcription bubble. In the case of intrinsic termination, the external force for forward translocation is derived from annealing the base-paired stem of the terminator hairpin. The nucleation of annealing occurs although RNAP is paused over the U-stretch, and the weak A/U DNA/RNA hybrid is disrupted. This proposed mechanism is consistent with the requirements for a strong G/C hairpin and weak A/U hybrid for efficient intrinsic termination. For factor-dependent termination, the trans-acting termination factors use energy from ATP hydrolysis to move downstream along the nucleic acid and push RNAP forward. Forward movement of a paused, blocked, or arrested polymerase such that elongation cannot occur also results in hybrid destabilization and termination of transcription. Factor-dependent termination can release transcripts from DNA/RNA hybrids containing residues other than U in the RNA. This lack of a requirement for a weak A/U hybrid suggests that greater force is generated from ATP hydrolysis than by base pairing in an intrinsic terminator stem-loop.

**FIGURE 7. Effects of antisense oligonucleotides on transcript release from vRNAP.** A, diagram of antisense oligonucleotides used in these experiments. The top sequence represents DNA template strand, and the dashed line and sequence represent the nascent RNA. TECs were halted at the end of the A-less cassette such that the 3′ end of the nascent RNA is C77 with the DNA/RNA hybrid extending 8–9 nts upstream from the active site. The solid black lines represent the antisense oligonucleotides with the numbers on the left corresponding to the region on the nascent RNA to which they are complementary. The 3′-most 5 nts in 51–65-SMM are not complementary to the RNA. B, phosphorimage of polyacrylamide gel analysis of in vitro transcript release assays. Where indicated, reactions contained one of the oligonucleotides depicted in A. The number at the top of the gel indicates the downstream-most residue on the RNA to which the oligonucleotide was complementary. Oligonucleotides were added at the start of Step 2 of transcription. Transcripts that were released in the supernatant (S) were separated from template-bound transcripts (B). Reactions were performed in the absence of dATP and ATP so that NPH I was not active. The percent transcript release is indicated below the B and S pair of lanes for each reaction. C, phosphorimage of polyacrylamide gel analysis of an in vitro transcript release assay in the presence of EcoRI* bound to DNA template at various locations downstream of vRNAP halted at the end of the A-less cassette (C77). Released transcripts (S) were separated from template-bound transcripts (B) and analyzed on 12% polyacrylamide gels. The antisense oligonucleotide complementary to +51–75 was added to Step 2 of each reaction where indicated. The estimated distance in nucleotides (0, 2, or 6 nts) between the downstream edge of vRNAP and the upstream edge of the EcoRI* block is indicated; for each reaction a dash indicates no oligo or EcoRI* was added. The percent transcript release is indicated below the B and S pair of lanes for each reaction D, bar graph representation of the percent transcript released by the oligonucleotide in the presence of either CTP and UTP (C/U) or Ido-CTP and Ido-UTP (IC/BrU) in the nascent transcript. Released transcripts (S) were separated from template-bound transcripts (B) and analyzed on 12% polyacrylamide gels. The percent transcript release is indicated below the B and S pair of lanes for each reaction.
Forward Translocation in Vaccinia Transcription Termination

The eukaryotic RNAP I and III terminate on U-rich sequences similar to bacterial intrinsic termination, although these U-runs are not preceded by hairpins. In animal cells, transcript release at U-stretches requires an accessory protein that does not demonstrate ATPase activity, so the mechanism of RNA release is unclear (43, 44). Termination with RNAP II lacks U-rich regions near the site of termination and is tightly associated with cleavage and polyadenylation of the mRNA (45–47). Two models have been proposed to explain these observations as follows: the torpedo model, which posits that transcript cleavage at the poly(A) site allows entry of an exonuclease (Rat1 or Xrn2) (48, 49), and the allosteric model, which suggests that the poly(A) site induces a conformational change in the TEC to promote termination (50–52). The results of this study suggest that eukaryotic RNAPs may use the mechanism of forward translocation, like bacteria, to cause transcript termination.

The E. coli transcription repair coupling factor Mfd (mutation frequency decline) releases RNAP stalled at DNA lesions and recruits DNA repair factors (41, 42). Mfd is also an ATPase belonging to the DEXH-box family of helicases. As with NPH I, helicase activity has not been demonstrated, although Mfd has been shown to translocate along double-stranded DNA (41, 53). Mfd uses ATP hydrolysis to move RNAP forward on the DNA template so as to stimulate transcript release and recycle the stalled polymerase (41, 42). Because Mfd and NPH I both belong to the DEXH superfamily of proteins and perform similar functions, we tested whether NPH I also uses forward translocation to induce transcription termination.

Several observations presented here indicate that NPH I uses forward translocation to induce transcript release with vaccinia vRNAP. Blocking the downstream movement of vRNAP with a roadblock protein (EcoRI*) inhibits NPH I-mediated transcript release (Fig. 6). Furthermore, transcript release and termination are inhibited by the presence of iodo-CTP and bromo-UTP (Fig. 5). The simplest explanation for these observations is that the presence of IC and BrU in the nascent transcript stabilizes the RNA/DNA hybrid and inhibits the ability of NPH I to shear the hybrid. However, it is also possible that the presence of these analogues in the transcript interferes with other aspects of termination beyond inhibiting NPH I-mediated release. For example, incorporation of BrU into the U$_2$NU termination signal is known to disrupt the interaction between VTF and U$_3$NU (14, 35).

Deng and Shuman (16) previously reported that transcript release from TECs stalled at the end of an A-less cassette requires ATP, NPH I, VTF, and the U$_2$NU termination signal. These results led to the suggestion that the requirements for N-MR were the same as for termination. However, in this report, we show that when dATP is employed as the substrate for NPH I, transcript release is independent of VTF and U$_3$NU. This observation is consistent with results shown by Piacente et al. (24), where NPH I released transcripts in a VTF- and U$_2$NU-independent manner at the end of the A-less cassette when stimulated by dATP. The rate of hydrolysis of dATP and ATP by NPH I was determined to be 11,000 and 9,000 mol of ATP hydrolyzed per mol of NPH I/min, respectively (data not shown), and because both ATP and dATP have been shown to be equally effective as a substrate for NPH I (15, 23), the difference in N-MR between ATP and dATP is likely because ATP is a substrate of vRNAP, whereas dATP is not. When RNAP is stalled at the end of the A-less cassette of Ter29 (Fig. 2), the next four templated residues in the RNA are A residues; hence the presence of ATP allows the polymerase to elongate. In this circumstance, when NPH I induces forward translocation, the nascent transcript is extended such that the DNA/RNA hybrid length is maintained and destabilization does not occur. Under these circumstances, transcript release does not occur when vRNAP reaches the end of the run of four A residues. However, addition of VTF and U$_3$NU prevented vRNAP elongation and facilitated NPH I-mediated release of the transcripts.

Because transcription termination normally occurs with an actively transcribing vRNAP, the above results suggest that the role of VTF/U$_3$NU in transcript release is to slow or pause vRNAP to allow for N-MR. This potential role of VTF/U$_3$NU is further supported by the results reported in Piacente et al. (24) where NPH I released transcripts independent of VTF and U$_3$NU in the presence of low (10 μM) NTP levels when vRNAP is elongating slowly. Notably Piacente et al. (24) observed NPH I-mediated transcript release at various discrete positions during transcription, including the usual site of termination (approximately +75 nts). One interpretation of these results would be that when vRNAP is limited for NTPs, transcription elongation is slow and the TEC may pause or stall due to nucleotide deprivation at which point NPH I-mediated forward translocation would induce transcript release. Therefore, at low NTP levels that slow transcription elongation, NPH I-mediated transcript release no longer requires VTF or U$_3$NU, whereas at high NTP levels with rapid elongation, transcript release requires VTF and U$_3$NU. We have also observed NPH I-mediated transcript release independent of VTF when vRNAP was artificially stalled at various locations on the DNA template using the EcoRI* roadblock protein (data not shown).

NPH I can also act as a positive elongation factor, as evidenced by facilitating transcription elongation with TECs stalled in a run of U residues (16). In this report, we provide additional evidence that NPH I is a positive elongation factor due to its ability to prevent/recover backtracked TECs (Fig. 4). The positive elongation behavior of NPH I is also consistent with a forward translocation mechanism of action. When a TEC backtracks, NPH I mediates forward movement of vRNAP downstream so as to realign the 3’ end of the transcript with the vRNAP active site. In this case, forward translocation does not result in transcript release because the DNA/RNA hybrid length is not shortened.

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