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Vaccinia Virus Gene *A18R* DNA Helicase Is a Transcript Release Factor*

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Prior phenotypic analysis of a vaccinia virus gene *A18R* mutant, Cts23, showed the synthesis of longer than wild type (Wt) length viral transcripts during the intermediate stage of infection, indicating that the A18R protein may act as a negative transcription elongation factor. The purpose of the work described here was to determine a biochemical activity for the A18R protein. Pulse-labeled transcription complexes established from intermediate virus promoters on bead-bound DNA templates were assayed for transcript release during an elongation step that contained nucleotides and various proteins. Pulse-labeled transcription complexes elongated in the presence of only nucleotides were unable to release nascent RNA. The addition of Wt extract during the elongation phase resulted in release of the nascent transcript, indicating that additional factors present in the Wt extract are capable of inducing transcript release. Extract from Cts23 or mock-infected cells was unable to induce release. The lack of release upon addition of Cts23 extract suggests that A18R is involved in release of nascent RNA. By itself, purified polyhistidine-tagged A18R protein (His-A18R) was unable to induce release; however, release did occur in the presence of purified His-A18R protein plus extract from either Cts23 or mock-infected cells. These data taken together indicate that A18R is necessary but not sufficient for release of nascent transcripts. We have also demonstrated that the combination of A18R protein and mock extract induces transcript release in an ATP-dependent manner, consistent with the fact that the A18R protein is an ATP-dependent helicase. Further analysis revealed that the release activity is not restricted to a vaccinia intermediate promoter but is observed using pulse-labeled transcription complexes initiated from all three viral gene class promoters. Therefore, we conclude that A18R and an as yet unidentified cellular factor(s) are required for the *in vitro* release of nascent RNA from a vaccinia virus transcription elongation complex.

Elongation and termination are key control points in both prokaryotic and eukaryotic transcription (1). Transcriptional events such as pausing, arrest, and termination are regulated by cis- and trans-acting factors that decide the fate of a given transcript, either elongation or termination. A paused complex, in which the 3′ end of the nascent RNA is retained in the polymerase catalytic site, can be induced by a DNA sequence-specific element, such as a T-rich sequence, or blockage of the RNA polymerase by so-called negative elongation factors. Elongation of a paused complex may resume either spontaneously or in response to positive elongation factors. An arrested complex, in which the catalytic site of the polymerase has slipped backwards and out of context with the 3′ end of the nascent transcript, must cleave the nascent RNA to return the 3′ end to the catalytic site in order to relieve arrest. Cleavage is an endogenous activity of the RNA polymerase but is activated in response to trans-acting factors. A transcription complex that releases its nascent transcript and dissociates from the DNA template is considered terminated. Termination may or may not require both cis-acting nucleic acid sequence elements and trans-acting factors. For example, the murine factor TTF-I binds to a specific DNA sequence and blocks elongating RNA polymerase I allowing another factor, PTRF (polymerase I transcript release factor), to induce transcription termination (2). In contrast, *Drosophila* factor 2 induces transcription termination in a sequence-independent manner (3, 4).

Vaccinia virus has historically served as a superb model for transcription (5). The prototypic Orthopoxvirus has a linear double-stranded DNA genome of 192,000 base pairs, which it replicates in the cytoplasm of the infected host cell. Due to the cytoplasmic site of infection, the virus encodes the majority of the enzymatic machinery necessary for both viral RNA and DNA metabolism. During infection, viral genes are expressed in a transcriptional cascade encompassing three stages as follows: early, intermediate, and late. Each stage requires trans-acting factors for transcription initiation that are synthesized in the previous stage thus providing the basis for sequential regulation. Biochemical and biological experiments during the past few years have shown elongation and termination of all three transcriptional stages are also regulated events.

The general features of vaccinia early gene transcription elongation and termination are fairly well understood. Early gene mRNA 3′ ends are formed by termination and not endonucleolytic cleavage (6, 7). Termination depends on extrusion of a UUUUUNU RNA signal from the ternary complex, as well as ATP hydrolysis (8). The model for early termination proposes that vaccinia termination factor (identical to the vaccinia virus capping enzyme) is poised to scan the RNA for the termination signal. Recognition of the signal by vaccinia termination factor activates the ATPase activity of NPH-I, a virus-coded DNA-dependent ATPase, resulting in release of the nascent transcript from the elongation complex (9, 10). Termination occurs 20–50 nt downstream of the termination signal (11, 12).

Less is understood concerning intermediate and late vaccinia transcription elongation and termination, although these processes differ from early transcription and are clearly regulated. Genetic experiments imply the existence of both positive and negative virus-coded intermediate and late transcription elongation factors; however, these factors have not been characterized biochemically (13–16). The elongation complexes from intermediate and late gene promoters do not recognize early

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termination signals, indicating that the factors necessary for intermediate and late termination may be different from those utilized during early transcription termination. Intermediate and late transcripts are also 3′ heterogeneous for any given gene, which may indicate that there is no sequence-specific termination signal as found in early gene termination.

Based on the phenotypic analysis of Cts23, a temperature-sensitive mutant in the vaccinia gene *A18R*, we propose that the A18R protein functions as a negative transcription elongation factor or a termination factor. Analysis of several vaccinia genes using Northern blots, RNase protection, and reverse transcriptase-PCR[1] analysis determined that mutations in the gene *A18R* result in readthrough transcription from intermediate promoters into downstream genes (16). These transcripts are longer than those synthesized during a Wt infection. Previous analysis of the *A18R* gene determined that it encodes a 56-kDa protein that is expressed throughout infection and packaged in virions (17). The A18R protein is both a DNA helicase and a DNA-dependent ATPase, not inconsistent with a role as a termination factor (18, 19).

To test the hypothesis that A18R is a transcription termination factor, intermediate promoter-specific, pulse-labeled transcription complexes established on bead-bound DNA templates were assayed for transcript release during an elongation step that contained nucleotides and various proteins. Release was analyzed by comparing transcripts present in the supernatant to transcripts in the bead-bound fraction. Extract from Wt-infected cells, but not mock- or Cts23-infected cells, stimulated transcript release. We were also able to demonstrate transcript release using a combination of extract from mock-infected cells plus purified A18R protein. The release activity is dependent upon ATP hydrolysis, arrest of the transcription elongation complex, presence of the proteins prior to arrest of the complex, and is active on all three classes of promoters.

EXPERIMENTAL PROCEDURES

Eukaryotic Cells, Viruses, and Bacterial Hosts—A549 cells, wild type vaccinia strain WR, the A18R temperature-sensitive mutant Cts23, and the conditions for their growth, infection, and plaque assay have been described previously (20–22). *Escherichia coli* DE3 pLysS contains an isopropyl-1-thio-β-D-inducible chromosomal copy of the bacteriophage T7 RNA polymerase gene (23).

Plasmids—All plasmids used for transcription are based on pC_2AT19 (24) containing a 375-nt G-less cassette cloned into pUC13 with the total size approximately 3 kilobase pairs. pG8G, pVGFG, and pCFW10 contain upstream of the 375-nt G-less cassette promoters from the intermediate vaccinia virus gene *G8R*, the early vaccinia gene *C11R*, and the late vaccinia gene *F17R* (20, 25), respectively. pSB24 contains a synthetic early promoter upstream from the 375-nt G-less cassette (26).

pG8GX is a derivative of pG8G that contains the vaccinia gene *G8R* intermediate promoter upstream of a 3′-truncated, 94-nt G-less cassette derived from pC_2AT19. The *G8R* promoter and the 5′ 92 nt of the pC_2AT19 G-less cassette were PCR-amplified from pG8G using the M13-40 universal sequencing primer as the upstream primer and a downstream primer that contained nucleotides 73–92 of the G-less cassette flanked with a *Sma*I site, a *Sca*I site, and a *Bam*HI site. The PCR-amplified fragment was cleaved with *Eco*RI (upstream) and *Bam*HI (downstream) and cloned into the vector portion of pC_2AT19, which had also been cleaved with *Eco*RI and *Bam*HI. The *Sma*I site at the 3′ end of the resulting truncated G-less cassette serves to arrest efficiently transcription of the G-less cassette, and the downstream *Sca*I site was used for identification of the desired clone. Accurate transcription of the pG8GX G-less cassette should yield an RNA of approximately 94 nt in length.

p16A18 (19) contains the vaccinia virus gene *A18R* coding sequence inserted in frame downstream from an amino-terminal polyhistidine tag in the vector pET16b (Novagen).

Infected Cell Extracts for Transcription—Confluent 100-mm dishes of A549 cells were either mock-infected or infected with vaccinia virus with a multiplicity of infection of 15 and incubated at 40 °C for 16 h in the presence of 10 mM hydroxyurea or in the absence of drug. Extracts were prepared as described (20). Briefly, vaccinia-infected cell monolayers were permeabilized with lysolecithin, harvested, treated with micrococcal nuclease, clarified by centrifugation, and stored at −70 °C. Total protein concentration was determined by the Bradford protein assay (Bio-Rad).

Immobilized DNA Templates—All templates used for transcription were immobilized by binding linearized plasmid DNA to paramagnetic beads. One set of immobilized templates, including NpG8G, NpSB24, and NpCFW10, were generated by linearization with *Nde*I, which cleaves the DNA template 220 nt upstream from the promoter. The resulting templates contain a 375-nt G-less cassette and approximately 2400-nt DNA downstream from the G-less cassette. Two additional shorter templates, N/VpG8G and N/VpG8GX, were constructed by restriction digest with *Nde*I and *Vsp*I. The resulting templates contain 220 base pairs DNA upstream from the G8R intermediate promoter and either 540 or 260 base pairs downstream for transcription (Fig. 1*B*). In all cases the cleaved DNA fragments were end-filled with Klenow, dCTP, dGTP, dATP, and biotin-16-dUTP (Roche Molecular Biochemicals). The biotinylated DNA was separated from the free nucleotides using the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). The DNA was eluted from the column in 100 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and adjusted to 1 M NaCl. DNA samples were then incubated with streptavidin-conjugated Dynabeads M280 (Dynal) in 1 M NaCl/TE for 30 min at 42 °C to generate bead-bound templates. Beads with bound DNA were concentrated using a magnet and washed twice in 1 M NaCl/TE, followed by two washes in TE. The bead-bound DNA was stored in TE at 4 °C.

In Vitro Transcript Release Assay—Transcription reactions were done in three phases, initiation, pulse, and chase. Reactions (25 μl) contained a final concentration of 25 mM HEPES, pH 7.4, 4.5% glycerol, 80 mM KOAc, 5 mM $MgCl_2$, 1.6 mM DTT, 1 mM ATP, 5 μl of bead-bound DNA template, and 15 μl of extract from hydroxyurea-treated wild type vaccinia-infected cells. Reactions were incubated at 30 °C for 10 min to form initiation complexes. The pulse phase was initiated by adding 3 μl of a solution containing 11 mM ATP, 11 mM GTP, 6 mM UTP, and 6 μCi of [α-P^{32}]CTP (~3000 Ci/mmol stock) such that the final concentration is 2.1 mM ATP, 1.1 mM GTP, 0.6 mM UTP, 22.3 mM HEPES, pH 7.4, 4% glycerol, 71.4 mM KOAc, 4.5 mM $MgCl_2$, and 1.4 mM DTT in a total of 28 μl. These reactions were then incubated at 30 °C for 30 s. The reactions were stopped by placing the tube on a magnet on ice. The pellets were washed with 1–1.5 pulse reaction volumes of high salt transcription buffer (5 mM $MgCl_2$, 25 mM HEPES, pH 7.4, 1.6 mM DTT, 1 M KOAc, and 7.5% glycerol), followed by three washes in 1–1.5 pulse reaction volumes of low salt transcription buffer (5 mM $MgCl_2$, 25 mM HEPES, pH 7.4, 1.6 mM DTT, 80 mM KOAc, 200 μg/ml bovine serum albumin, and 7.5% glycerol). The chase phase was done by adding to the resuspended complexes a mixture of NTPs, extract, and proteins in a final volume of 25 μl containing 25 mM HEPES, pH 7.4, 4.5% glycerol, 80 mM KOAc, 5 mM $MgCl_2$, 1.6 mM DTT, 600 μM ATP, 600 μM GTP or 10 μM 3′-OMeGTP, 600 μM UTP, 1.2 mM CTP, 20 units RNasin, and purified protein or extract as indicated. Chase reactions were performed at 30 °C for various times. The beads were concentrated using a magnet, and the 25-μl supernatant was removed to a separate tube. One hundred seventy five microliters of "PK mix" (114 mM Tris-HCl, pH 7.5, 14 mM EDTA, 150 mM NaCl, 1.14% SDS, 40 μg of glycogen, 230 μg/ml proteinase K) was added, and reactions were incubated at 37 °C for 30 min. Reactions were extracted once with 175 μl of phenol/chloroform. Nucleic acids were precipitated by addition of 50 μl 10 M ammonium acetate and 150 μl isopropyl alcohol, incubation at room temperature for 30 min, and centrifugation for 20 min. Pellets were washed once with 70% ethanol, dried, and resuspended in 10 μl of formamide loading buffer. Samples were denatured at 90 °C for 3 min and loaded on a 6% 8 M urea-PAGE. Gels were fixed, dried, and analyzed by autoradiography and phosphorimagery. Released transcripts were expressed as a percentage derived by dividing the quantity of transcripts in the supernatant by the total quantity of transcripts in both the supernatant and associated with the beads.

Chromatography and Fractionation—Extract from Wt or Cts23-infected A549 cells was chromatographed on 2-ml columns of phosphocellulose (Whatman) or Q-Sepharose (Amersham Pharmacia Biotech) equilibrated in Buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.01% Nonidet P-40, 1 mM DTT, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 μg/μl leupeptin, and 0.7 μg/μl pepstatin A). All steps were

[1] The abbreviations used are: PCR, polymerase chain reaction; nt, nucleotide; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; Wt, wild type; AMPPNP, adenosine 5′-(β,γ-imino)triphosphate.

performed at 4 °C. Extract was loaded on the column, and the column was washed in 4 ml of Buffer A, and 0.5 ml of flow-through fractions were collected. Bound proteins were eluted stepwise with 4 ml each of Buffer A containing 0.25, 0.5, and 1 M NaCl, and 0.5-ml fractions were collected. Peak fractions were identified using the Bradford protein assay, pooled, and dialyzed overnight against Buffer A containing 50 mM NaCl. The fractions were stored at −20 °C.

Induction and Preparation of Extract from E. coli—An overnight culture of pLysS cells harboring the p16A18 plasmid was used to inoculate 1 liter of L-broth, containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. The culture was incubated at 37 °C to an A_{600} of 0.5. Isopropyl-1-thio-β-D-GALACTOPYRANOSIDE was added to a final concentration of 1 mM, and the culture was incubated at 37 °C for 4 h. The cells were pelleted and stored at −70 °C overnight. All subsequent procedures were performed at 4 °C. The thawed bacterial pellet was resuspended in 50 ml of lysis buffer (50 mM Tris, pH 7.5, 0.15 M NaCl, 10% sucrose) plus a final concentration of 50 μg/ml lysozyme and 0.1% Triton X-100. The cells were sonicated at 4 °C for eight sequences consisting of 15 s on and 45 s off. Insoluble material was removed by centrifugation for 30 min at 18,000 rpm in a Sorvall SS34 rotor at 4 °C. For purification of the soluble A18R protein, the supernatant was then chromatographed on a His-Bind (Novagen) column and phosphocellulose column as described below.

His-Bind Column and Phosphocellulose Column—The supernatant was mixed for 1 h with 2 ml of nickel-nitrilotriacetic acid-agarose resin (Qiagen) that had been equilibrated with lysis buffer. The slurries were poured into a column and washed sequentially with 20 ml of lysis buffer, 20 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, 5% glycerol), and 20 ml of wash buffer 1 (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, 5% glycerol). Bound proteins were eluted with 20 ml of wash buffer 2 (200 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, 5% glycerol) collecting 1-ml fractions. Peak fractions were identified using the Bradford protein assay (Bio-Rad), pooled, and dialyzed overnight against 1 liter of Buffer A. The dialysate was applied to a 2-ml column of phosphocellulose that had been equilibrated with Buffer A. The column was washed with 5 ml of Buffer A containing 250 mM NaCl. Bound proteins were eluted with 10 ml of Buffer A containing 500 mM NaCl collecting 0.5-ml fractions. Peak fractions were identified using the Bradford protein assay, pooled and dialyzed overnight against 4 changes, 1 liter each, of a solution containing 40 mM Tris-HCl, pH 8, 20 mM KCl, and 40% glycerol. The enzyme was stored at −20 °C. The His-A18R protein preparation was greater than 90% pure as judged by PAGE and displayed DNA-dependent ATPase activity of 10,000 nmol of ATP hydrolyzed per min per μg of protein, equivalent to previously reported preparations (19).

Polyhistidine-tagged vaccinia virus J3R protein, prepared in a fashion similar to His-A18R, was a gift from Dr. Ying Xiang (University of Florida).

Western Blot Analysis—Samples were separated by electrophoresis on 10% SDS-PAGE. The proteins were transferred to nitrocellulose in 25 mM Tris-HCl, 192 mM glycine, 20% methanol at 4 °C overnight. Nitrocellulose filters were incubated with monoclonal anti-A18 primary antibody (1:10,000) (15), and the bound antibody was detected using polyclonal anti-mouse horseradish peroxidase-conjugated antibody (1:5000; Amersham Pharmacia Biotech) and enhanced chemiluminescence. Western blotting reagents (Amersham Pharmacia Biotech) were used as described by the manufacturer.

RESULTS

To measure the activity of the vaccinia virus A18R protein *in vitro*, we developed a transcription elongation assay based on a previously described crude system for study of vaccinia early, intermediate, and late gene transcription initiation (20). Previous experiments showed that crude extracts prepared from cells infected under normal conditions are competent for transcription of early, intermediate, and late gene promoters. Since intermediate and late viral gene expressions are coupled to viral DNA replication, treatment of infected cells with a DNA replication inhibitor such as hydroxyurea permits synthesis of only early gene products, including intermediate transcription factors. Thus extracts prepared from cells infected in the presence of hydroxyurea are competent for transcription of intermediate promoters only (20). For most experiments, we chose to use hydroxyurea-treated, intermediate promoter-specific extract for two reasons. First, the best evidence that the A18R protein has elongation factor activity is based on *in vivo* studies of intermediate genes (16). Second, we wished to prepare extracts from A18R mutant infections under non-permissive conditions while at the same time circumventing undesirable pleiotropic effects of the A18R mutation. Readthrough transcription from convergent intermediate promoters during A18R mutant infections causes double-stranded RNA accumulation, induction of the cellular 2-5A pathway, and ultimately activation of RNase L (27, 28). Hydroxyurea treatment prevents 2-5A pathway activation by preventing intermediate transcription. Cells infected with A18R mutant virus at the non-permissive temperature produce less than 10% of the normal amount of A18R protein due to instability of the mutant protein (17). Thus preparation of extracts from A18R mutant-infected cells at the non-permissive temperature provides an A18R protein-deficient extract that is otherwise comparable to extract from cells infected with Wt virus under identical conditions.

Extract from hydroxyurea-treated vaccinia-infected cells was used to assay elongation and transcript release from linear bead-bound DNA templates containing a vaccinia intermediate promoter as follows. First, transcription complexes were assembled during a preincubation reaction containing Wt extract, bead-bound template, and ATP. Transcription was then initiated, and the nascent transcript was radiolabeled by the addition of [α-^{32}P]CTP, ATP, GTP, and UTP during a short, 30-s pulse reaction. The ternary complexes, consisting of the DNA template, the transcription apparatus, and the radiolabeled nascent RNA, were stripped of nonspecific proteins and unincorporated nucleotides during three washes in high salt transcription buffer followed by three washes in low salt transcription buffer. An elongation reaction was then done by adding to the resuspended complexes a chase mixture containing NTPs, extract, and proteins. Following the elongation reaction the beads were concentrated using a magnet; the supernatant was removed to a separate tube, and the labeled RNA in each fraction was analyzed on a denaturing polyacrylamide gel. Released transcripts were expressed as a percentage derived by dividing the quantity of transcripts in the supernatant by the total quantity of transcripts in both the supernatant and associated with the beads.

Transcription Is Specific for the Viral Promoter—As an initial test of the fidelity of the system, we sought to prove that the intermediate promoter was accurately recognized. Two bead-bound templates were designed such that transcription from the *G8R* promoter to the downstream end of the template would generate either 260 or 540 nt of RNA (Fig. 1*B*). Pulse-labeled elongation complexes were established and analyzed on a denaturing polyacrylamide gel (Fig. 1*A, lanes 1* and *10*). The transcripts were approximately 100 nt in length and were cut off on the autoradiograph shown. Elongation was continued upon addition of ribonucleotides during the chase phase and the transcripts synthesized from each template were of the appropriate length, either 260 or 540 nt (Fig. 1*A, lanes 2* and *11*). At the end of the chase phase, the bead-bound template was separated from the supernatant using a magnet. Comparison of Fig. 1*A, lanes 2* and *3* and *lanes 11* and *12*, indicates that transcripts synthesized during a nucleotides-only chase reaction are not released into the supernatant but remain associated with the bead-bound template. Our protocol for generating elongation complexes required extensive washing with 1 M salt, and we questioned whether additional proteins could act on the isolated elongation complexes to induce release of the nascent transcript from the bead-bound template. Chase reactions were therefore performed in the presence of nucleotides plus extract from mock-, Wt-, or the A18R mutant Ts23-infected cells. The addition of extract from Wt-infected cells resulted in the re-

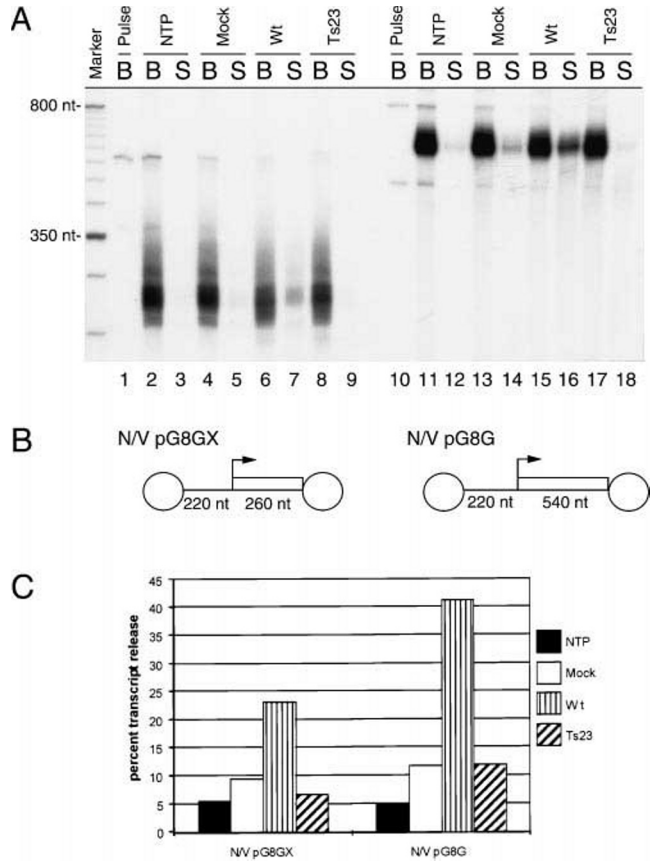

FIG. 1. **Transcription is promoter-specific.** *A*, autoradiogram of *in vitro* transcript release assay. Transcription complexes were formed from Wt extract on immobilized N/V pG8GX or N/V pG8G DNA that contain the vaccinia *G8R* intermediate promoter. Following a 30-s pulse reaction (*Pulse*), labeled complexes were washed in transcription buffer, and elongation was continued in the presence of 0.6 mM ATP, 0.6 mM GTP, 0.6 mM UTP, and 1.2 mM CTP alone (*NTP*) or with additional 7.5 μg mock extract (*Mock*), Wt extract (*Wt*), or Ts23 extract (*Ts23*) for 20 min. The bead-bound RNA (*B*) was separated from released RNA (*S*) using a magnet. These transcripts were analyzed by 6% 8 M urea-PAGE. Sizes, in nt, are shown on the *left. B*, diagram of the DNA templates used for transcription. The DNA template (*line*) contains a biotinylated ATP incorporated at both the 5′ and 3′ end, which anchors the DNA to a streptavidin-coated magnetic bead (*circles*). The bead is anchored 220 nt from the promoter at the 5′ end of the template. The transcription unit consists of the *G8R* intermediate promoter (*arrow*) fused to either 260 or 540 nt of downstream DNA. *C,* graphic representation of the percent transcript release for each reaction in *A*. Bound and released transcripts were quantitated using a PhosphorImager; the quantity of transcripts in the supernatant was divided by the quantity of transcripts on both the beads and in the supernatant and expressed as a percentage.

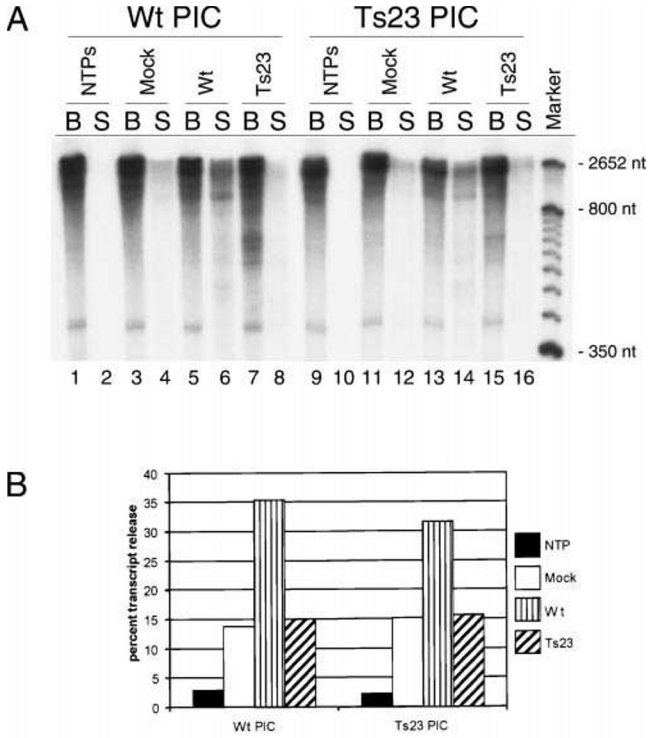

FIG. 2. **A18 is not required for initiation *in vitro*.** *A*, transcription complexes were formed on immobilized NpG8G DNA containing the vaccinia *G8R* intermediate promoter and extracts from either Wt- (*Wt PIC*) or Ts23 (*Ts23 PIC*)-infected cells. Transcription was performed as described in Fig. 1 and released transcripts (*S*) were separated from bound transcripts (*B*) and analyzed by 6% 8 M urea-PAGE. Sizes in nt are shown at the *right. B*, graphic representation of the percent transcript release for each reaction in *A*.

lease of transcripts during a 20-min chase from either template (Fig. 1*A*, compare *lanes 6* and *7*, and *lanes 15* and *16*). The percent transcript release was analyzed by phosphorimagery, Fig. 1*C*. Extract from neither mock-infected nor Ts23-infected cells was capable of generating a significant amount of released transcripts (Fig. 1*A, lanes 4* and *5, 8* and *9, 13* and *14*, and *17* and 18, and Fig. 1*C*). In summary, these experiments show that initiation *in vitro* occurs specifically at the viral intermediate promoter. These data also suggest that transcript release in Wt extract is due to the presence of A18R protein, which is absent in Ts23 extract.

Release Does Not Require the Presence of A18R during Initiation—In the previous experiment, Wt extract was used to generate the transcription complexes formed during the preincubation step. To determine whether factors specific to a Wt extract and present in the washed elongation complex contributed to release, we compared transcription complexes formed using either Wt or Ts23 extract during the preincubation and pulse steps (Fig. 2*A*, *Wt* or *Ts23 PIC*). Transcription complexes were formed on linearized bead-bound NpG8G, a template that contains approximately 3 kilobase pairs of sequence downstream from the G8R promoter. These complexes were chased in the presence of unlabeled ribonucleotides or nucleotides plus mock, Wt, or Ts23 extract (Fig. 2*A*). Both complexes show similar levels of transcript release in response to the addition of Wt extract (Fig. 2*A*, compare *lanes 5* and *6, 13* and *14*, and Fig. 2*B*). Therefore, Wt or Ts23 extracts are equally competent for transcription complex assembly and initiation. Therefore, Wt extract was used to generate transcription complexes for all release assays.

Transcript Release Is Time- and Concentration-dependent—To determine the kinetics of release, we performed a chase time course. Pulse-labeled elongation complexes were formed, and samples were taken at various time points during elongation. Similar kinetics of elongation were observed with the addition of ribonucleotides alone or in combination with Wt or Ts23 extract (Fig. 3*A*). Release is detected with the addition of Wt extract (Fig. 3*A, lanes 13–24*), and the level of release increases linearly as a function of time (Fig. 3*B*). Longer incubation times do not result in more than 60% release. Ts23 extract also resulted in a linear increase in release activity with time that was measurably above the nucleotides-only control but significantly less than Wt (Fig. 3*A, lanes 25–36,* and Fig. 3*B*). The lower level of release observed with addition of Ts23 extract could represent nonspecific release or result from the lower level of A18R protein in Ts23 extract. In summary, release activity is significantly diminished in a Ts23 extract throughout a time course substantiating our hypothesis that release is specific to A18R protein.

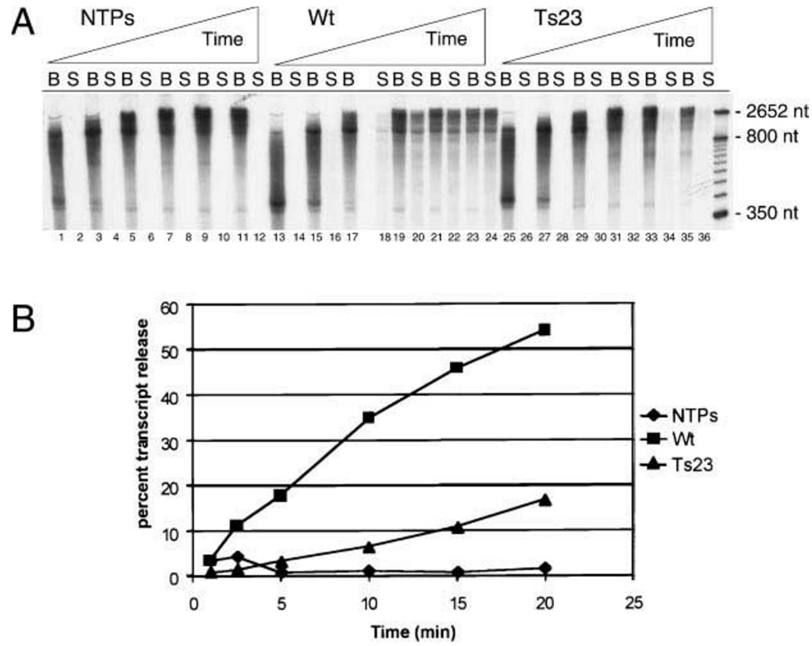

FIG. 3. **Time course of elongation in a chase reaction.** *A*, pulse-labeled transcription elongation complexes were formed on NpG8G bead-bound template using extract from Wt-infected cells. Complexes were washed in 1 M transcription buffer, and transcription was continued in the presence of 0.6 mM ATP, 0.6 mM GTP, 0.6 mM UTP, and 1.2 mM CTP alone (*NTPs*) or in addition to 30 μg of Wt extract (*Wt*) or Ts23 extract (*Ts23*) for 1, 2.5, 5, 10, 15, and 20 min. Released transcripts in the supernatant (*S*) and bound transcripts associated with the bead-bound template (*B*) were separated and analyzed by denaturing 6% PAGE. *B*, graphic representation of the percent transcript release for each reaction in *A*.

We next performed an extract titration to determine the optimal quantity of extract for efficient release. Transcription complexes were formed from Wt extract, washed, and increasing concentrations of mock, Wt, or Ts23 extract were tested in combination with ribonucleotides for release during a 20-min chase reaction. Increased transcript release occurred as the quantity of Wt extract was increased (Fig. 4*A, lanes 11–18*, and Fig. 4*B*); however, no effect on release was observed with increasing quantities of either mock or Ts23 extract (Fig. 4*A, lanes 3–10* and *lanes 21–28,* and Fig. 4*B*). These results further support the hypothesis that A18R is important for transcript release.

Transcript Release Is Complemented by Crude Fractions from Wt Extract—In an attempt to correlate the release activity achieved by the addition of Wt extract with the presence of A18R protein, a crude fractionation protocol was employed. Extracts were prepared from either Wt- or Ts23-infected cells and fractionated on phosphocellulose and Q-Sepharose columns separately. Columns were eluted stepwise with 0.25, 0.5, and 1 M NaCl. Each Wt extract fraction was analyzed by PAGE (data not shown) and by Western blot analysis using an anti-A18 monoclonal antibody (Fig. 5, *B* and *C*). As demonstrated by Western blot, A18R protein fractionated into the 0.5 M phosphocellulose fraction and the 0.25 M Q-Sepharose fraction (Fig. 5*B*, 0.5 M and Fig. 5*C*, 0.25 M). Each fraction was assayed for release during a chase reaction from pulse-labeled elongation complexes (Fig. 5*A*). As controls, chase reactions containing ribonucleotides alone or ribonucleotides plus mock, Wt, or Ts23 extract were performed (Fig. 5*A, lanes 1–8* and *lanes 13* and *14*). As previously shown, only the addition of Wt extract is capable of inducing transcript release (Fig. 5*A, lanes 5* and *6* and *lanes 13* and *14*, and Fig. 5*D*). Two of the column fractions were capable of inducing release, the 0.5 M phosphocellulose fraction and the 0.25 M Q-Sepharose fraction (Fig. 5*A, lanes 23* and *24* and *lanes 31* and *32*). These same fractions contain A18R protein as judged by Western blot analysis (Fig. 5*B*, 0.5 M, and Fig. 6*C*, 0.25 M). The phosphocellulose wash fraction, Fig. 5*A, lanes 19* and *20,* also showed release in this experiment. This result was not reproducible in subsequent release experiments done with the same material. For comparison, Ts23 extract was also fractionated by the same protocol (data not shown). A18R protein was not detected by Western blot in extract from Ts23-infected cells (Fig. 5*B*, *E122297*) nor any Ts23 extract fractions from the phosphocellulose or Q-Sepharose columns (data not shown). In addition, no significant release was detected with the addition of fractions from Ts23 extract. The fractionation protocol described here provides circumstantial evidence for the role of A18R protein in transcript release. However, these are crude fractions that contain many more proteins than just A18R. Conclusive evidence for the role of A18R must be obtained with a purified fraction or purified protein.

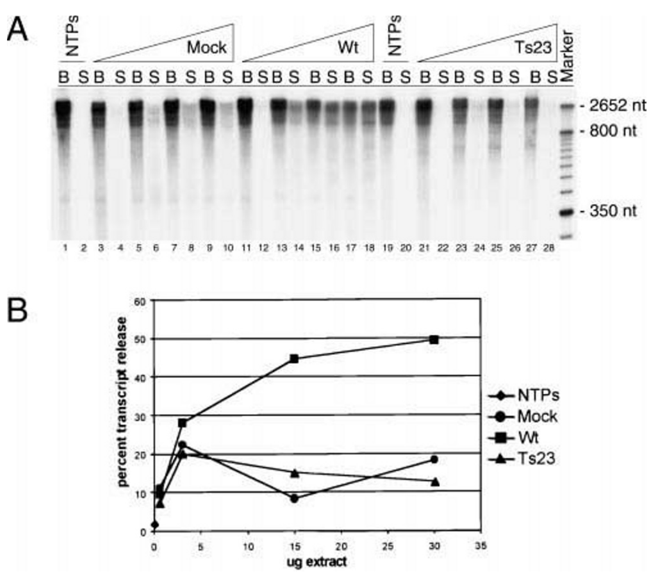

FIG. 4. **Add-back extract titration.** *A*, elongation complexes were generated as detailed in Fig. 3 (*Pulse*) and washed in 1 M transcription buffer, and transcription was continued for 20 min in the presence of 0.6 mM ATP, 0.6 mM GTP, 0.6 mM UTP, and 1.2 mM CTP alone (*NTPs*). Other reactions were supplemented with increasing concentrations of mock extract (*Mock*), Wt extract (*Wt*), or Ts23 extract (*Ts23*), as follows: 0.5 μg (*lanes 4* and *5, 12* and *13,* and *23* and *24*), 3 μg (*lanes 6* and *7, 14* and *15,* and *25* and *26*), 15 μg (*lanes 8* and *9, 16* and *17,* and *27* and *28*), 30 μg (*lanes 10* and *11, 18* and *19,* and *29* and *30*). *B*, bound; *S*, supernatant. *B*, percent transcript release plotted against the quantity of mock, Wt, or Ts23 extract.

Release Occurs from a Stalled Elongation Complex and Can

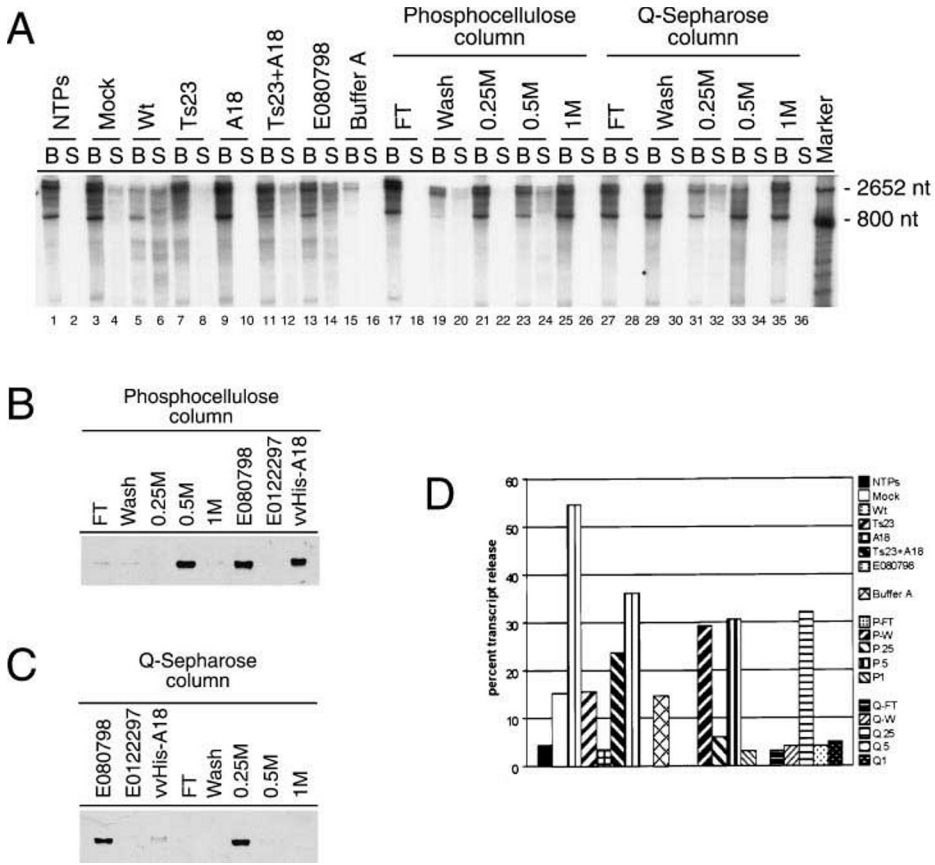

FIG. 5. **Wt extract fractionation.** *A*, pulse-labeled elongation complexes were generated as detailed in Fig. 3. Transcript release was assayed with the addition of 0.6 mM ATP, GTP, UTP, and 1.2 mM CTP (*NTPs*) or NTPs and 30 μg of mock (*Mock*), Wt (*Wt* and *E080798*), or Ts23 (*Ts23*) extract, 1.32 μg of vaccinia virus (*vv*) His-A18 protein (*A18*), or 5 μg of each fraction from the phosphocellulose and Q-Sepharose columns during a 20-min chase reaction. E080798 was the extract fractionated on the phosphocellulose and Q-Sepharose columns. *B*, bound; *S*, supernatant. *B* and *C*, Western blot analysis. Monoclonal α-A18R antibody was used to probe a 10% SDS-PAGE containing 3.125 μg of each sample from the phosphocellulose and Q-Sepharose columns, 7.5 μg either Wt extract (*E080798*) or Ts23 extract (*E122297*), and 0.3 μg of purified *vvHis-A18* protein. *D*, graphic representation of the percent transcript release for each sample in *A*.

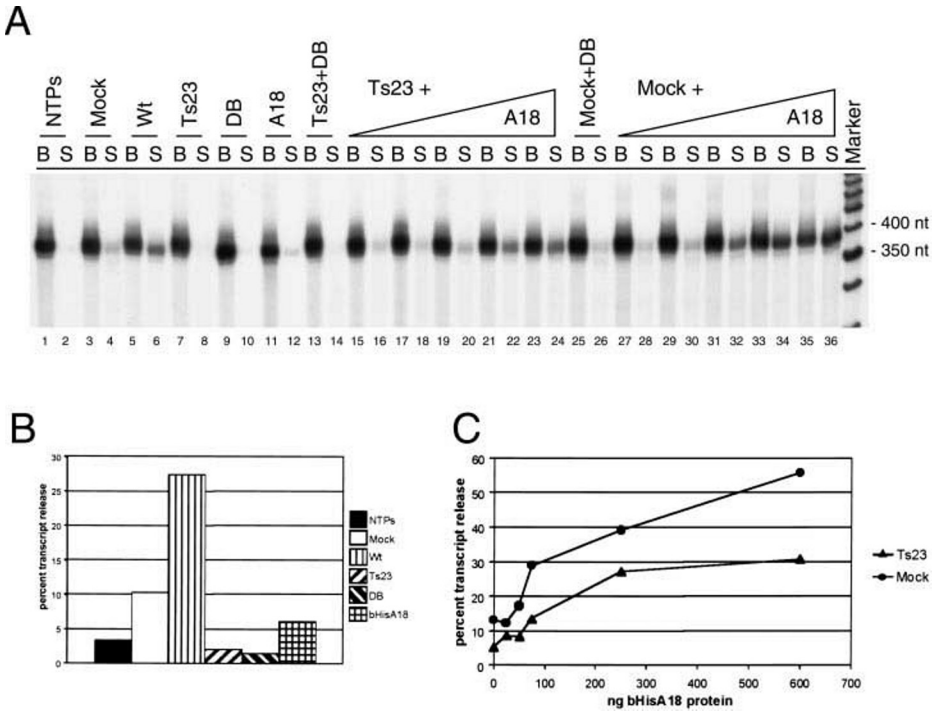

FIG. 6. **Release occurs from a stalled elongation complex and can be complemented by His-A18 and a cellular factor.** *A*, pulse-labeled transcription elongation complexes were formed on NpG8G bead-bound template using extract from Wt-infected cells. Complexes were washed in 1 M transcription buffer, and transcription elongation was continued to the end of the G-less cassette using 0.6 mM ATP, UTP, 1.2 mM CTP, and 0.01 mM 3′-OMeGTP alone (*NTPs*), or in addition to 30 μg of mock-infected extract (*Mock*), Wt extract (*Wt*), or Ts23 extract (*Ts23*). Transcripts synthesized in the presence of 3′-OMeGTP are approximately 400 nt in length. Purified recombinant His-A18 protein was used at 300 ng either alone (*A18*) or in combination with Ts23 or mock extract. *DB*, A18 storage buffer; *B*, bound; *S*, supernatant. *B* and *C*, graphic representation of the percent transcript release for each sample in *A*.

Be Complemented by His-A18R and a Cellular Factor—In all experiments described above release occurs predominantly at the downstream end of the template where the template is joined to a paramagnetic bead. In order to eliminate the possibility that the observed release is an artifact due to the presence of the bead, we conducted experiments designed to promote release in the middle of a DNA template. We refer to this protocol as a "mid-template" assay. This assay is designed to reflect the situation *in vivo* where a transcription complex will terminate despite the presence of additional template downstream. We accomplished this by arresting transcription at the end of a 375-nt G-less cassette downstream from the intermediate *G8R* promoter present within the 3-kilobase pair template NpG8G. Pulse-labeled elongation complexes formed on NpG8G were washed and elongated either in the absence of GTP (with all other nucleotides present) (data not shown) or in

the presence of 3′-OMeGTP and all other ribonucleotides (Fig. 6*A*) with additional proteins provided as indicated. The addition of 3′-OMeGTP arrests the elongation complex at the end of the G-less cassette where the first GTP would be incorporated (Fig. 6*A, lane 1*) resulting in the synthesis of an approximately 400-nt transcript. Addition of Wt extract during the chase reaction resulted in release of the transcript at the end of the G-less cassette (Fig. 6*A, lanes 5* and *6*). Release did not occur with mock or Ts23 extract (Fig. 6*A, lanes* 3 and *4* and *lanes 7* and *8*). Similar results were obtained when the complex was elongated in the absence of GTP (data not shown). In other experiments not shown, we attempted to induce release by first elongating to the end of the G-less cassette in the absence of added extract and then adding extract to the arrested complex. We also tried to induce mid-template release by slowing elongation using reduced concentrations of UTP. In neither protocol did we observe significant mid-template release. These results show definitively that release can be induced in the middle of the template but strongly suggest that release can only be accomplished on a complex that is stalled. Furthermore, the results indicate that in order to observe release, release factors need to be present during elongation, before the polymerase stalls.

In order to determine definitively whether A18R is required for transcript release, we attempted to complement the defect in release activity observed in Ts23 extracts by addition of purified A18R protein. Pulse-labeled elongation complexes were formed and assayed during the chase phase with His-A18R protein. His-A18R was expressed in *E. coli* and purified over nickel and phosphocellulose columns as described under "Experimental Procedures." The addition of ribonucleotides, Ts23 extract, or purified His-A18R protein alone to the chase was not sufficient for transcript release (Fig. 6*A, lanes 11* and *12*, and Fig. 6*B*). Addition of increasing amounts of His-A18R protein to the Ts23 extract resulted in increasing release equivalent to the levels of His-A18R protein (Fig. 6*A, lanes 15–24*, and Fig. 6*C*). As a control, a similar titration of purified His-J3R protein (J3R is a vaccinia 2′-*O*-methyltransferase and poly(A) polymerase processivity factor) expressed in *E. coli* was tested in combination with Ts23 extract (data not shown). The transcription complexes did not release the nascent RNA in the presence of His-J3R protein. These results demonstrate that the release defect observed in Ts23 extract can be complemented by purified A18R protein.

The results described above show that purified A18R protein is necessary but not sufficient for transcript release. To determine whether the additional factors required for release are viral or cellular in nature, extract from mock-infected cells was tested in the release assay. Mock extract alone does not produce a significant level of released transcripts (Fig. 6*A, lanes 3* and *4*). A titration of His-A18R in combination with mock extract induced more efficient release than His-A18R plus Ts23 extract (Fig. 6*A*, compare *lanes 27–36* and *lanes 15–24*, and Fig. 6*C*). The simplest explanation for these observations is that a cellular factor(s) is needed in addition to A18R for transcript release.

Release Requires ATP Hydrolysis—It has been shown previously that A18R possesses a DNA-dependent ATPase activity and that the enzyme can readily use dATP as a substrate rather than ATP (19). We therefore hypothesize that any stage of transcription that requires A18R would also be ATP-dependent. Assessing the role of ATP hydrolysis in transcription is complicated by the requirement for ATP as a substrate for the polymerase during elongation. Therefore, we examined the ATP dependence of the release activity by replacing the ATP in the chase reaction of the mid-template assay with the non-hydrolyzable ATP analog, AMPPNP. AMPPNP can be used as a substrate for the vaccinia RNA polymerase, and substitution results in efficient synthesis of long transcripts (Fig. 7*A*, compare *lanes 1* and *3*). Substitution of ATP with dATP, a hydrolyzable ATP analog that cannot be efficiently used for synthesis, yielded transcripts that are much shorter in length (Fig. 7*A*, compare *lanes 1* and *5*). Transcription elongation in the presence of dATP can be rescued with the provision of AMPPNP (Fig. 7*A, lane 7*). The combination of dATP and AMPPNP satisfies the energy requirement and provides a nucleotide capable of being incorporated into the nascent RNA chain. We then assayed the effect of AMPPNP substitution on release in combination with mock extract (Fig. 7*A*, *Mock*), Wt extract (Fig. 7*A, Wt*), or mock extract plus His-A18R protein (Fig. 7*A, Mock+A18*). As controls, the level of release in response to a given extract was assayed using ATP or dATP alone or the combination of AMPPNP and dATP and quantitated as described previously (Fig. 7*A, lanes 9* and *10, 15* and *16, 17* and *18, 23* and *24, 25* and *26*, and *31* and *32*, and Fig. 7*B*). Since the extracts added during the chase reactions contain some endogenous ATP, substitution of ATP with dATP in these controls did not restrict elongation as much as in chase reactions done in the presence of nucleotides alone. Substitution of ATP with AMPPNP did not have an effect on the low level of release detected in the presence of mock extract (Fig. 7*A*, compare *lanes 9* and *10* and *lanes 11* and *12,* and Fig. 7*B*). On the other hand, replacing ATP with AMPPNP severely inhibits transcript release when assayed with Wt extract (Fig. 7*A*, compare *lanes 17* and *18* and *lanes 19* and *20*, and Fig. 7*B*) or mock extract plus His-A18R protein (Fig. 7*A*, compare *lanes 25* and *26* and *lanes 27* and *28*, and Fig. 7*B*). Therefore, we conclude that A18R-catalyzed transcript release is an ATP-dependent event.

A18R-dependent Transcript Release Occurs from All Vaccinia Promoters—The previous experiments were all carried out using a template that contained a vaccinia intermediate promoter. However, further characterization of the mechanism by which A18R functions in transcription termination requires analysis of the specificity of the release activity for the different stages of transcription. We therefore performed A18R-dependent transcript release assays using templates that contain a promoter from each of the three stages of transcription. Each template is precisely analogous to the intermediate promoter containing template described above and contains a 375-nt G-less cassette downstream from an early, intermediate, or late gene promoter. In order to assay transcription from early and late promoter-driven initiation complexes, we used extract from Wt-infected cells that were not treated with hydroxyurea as a source of activity for forming pulse-labeled elongation complexes. Intermediate promoter-driven complexes can be formed using extract from either hydroxyurea-treated or non-hydroxyurea-treated Wt-infected cells. The mid-template assay was performed using complexes initiated from each promoter and elongated in the presence of ATP, CTP, UTP, and 3′-OMeGTP, as well as additional proteins. The ability of each complex to release the nascent RNA was determined using mock extract plus His-A18R protein. In the case of each promoter, release occurred only in the presence of both mock extract and His-A18R protein (Fig. 8*A, lanes 9* and *10, 19* and *20, 29* and *30, 39* and *40, 49* and *50*, and Fig. 8*B*). Although the absolute level of transcription in non-hydroxyurea extract is less than the hydroxyurea extract, the amounts of released RNA observed from the intermediate promoter template are equivalent (Fig. 8, *A* and *B*, *NpG8G(+)* and *NpG8G(−)*). Additionally, the absolute level of transcription using the various promoters is different, but release does occur and is specific for

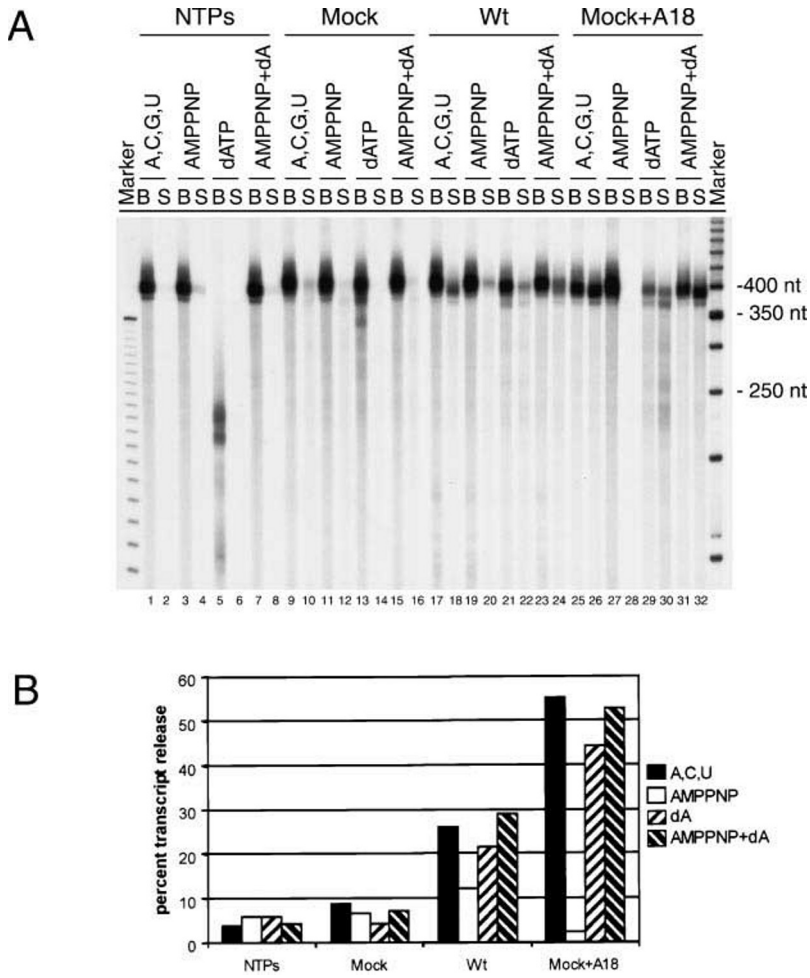

FIG. 7. **Transcript release requires ATP hydrolysis.** *A*, ternary complexes were formed and elongated as described in Fig. 6. The standard elongation reaction included 0.6 mM ATP, UTP, 0.01 mM 3′-OMeGTP, and 1.2 mM CTP (*A, C, G, U*). In other reactions, adenosine analogs AMPPNP (*AMPPNP*) and dATP (*dA*) replaced ATP as indicated, each at 0.6 mM concentration. Released transcripts (*S*) were separated from bound transcripts (*B*) and analyzed as described previously. *B*, released and bound transcripts are expressed as percent transcript release.

the presence of mock extract plus His-A18R protein. These results indicate that the activity provided by mock extract and His-A18R protein acts on complexes initiated from all three promoters, early, intermediate, and late, implying that A18R could serve as a release factor at each stage *in vivo*.

DISCUSSION

Previous research has implicated the vaccinia virus *A18R* gene product in 3′ end formation of vaccinia virus intermediate stage transcripts. Specifically, mutations in the *A18R* gene result in synthesis of readthrough transcripts at intermediate times during infection implying that the A18R protein acts as a negative elongation or termination factor (16). We developed an immobilized template assay to study the effects of the A18R protein on elongation and release of nascent RNA from vaccinia virus transcription complexes. The results of this study allow us to draw several major conclusions. First, nascent transcript release requires A18R protein and an additional activity that can be provided by either mock extract or extract from A18R mutant (Cts23)-infected cells. Second, the A18R protein and/or the cellular factor must be present during elongation in order for release to occur. Third, release requires a stalled transcription elongation complex. Fourth, transcript release requires ATP hydrolysis. Finally, the transcript release activity provided by mock extract and A18R protein, or Cts23 extract and A18R protein, can catalyze release of transcripts synthesized from promoters representing all stages of vaccinia transcription.

A18R protein alone cannot induce transcript release but requires an additional factor that can be provided by extract from either mock or A18R mutant-infected cells. The activity is heat-labile as demonstrated by the abolishment of transcript release activity after heating the extract for 10 min at 65 °C.[2] The simplest explanation for these observations is that a cellular factor(s) is needed in addition to A18R for transcript release. Another possible explanation is that the factor(s) from Cts23-infected cells and the factor(s) from mock-infected cells are different. Extract from mock-infected cells could be providing an analogous activity or an activity that abolishes the need for the viral factor. The proof of either hypothesis requires that this factor be purified and identified from uninfected cell extract and potentially Cts23-infected cell extract. Participation of cellular factors in vaccinia virus transcription is not without precedence. Two groups have reported evidence for the requirement of cellular factors, VLTF-X and LPBP, for late transcription initiation (29–31). Whether these are the same factor or two different factors is not known. An additional factor, VITF-2, is provided by the nucleus of uninfected cells and is required for intermediate transcription initiation *in vitro* (32). Taking into account the fact that A18R and the cellular factor act on polymerase complexes initiated from all three stages of transcription, either or both of the previously mentioned cellular factors could be the activity we have discovered. Transcript release was also complemented with partially purified fractions from Wt-infected cells. Additional mock extract was not required for release to occur, providing evidence that the factor was present in the fractions. Therefore, the factor either cofractionated with A18R due to intrinsic properties or it was bound to A18R.

We have observed that the A18R protein and/or the cellular

[2] C. A. Lackner and R. C. Condit, unpublished observations.

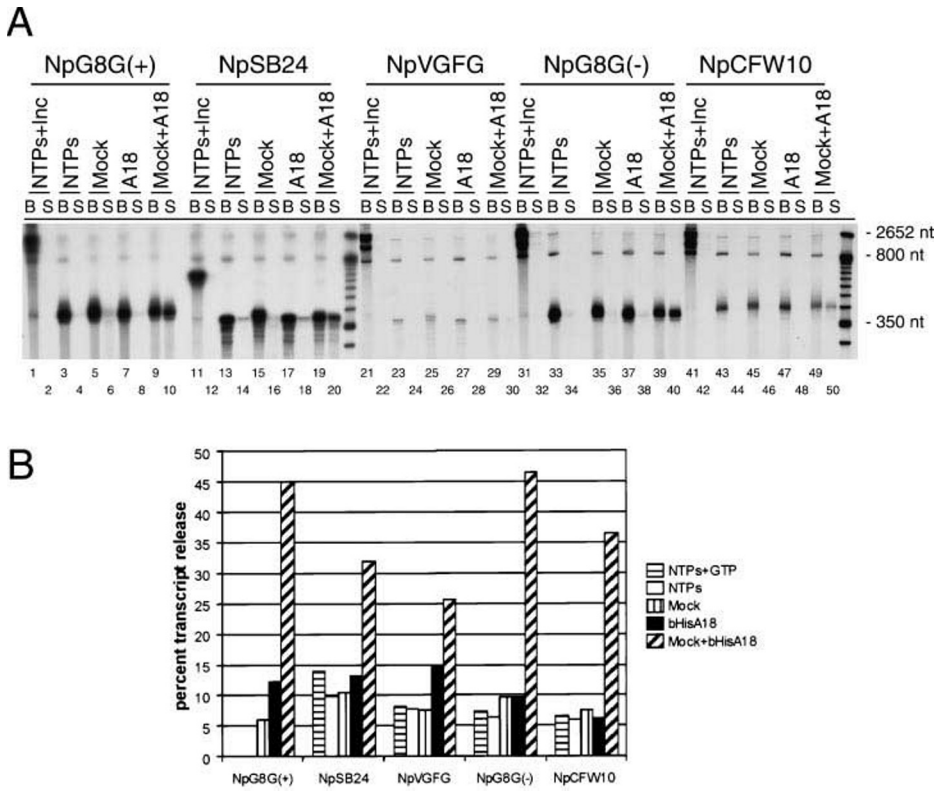

FIG. 8. **A18-dependent transcript release occurs from all vaccinia promoters.** *A*, transcription elongation complexes were formed as described in Fig. 6 using templates containing an early promoter (*NpSB24* and *NpVGFG*), an intermediate promoter (*NpG8G*), or a late promoter (*NpCFW10*). The (+) indicates that the transcription complexes were generated using extract from hydroxyurea-treated Wt-infected cells. The (−) indicates that the transcription complexes were generated using extract from non-hydroxyurea-treated Wt-infected cells. *B*, bound; *S*, released. *B*, released and bound transcripts are expressed as percent transcript release.

factor must be present during elongation in order for transcript release to occur. The factors need not be present during initiation, because washed elongation complexes that are incapable of transcript release can be induced to release RNA by subsequent addition of A18R protein and the cellular factor. Whether both A18R and the cellular factor must be present during elongation remains to be determined. These results suggest that at least one of the two factors may become associated with the elongation complex after initiation and "ride" the complex, poised for arrest and termination. This phenomenon is not without precedent. For example, the eukaryotic elongation factors TFIIF (33), Elongin (34), and ELL (35) and the prokaryotic elongation factors GreA (36), Q (37), and N (38) must all form an association with their cognate elongation complex as a prerequisite to activity (1).

We have observed that the elongation complex must be stalled in order for transcript release to occur. Specifically, detection of transcript release *in vitro* requires arrest of the polymerase induced by transcribing to a bead attached to the downstream end of a DNA template or by transcribing to the end of a G-less cassette in the absence of GTP or in the presence of 3′-OMeGTP. We have tested elongation through both bacterial plasmid sequences and authentic viral sequences, and we have observed no effect of specific nucleic acid sequence on transcript release *in vitro*. *In vivo*, transcripts synthesized from either intermediate or late genes are heterogeneous in length, consistent with a sequence-independent termination event. The possibility exists that other factors *in vivo* may act to either pause or arrest the transcription complex prior to termination. Thus termination of post-replicative transcription in vaccinia may resemble murine RNA polymerase I termination, where transcription is blocked by TTF-I prior to transcript release catalyzed by PTRF (2, 39).

A18R shares a requirement for ATP hydrolysis with several transcription termination factors from both prokaryotic and eukaryotic systems (1, 19). These factors include Rho (40), La (41), factor 2 (42), and NPH-I (43). Each protein requires a different nucleic acid cofactor for its activity. The A18R ATPase activity is stimulated by single-stranded DNA, double-stranded DNA, and DNA-RNA hybrids, similar to NPH-I, factor 2, and La, respectively. Of these termination factors only Rho and A18R have identified helicase activity. The other proteins contain helicase motifs; however, no helicase activity has been described. The weak helicase activity of A18R is capable of unwinding a DNA duplex that is 20 nt or less (18). The protein is also capable of binding single-stranded DNA in the absence of ATP. Whereas our results demonstrate that transcript release is dependent upon ATP hydrolysis, this could be the activity of A18R or the unidentified cellular factor. To determine the ATP-dependent factor, we attempted to purify A18R mutant protein (D206N) for analysis in the transcript release assay. This mutation results in a single amino acid change within the Walker B box sequence proposed to be associated with ATP binding and should have the effect of reducing the ATPase activity of A18R. This mutation destabilized the protein preventing its purification for use in the transcript release assay. Based on these findings we propose that during elongation the A18R protein binds to the single-stranded non-template DNA strand in the region of the transcription bubble (44) and awaits activation of both ATPase and helicase activities to induce transcript release. This activity would be similar to that proposed for NPH-I, the energy coupling factor required for vaccinia early gene transcription termination. NPH-I is postulated to bind to the non-template DNA strand within the transcription bubble. Recognition of the early termination signal by vaccinia termination factor would activate the ATPase activity of NPH-I resulting in the release of the nascent RNA (9, 10).

The observation that the A18R protein catalyzes transcript release from early as well as late transcription complexes suggests that A18R acts on all three classes of transcription *in vivo*. Early transcription elongation complexes are significantly different than intermediate and late transcription complexes both in structure and function (5). Early transcription is catalyzed entirely by enzymes packaged in the virion and presum-

ably occurs within uncoated viral core particles *in vivo*. By contrast, intermediate and late transcription occurs in viral DNA-containing cytoplasmic centers of replication called virosomes. Early complexes contain RAP94, a viral factor that is required for early transcription initiation and that remains strongly associated with the early elongation complex, while intermediate and late transcription complexes probably lack this factor. Early transcripts are homogeneous in length, resulting from recognition of a cis-acting RNA sequence by the vaccinia virus capping enzyme and NPH-I, whereas intermediate and late transcripts are heterogeneous in length. Nevertheless, the fact that the A18R protein is synthesized throughout infection and packaged within virions supports a role for A18R during early transcription. A18R mutants do not affect early viral transcription *in vivo* (28), but this is not atypical for mutations in vaccinia virion enzymes. For example, temperature-sensitive mutants in the virion early transcription initiation factor VETF (45), the RNA helicase NPH-2 (46), the mRNA capping enzyme (47), and the RNA polymerase (48, 49) have shown no pronounced effect on early transcription *in vivo*. Although the mechanism of early transcription termination is reasonably well understood, a role for A18R as an early transcript release factor has not been ruled out.

Genetic and biochemical evidence suggests that both vaccinia intermediate and late gene transcription elongation are regulated by several viral genes in addition to *A18R*. The fact that both intermediate and late transcripts possess heterogeneous 3′ ends implies a similar mechanism of transcription termination for both gene classes. Mutation of either gene *G2R* (14) or *J3R*[3] results in synthesis of 3′-truncated intermediate and late viral mRNAs, implying that each of these gene products exerts positive transcription elongation factor activity on both intermediate and late viral genes. Mutations in either *G2R* (13) or *J3R*[4] suppress *A18R* mutations, strongly suggesting that all three genes function in the same pathway. Interestingly, the *J3R* gene product has previously been shown to encode a protein with both 2′-*O*-methyltransferase and poly(A) polymerase processivity activities (50); no distinct biochemical activity has yet been identified for the G2R protein. One additional protein, the viral *H5R* gene product, has been shown to associate directly with the G2R protein (15). The H5R protein is an abundant phosphoprotein found associated with virosomes (51), and it has been shown to stimulate late viral transcription *in vitro* (52). Finally, evidence exists that the A18R, G2R, and H5R proteins are all associated either directly or indirectly as a complex *in vivo* (15). In summary, the evidence to date suggests that intermediate and late gene transcription elongation in vaccinia is controlled by a complex of viral and cellular factors possessing both positive and negative elongation factor activities. The powerful combination of genetics and the *in vitro* system described here provide us with an opportunity to investigate the activities of the individual components of this transcription elongation complex. Vaccinia has often served as a valuable model system for transcription in higher eukaryotes, and thus these studies of vaccinia transcription elongation and termination may provide insight into the same processes in mammalian cells.

[3] Y. Xiang, D. Latner, and R. C. Condit, manuscript in preparation.

[4] D. Latner, Y. Xiang, J. P. Condit, J. I. Lewis, and R. C. Condit, manuscript in preparation.

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