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Purification and Properties of the Vaccinia Virus mRNA Processing Factor*

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Susan M. D'Costa[1], **Travis W. Bainbridge, and Richard C. Condit**

From the Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida 32610-0266

The mRNAs encoding the vaccinia virus F17 protein and the cowpox A-type inclusion protein are known to possess sequence-homogeneous 3′ ends, generated by a post-transcriptional cleavage event. By using partially purified extracts, we have previously shown that the same factor probably cleaves both the F17 and A-type inclusion protein transcripts and that the cleavage factor is either virus-coded or virus-induced during the post-replicative phase of virus replication. In this study, we have purified the cleavage factor from vaccinia-infected HeLa cells using column chromatography and gel filtration. The factor eluted from the gel filtration column with an apparent molecular mass of ~440 kDa. Mass spectrometric analyses of the proteins present in the peak active fractions revealed the presence of at least one vaccinia protein with a high degree of certainty, the H5R gene product. To extend this finding, extracts were prepared from HeLa cells infected with vaccinia virus overexpressing His-tagged H5, chromatographed on a nickel affinity column, and eluted using an imidazole gradient. Cleavage activity eluted with the peak of His-tagged H5. Gel filtration of the affinity-purified material further demonstrated that cleavage activity and His-tagged H5 co-chromatographed with an apparent molecular mass of 463 kDa. We therefore conclude that H5 is specifically associated with post-transcriptional cleavage of F17R transcripts. In addition, we show that dephosphorylation of a cleavage competent extract with a nonspecific phosphatase abolishes cleavage activity implying a role for phosphorylation in cleavage activity.

Poxviruses are unique among the DNA viruses because they carry out their entire replication cycle in the cytoplasm of the infected cell. Poxviruses therefore encode most of the machinery required for viral replication and transcription (1). Vaccinia virus is the prototypical and therefore best studied member of the Poxviridae family. Vaccinia transcription is temporally regulated by several viral and possibly some host factors. Vaccinia encodes a multisubunit RNA polymerase that bears similarity to eukaryotic RNA polymerases but, importantly, lacks a C-terminal domain. Early virus gene transcription precedes viral DNA replication. Intermediate and late (collectively termed post-replicative) gene transcription is coupled to and therefore occurs concomitantly with DNA replication.

Vaccinia virus employs different mechanisms to mediate 3′ end formation of early and post-replicative viral mRNAs (2). Early transcripts are homogeneous in sequence at their 3′ ends. This homogeneity results from a transcription termination event mediated by a specific *cis*-acting sequence in the nascent RNA (5′UUUUUNU3′) and several virus-coded *trans*-acting early gene-specific termination factors (3–9). By contrast, post-replicative mRNAs are extremely heterogeneous in sequence at their 3′ ends such that each post-replicative gene produces a family of transcripts ~1–4 kb in length (10). These transcripts are polyadenylated by a relatively promiscuous virus-coded poly(A) polymerase. To date, no *cis*-acting sequence has been identified that signals late transcription termination. The *cis*-acting signal that is operative at early times is not active at late times even when present in late transcripts. If a post-replicative *cis*-acting transcription termination sequence exists, it must be both abundant and inefficient to account for the observed RNA 3′ end heterogeneity. Nevertheless, genetic and biochemical experiments have identified several *trans*-acting factors that influence post-replicative mRNA 3′ end formation by modulating transcription elongation and/or termination. These factors include the products of vaccinia genes *A18R*, *G2R*, and *J3R* (11–15). Existing evidence suggests that A18 is a transcription termination factor; A18 catalyzes release of nascent RNA from transcription elongation complexes *in vitro*, and mutants in the *A18R* gene produce longer than normal post-replicative mRNAs *in vivo* (12). In contrast, mutants in *G2R* and *J3R* produce 3′-truncated post-replicative RNAs *in vivo*, suggesting that they are positive transcription elongation factors (11, 13, 15–17). The vaccinia protein H5 may also play a role in transcription elongation. H5 has been shown to stimulate late transcription *in vitro* (18). In addition, H5 interacts with the late transcription elongation factor G2 (19) and with the late transcription initiation factors A2 and G8 (20). Finally and most importantly, an IBT^r mutant has recently been isolated in the *H5R* gene (21). Isatin β-thiosemicarbazone (IBT)[2] is an anti-poxvirus drug that is known to promote readthrough transcription in post-replicative vaccinia genes. Several IBT-dependent and -resistant mutants have been identified previously in other vaccinia proteins that influence late transcription elongation, including two of the largest vaccinia RNA polymerase subunits (J6 and A24) and the positive transcription elongation factors

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[1] To whom correspondence should be addressed: P. O. Box 100266, JHMHC, University of Florida, Gainesville, FL 32610. Fax: 352-392-3133; E-mail: ihssd@ufl.edu.

[2] The abbreviations used are: IBT, isatin β-thiosemicarbazone; ATI, A-type inclusion; NE, nuclear extract; SAP, shrimp alkaline phosphatase; pol, polymerase; FT, flow-through.

(G2 and J3), thus establishing a strong association between IBT resistance and control of transcription elongation (15, 22–24).

Post-transcriptional site-specific cleavage is linked with 3′ end formation of at least some vaccinia late transcripts. At least four late orthopoxvirus transcripts possess sequence-homogeneous 3′ ends, including the *ATI*, *F17R*, *A24R* gene transcripts and transcripts arising from telomere junctions (25–29). Of these, the 3′ ends of the *ATI* and *F17R* gene transcripts result from a post-transcriptional site-specific endoribonucleolytic reaction (28, 29). The mechanism of 3′ end formation of the telomeric transcripts and the A24 transcripts has not been determined. The *cis*-acting sequence required for processing of the ATI transcript includes 43 nucleotides spanning the cleavage site, and the sequence responsible for cleavage in both the *F17R* and *ATI* gene transcripts is orientation-specific. The *F17R* gene cleavage site has not been fine-mapped; however, no obvious sequence or structural homology is evident between the sequences flanking the *F17R* and *ATI* gene cleavage sites. Partially purified extracts cleave both the ATI and F17 transcripts *in vitro*, suggesting the involvement of the same cleavage factor for both transcripts (29). The cleavage factor is either virus-coded or -induced at late times post-infection; "early" extracts prepared in the presence of DNA replication inhibitors and virion-soluble extracts do not possess cleavage activity.

To identify the factor required for post-transcriptional site-specific cleavage of late nascent mRNA transcripts, we have purified the cleavage factor from vaccinia-infected HeLa S3 cells. This study elucidates this purification and confirms unequivocally that the product of the *H5R* gene is specifically associated with cleavage activity. The results carry implications concerning the mechanism of vaccinia virus post-replicative mRNA 3′ end formation in general, and the role of H5 in vaccinia replication in particular.

EXPERIMENTAL PROCEDURES

Eukaryotic Cells, Viruses, and Bacterial Hosts

BSC40 cells, wild type vaccinia strain WR, recombinant vaccinia virus, VVTMA18, VVT7, and the conditions for their growth and infection have been described previously (30–34). HeLa S3 cells (gift from Dr. J. B. Flanegan, University of Florida) were grown in suspension culture in Eagle's minimal essential medium (Joklik's modification) containing 5% calf serum and 2% fetal calf serum. Vaccinia virus VVT7 (vTF7.5) was obtained from Dr. Bernard Moss and described previously (33). *Escherichia coli* BL21(DE3) harboring the plasmid pET14b and containing the H5 open reading frame was a gift from Dr. Paula Traktman.

VVTMhisH5 (vvhisH5) is a recombinant vaccinia virus that contains a polyhistidine-H5R fusion within the thymidine kinase gene, driven by a T7 RNA polymerase promoter and an encephalomyocarditis internal ribosome entry site element. For the construction of VVTMHisH5, wild type vaccinia-virus infected cells were transfected with the plasmid pTMHisH5 (see below). Thymidine kinase-negative recombinants were selected, and their structure was confirmed by PCR analysis using appropriate primers.

Plasmid Constructs

pTMHisH5 was constructed by cloning the H5 coding sequence as an NdeI-BamHI fragment from pGADN-H5 into pTM1his cleaved with NdeI and BamHI (19, 35).

Purification of Vaccinia mRNA Processing Factor

Preparation of Nuclear Extracts from Infected Cell Lysates—Six-liter batches of HeLa S3 cells (5×10^5 cells/ml; 48 liters total) were sedimented by centrifugation, resuspended in 1/10th the original volume (600 ml; fresh medium), and infected with wild type (strain WR) vaccinia virus at 5 plaque-forming units/cell for ½-h at 37 °C. After adsorption, infected cells were diluted into their original volume with spent media, and the infection was allowed to proceed for 18 h at 37 °C. Infected cells were then harvested by centrifugation, washed once with phosphate-buffered saline, re-centrifuged, and pellets were frozen at −80 °C. Preliminary experiments established that most of the cleavage activity was associated with the nuclear fraction. To prepare nuclear extracts, cell pellets were thawed on ice, resuspended in two packed cell volumes of buffer A (25 mM Tris-HCl, pH 8, 1 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, protease inhibitor cocktail (Sigma)) containing 10 mM NaCl, and allowed to swell for 10 min. Cells were then Dounce-homogenized, and the nuclei were separated from the cytoplasmic fraction by centrifugation at $3200 \times g$ for 30 min at 4 °C. The nuclei were resuspended in 1 packed cell volume of Buffer A, 10 mM NaCl, and subjected to a second round of homogenization and centrifugation. The nuclear pellet was then resuspended in ½ packed nuclear volume of Buffer A, 10 mM NaCl. Buffer A, 20% glycerol, 500 mM NaCl was then added dropwise with stirring, and the homogenate (now in Buffer A containing 10% glycerol and 250 mM NaCl) was kept stirring on ice for 30 min. This homogenate was then sonicated four times for 15 s with a Sonics & Materials sonicator, model VC-250, and clarified at $100{,}000 \times g$ for 2 h in a swinging bucket rotor to generate nuclear extract (NE). Total protein concentration was determined using the Bradford protein assay (Bio-Rad).

Overexpression of H5 and A18 was achieved by co-infecting HeLa cells with vTF7.5 and either VVTMHisH5 or VVTMHisA18 (multiplicity of infection = 5). Nuclear extracts for purified his-H5 and his-A18 were prepared similar to wild type infections with the exception that nuclear pellets were resuspended in Buffer A such that the final concentration of NaCl in these homogenates was 400 mM. Expression of His-tagged H5 from bacterial cultures was done as described elsewhere (34).

Purification Scheme—We carried out *in vitro* cleavage assays (as described below) to monitor the chromatographic behavior of the cleavage factor. All purification steps with the exception of DEAE-cellulose chromatography were performed on a Bio-Rad BioLogic DuoFlow at 4 °C. DEAE-cellulose chromatography was carried out at 4 °C using a Bio-Rad Econo Pump run at maximum flow rate (1.6 ml/min). Buffer A indicated here is the same as that used for the preparation of extracts except that it contains 10% glycerol unless otherwise indicated. NE (175 ml; 800 mg) was applied to a 60-ml DEAE-cellulose (DE52, Whatman) column equilibrated with Buffer A containing 250 mM NaCl. The flow-through (FT) was collected and the column was

washed with 100 ml of starting buffer. The wash fraction was collected and pooled with the FT (DEAE pool). The DEAE pool (270 ml; 600 mg) was then applied to an 80-ml SP-Sepharose (GE Healthcare) column equilibrated with Buffer A containing 250 mM NaCl. After a wash with 400 ml of the starting buffer, the column was eluted in a stepwise fashion with 800 mM NaCl in Buffer A (800 ml). Ten-ml fractions were collected and assayed for cleavage using the *in vitro* cleavage assay described below. Fractions containing cleavage activity were pooled (SP-active; 20 ml; 55 mg) and dialyzed into Buffer B (20 mM KPO_4, pH 7.2; 200 mM NaCl; 5% glycerol). The dialyzed SP-active fraction (24 ml; 49 mg) was then applied to a 10-ml ceramic hydroxyapatite type II (Bio-Rad) column equilibrated with Buffer B. After a wash with 50 ml of starting buffer, the column was developed with a 50-ml linear gradient from 20 to 350 mM KPO_4, pH 7.2, in Buffer C (200 mM NaCl, 5% glycerol). One-ml fractions containing cleavage activity eluting between 190 and 240 mM potassium phosphate were pooled and dialyzed (HAP-active; 11 ml; 4 mg) into Buffer A containing 5% glycerol and 400 mM NaCl. HAP-active was then concentrated using an Amicon concentrator (Ultracel 100K; Millipore) to 175 μl (2.5 mg) and applied to a Superdex 200 gel filtration column (10 × 30 cm; GE Healthcare) equilibrated in Buffer A containing 5% glycerol and 400 mM NaCl. Molecular weight standards (high and low molecular weight standards; Amersham Biosciences) were used to calibrate the gel filtration column.

Purification of His-H5 Protein

The purification scheme for vaccinia His-tagged proteins (hisH5 and hisA18) was as follows: HeLa NE (200 mg, 12 liters) was applied to a 5-ml DEAE-cellulose column equilibrated with Buffer A containing 400 mM NaCl. The flow-through was collected; the column washed with 10 ml of the starting buffer, and the two fractions were pooled (DEAE pool). The salt concentration in the DEAE pool fraction was adjusted to 1 M and applied to a 5-ml nickel affinity column (His-trap; GE Healthcare) equilibrated with Buffer A containing 1 M NaCl. After a 25-ml wash with the starting buffer, the column was washed with 100 ml of Buffer A containing 1 M NaCl and 150 mM imidazole. The His-trap column was then developed with a 50-ml linear gradient from 150 to 300 mM imidazole in Buffer A. (A 50-ml step of 300 mM imidazole in Buffer A was used to elute hisA18.) The H5 protein eluted from the His-trap column was pooled, concentrated using an Amicon concentrator (Ultracel 5K; Millipore) to 2.5 ml (2.5 mg), and applied to a Superose 300 gel filtration column (GE Healthcare) equilibrated in Buffer A containing 5% glycerol and 400 mM NaCl. Molecular weight standards (high and low molecular weight standards; Amersham Biosciences) were used to calibrate the gel filtration column.

Alternatively, HeLa NE from 3 liters of vvhisH5- or vvhisA18-infected cells was applied to a 5-ml DEAE-cellulose column under the same conditions described above. DEAE pool from these fractionations was then applied to a 1-ml His-trap column in 400 mM NaCl. The column was successively washed with 20 ml of starting buffer (or buffer containing 0.5 M NaCl), 20 ml of buffer containing 0.4 M NaCl (or 0.5 M NaCl) and 150 mM imidazole, and finally developed with 20 ml of elution buffer containing 0.4 M NaCl (or 0.5 M NaCl) and 500 mM imidazole.

In Vitro Cleavage Assay

Plasmid pGF17stp, which contains the 3′ end of the F17R transcript was used as template for synthesizing the ^{32}P-labeled RNA substrate used for the *in vitro* cleavage assay as described previously (29). Cleavage assays were done as described previously with the following modifications. Briefly, 10 fmol of the substrate was denatured at 90–95 °C for 10 min and incubated with either infected cell extracts or fractions obtained during purification. Assays were typically carried out at 30 °C for 10 min in the presence of 40 mM Tris-HCl, pH 7.5, 300 mM NaCl, 7.5 mM $MgCl_2$, and 2.5 μg of bovine serum albumin in a final volume of 25 μl. Samples were then treated with 2 μl of "PK mix" (100 mM Tris-HCl, pH 7.5, 12.4 mM EDTA, 150 mM NaCl, 1% SDS, 50 μg of proteinase K) at 37 °C for at least 15 min. An equal volume (27 μl) of formamide-loading buffer was then added to the PK-treated samples. Samples were denatured at 90–95 °C for 5 min and loaded on a 6% polyacrylamide gel containing 8 M urea (Sequagel, National Diagnostics). Gels were fixed, dried, and visualized by autoradiography.

For the phosphatase experiments, the cleavage assay was carried out as described above but in the presence of 2 or 4 units of shrimp alkaline phosphatase (SAP; Promega) and 1× SAP buffer. For the heat-inactivated control, 20 units of SAP was heated at 90 °C for 15 min, and 4 units of this heat-inactivated SAP was added to the cleavage reaction containing all of the above-mentioned reagents and 1× SAP buffer.

Immunoblot Assays

Samples were resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose filters in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Filters were incubated either with polyclonal antisera directed against H5 (1:20,000) (36) or monoclonal antisera directed against the histidine tag (1:2000, Novagen). The bound primary antibody was then detected using a horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse; 1:5000) and chemiluminescent reagents (GE Healthcare).

Mass Spectroscopy

Mass spectrometric analyses were carried out at Custom Biologics (Toronto, Canada). Protein band(s) of interest were carefully excised and prepared for in-gel digest with trypsin followed by liquid chromatography/tandem mass spectrometry analysis as described. Briefly, gel slices were de-stained in a 1:1 (v/v) mixture of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate and then washed several times with de-ionized water. Acetonitrile was added to shrink and dehydrate the gel pieces which were then reduced by replacing the acetonitrile with 10 mM dithiothreitol in 100 mM ammonium bicarbonate. After 1 h at 56 °C the dithiothreitol solution was replaced with acetonitrile to once again shrink and dehydrate the gel slices. Dehydrated gel slices were re-swollen by the addition of the alkylating agent, 55 mM iodoacetamide in 100 mM ammonium bicarbonate, and incubated in the dark for 45 min at room temperature. Excess iodoacetamide was removed by washing

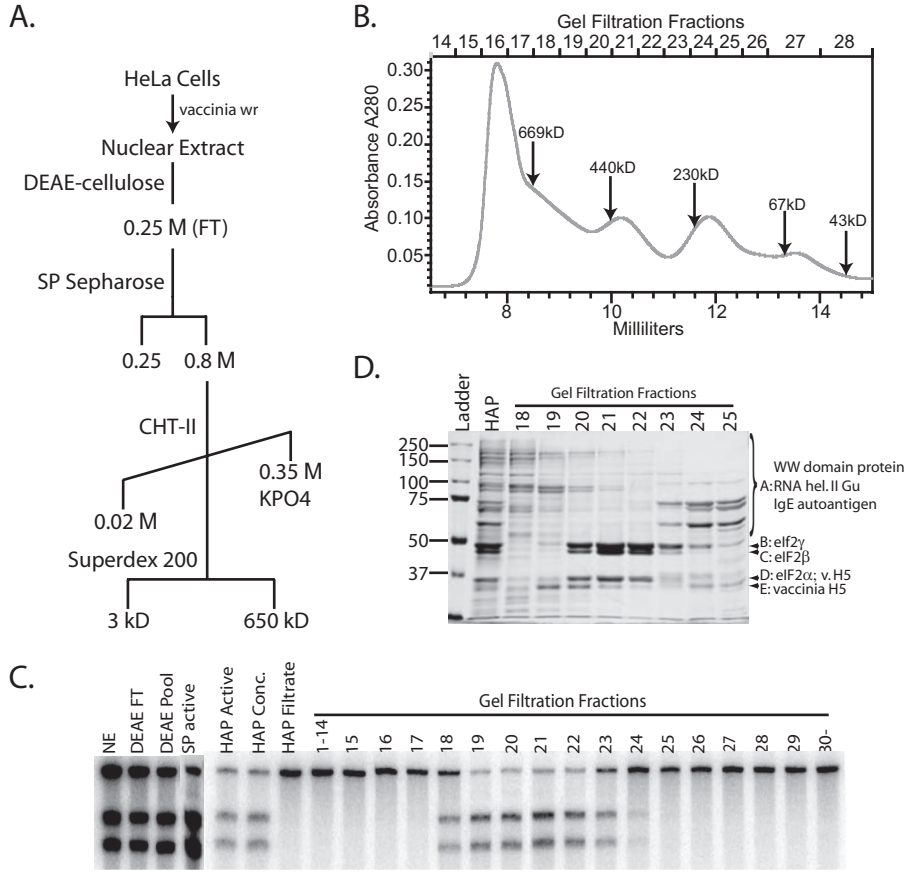

FIGURE 1. **Purification of the F17 mRNA processing factor.** *A,* purification scheme starting with a nuclear extract from vaccinia WR-infected HeLa cells is outlined specifying columns and fractionation protocols at the different steps. *Diagonal lines* indicate a linear elution gradient and *horizontal lines* indicate a step. The final step in the purification is Superdex 200 gel filtration column that separates globular proteins between 10 and 650 kDa. The cleavage factor eluted with a peak at 440 kDa. *CHT-II*, ceramic hydroxyapatite. *B,* A_{280} profile for Superdex 200. Absorbance, A_{280}, is indicated on the *left*. Fraction numbers are indicated at the *top*, and the volume (ml) at which each fraction eluted is indicated at the *bottom* of the plot. Fractions 1–26 were collected as 0.5-ml fractions, and fractions 27 to the end were collected as 1-ml fractions. *C,* autoradiograph of an *in vitro* cleavage assay showing representative fractions during purification. ^{32}P-Labeled F17 transcript (430 nucleotides) was incubated at 30 °C for 10 min with nuclear extracts or with fractions obtained at individual fractionation steps. The reaction was stopped with proteinase K and fractionated on a 6% polyacrylamide-urea gel. Pooled fractions containing cleavage activity at the various steps of the purification and all gel filtration fractions are shown. *NE,* nuclear extract; *DEAE FT,* flow-through from the DEAE cellulose column; *DEAE Pool,* FT and Wash from the DEAE-cellulose column; *SP active,* pooled and dialyzed active fractions from SP-Sepharose; *HAP active,* pooled and dialyzed active fractions from ceramic hydroxyapatite; *HAP conc,* concentrated (Amicon 100K) HAP active (applied to the gel filtration column); *HAP filtrate,* filtrate during concentration of HAP active. *D,* electrophoretic analysis of gel filtration fractions. Samples (40 μl) were electrophoresed through a 0.1% SDS, 8% polyacrylamide gel and subsequently silver-stained. Molecular weight ladder is indicated to the *left*. Bands A–E were cut and analyzed by mass spectrometry. The results of the mass spectrometric analysis are indicated on the *right*.

repeatedly with alternating aliquots of 100 mM ammonium bicarbonate and acetonitrile. Dehydrated gel pieces were air-dried at room temperature and then swelled on ice by the addition of digestion buffer containing 50 mM ammonium bicarbonate and 12.5 ng/μl of trypsin (Roche Applied Science, sequencing grade). After the gel pieces had completely swelled they were transferred to 37 °C and incubated overnight. Digestion buffer was transferred to a clean tube and 100 mM ammonium bicarbonate was added to the gel slices to extract the peptides by shaking at high speed for 45 min. Resulting proteolytic fragments were recovered by reverse phase purification. Eluted peptides were detected by a LCQ DECA XP mass spectrometer (Thermo, MA), equipped with an electrospray ionization source, a low flow metal needle assembly operating in data-dependent mode. The method consisted of two scan events, a full scan and a second data-dependent MS/MS scan. The dynamic mass range of the full scan was set at 300-3000 *m*/*z*. The resulting MS/MS data-dependent scan rejected known "contaminant" masses of 371.0, 391.0, 445.0, 462.0, 1221.89, 1321.9, 1421.9, 1521.8, 1521.9, 1621.9, 1721.9, and 1821.9 *m*/*z*. All other method parameters were default values set by the Xcaliber software version 1.2 (Thermo, MA).

RESULTS

Purification of the Cleavage Factor—Our preliminary analyses indicated that the bulk of cleavage activity is present at late times during vaccinia infection and fractionates with the nuclei of infected cells. We suspect that the cleavage factor is localized in virosomes and therefore partitions with nuclei during differential centrifugation of infected cell extracts. As detailed under "Experimental Procedures," we used an *in vitro* cleavage assay to test for fractions obtained during the various steps of purification, which could specifically cleave a radiolabeled RNA substrate. For reasons that we do not fully understand, we were unable to reproducibly achieve linearity in either a substrate or an enzyme titration in the cleavage assay. Therefore, we were unable to devise a quantitative assay or a unit definition for cleavage activity and have adopted the qualitative approach presented in this study.

The purification scheme outlined in Fig. 1 was established after numerous trial purifications using diverse chromatographic resins. Nuclear extracts were prepared from ~48 liters of HeLa cells infected with vaccinia virus, strain WR. These extracts were applied to a DEAE-cellulose column, and the flow-through (FT) from this column was collected. DEAE-cellulose chromatography served to remove any contaminating nucleic acids present in the crude extract and also clarify the extract before applying it to subsequent columns on the Bio-Rad Duoflow FPLC system. Further fractionation on SP-Sepharose and hydroxyapatite was carried out as outlined in Fig. 1 and detailed under "Experimental Procedures." The activity that eluted off the hydroxyapatite column was applied to a Superdex 200 gel filtration column as a final purification step. The gel filtration column also served to determine the molecu-

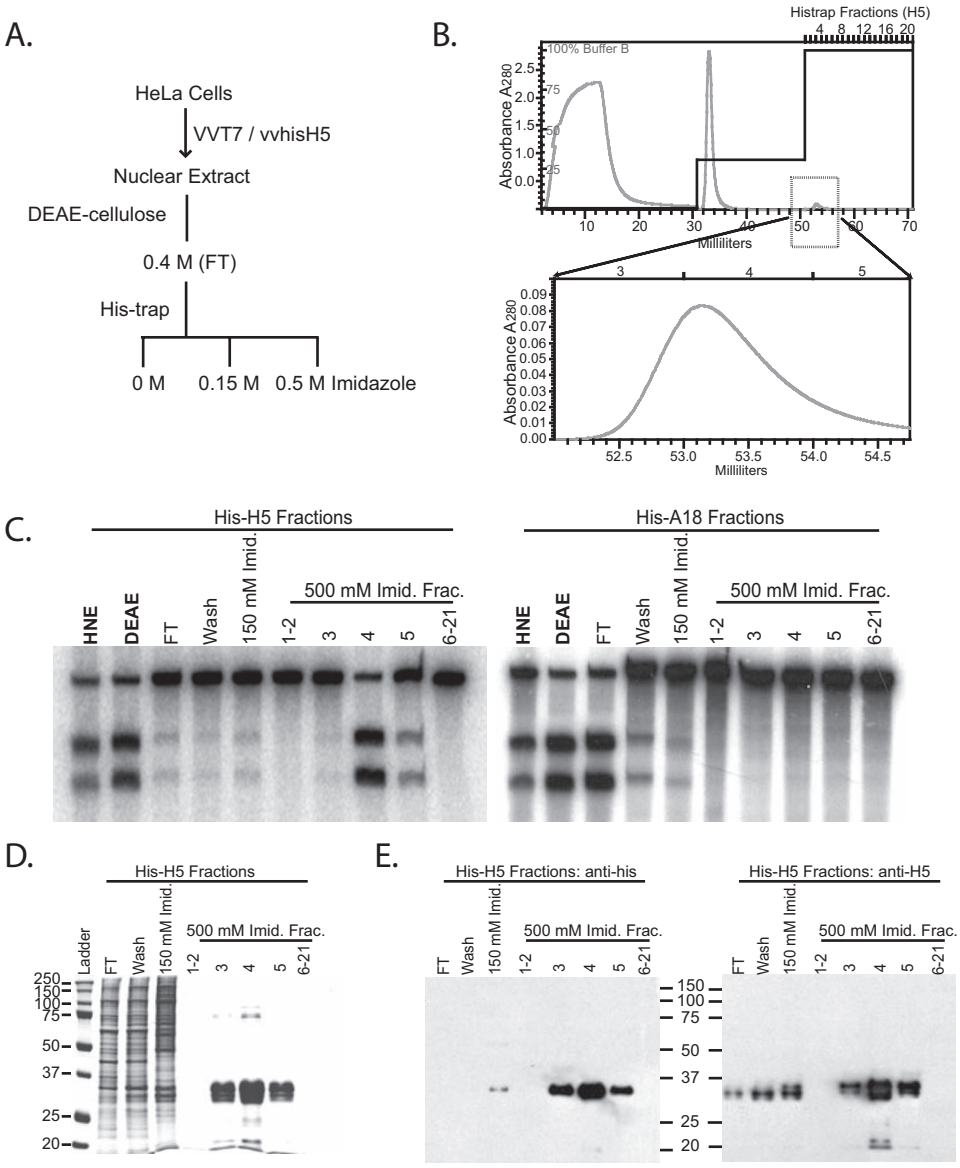

FIGURE 2. **F17 mRNA processing activity co-elutes with purified H5 protein.** *A,* purification scheme showing the fractionation of a nuclear extract from HeLa cells co-infected with VVT7 and vvhisH5 virus. *Horizontal bars* indicate a step elution. His-trap, nickel-affinity column (GE Healthcare). *B,* A_{280} profile of his-H5 purification on the His-trap column. Absorbance, A_{280}, is indicated on the *left*. Fraction numbers are indicated at the *top,* and the volume at which each fraction eluted (milliliters) is indicated at the *bottom* of the plot. The flow-through and 150 mM imidazole (*Imid.*) steps were collected as pools, and the 500 mM imidazole step was collected as 1-ml fractions. The peak of protein in the 500 mM imidazole step has been enlarged to show detail. *C,* autoradiographs of *in vitro* cleavage assays depicting the co-elution of cleavage activity with purified His-tagged H5 protein but not with his-A18 protein. ^{32}P-Labeled F17 transcript (430 nucleotides) was incubated at 30 °C for 10 min with fractions obtained at individual fractionation steps. The reaction was stopped with proteinase K and fractionated on a 6% polyacrylamide-urea gel. The peak of cleavage activity is in fraction 4 of the his-H5 purification, whereas the peak of cleavage activity is in the FT of the his-A18 purification. *DEAE, FT* and *Wash* (pooled) from the DEAE-cellulose column; *FT,* flow-through. *D,* electrophoretic analysis of His-trap active fractions. Samples (40 μl) were electrophoresed through a 0.1% SDS-11% polyacrylamide gel and subsequently stained with Coomassie Brilliant Blue. Molecular weight markers are indicated to the *left*. *E,* immunoblot analyses of his-H5 fractions. Samples (40 μl) were electrophoresed through duplicate 0.1% SDS, 11% polyacrylamide gels, transferred to nitrocellulose membrane overnight, and immunoblotted with anti-His antibody (*left*) or with anti-H5 antibody (*right*). Molecular weight markers are indicated in between the two blots. The peak of H5 protein is in fraction 4.

lar mass of the cleavage factor. The peak of cleavage factor activity (fraction 20) eluted with an apparent molecular mass of 440 kDa from the gel filtration column (Fig. 1, *B* and *C*). SDS-PAGE analysis of the gel filtration fractions indicated that at least four prominent protein bands were enriched in intensity in the cleavage-active fractions as compared with flanking fractions from the gel filtration column or peak fractions from earlier purification steps (Fig. 1*D*). Gel slices marked A–E excised from fraction 20 and analyzed by mass spectrometry revealed the presence of only one viral protein with a high degree of certainty, vaccinia H5 (Gel Slice D and E, ~35 kDa). In addition, as expected, a number of host proteins were identified in this fraction, including all three subunits of the translational initiation factor eIF2, and several host nuclear and nucleolar proteins (WW domain protein, nucleolar RNA helicase II Gu, IgE autoantigen). We have determined that eIF2 is not required for cleavage activity (data not shown). However, we are uncertain what role, if any, the host nuclear and nucleolar proteins may play in vaccinia late mRNA processing.

Purification of Overexpressed His-tagged H5 from Vaccinia-infected HeLa Cells—To confirm the role of H5 in late mRNA processing, we purified H5 from HeLa cells infected with a vaccinia virus overexpressing a His-tagged version of H5. We prepared nuclear extracts at late times postinfection from HeLa cells co-infected with VVT7 and vvhisH5. Fig. 2*A* describes the scheme for this purification. Using nickel-affinity chromatography, we purified His-tagged H5 (bulk of protein eluted in the 500 mM imidazole step) and observed that cleavage activity co-eluted with the H5 protein, suggesting that H5 is associated with cleavage activity (Fig. 2, *B–E*). To rule out the possibility that the co-elution of cleavage activity with His-tagged H5 was purely circumstantial, we purified His-tagged A18 from vaccinia-infected HeLa cells using a protocol similar to that used for the his-H5 purification. Cleavage activity eluted primarily in the flow-through fraction rather than in the 500 mM imidazole step that contained the bulk of A18 protein (Fig. 2*C*). These data confirmed the specific association of H5 with cleavage activity.

To determine the molecular weight of the affinity-purified, cleavage-competent H5, his-H5 was purified from 12 liters of HeLa cells infected and processed under the same conditions described above with the following modifications. The DEAE

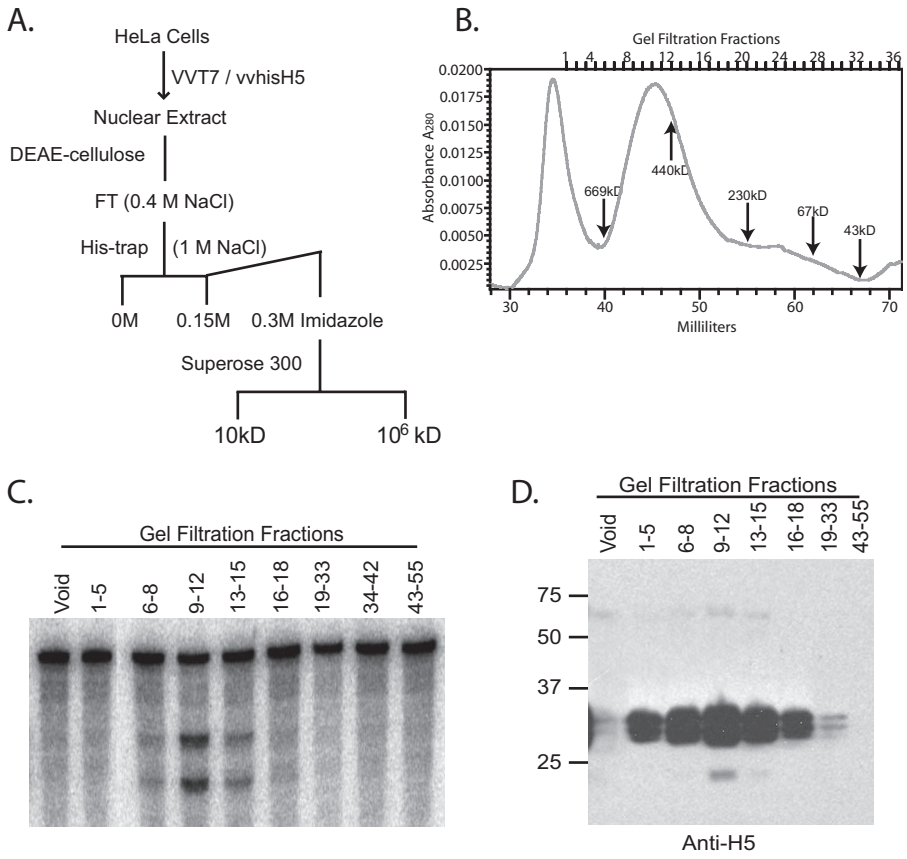

FIGURE 3. **His-tagged H5 co-elutes with cleavage activity with a peak at 463 kDa on gel filtration.** *A,* purification scheme showing the fractionation of a nuclear extract from 12 liters of HeLa cells co-infected with VT7 and VVTMHisH5 virus. *Diagonal lines* indicate a linear elution gradient, and *horizontal bars* indicate a step elution. His-tagged H5 co-eluted with cleavage activity with a peak at 463 kDa on Superose 300 gel filtration column that separates globular proteins between 10 and 10^6 kDa. *B,* A_{280} profile from Superose 300. The peak of activity from the His-trap column was pooled and concentrated (Amicon 5K) before applying to Superose 300 gel filtration column. Absorbance, A_{280}, is indicated on the *left*. Fraction numbers are indicated at the *top,* and the volume at which each fraction eluted is indicated at the *bottom* of the plot. Each fraction represents 1 ml of the elution volume. *C,* autoradiograph of an *in vitro* cleavage assay depicting the co-elution of cleavage activity with purified His-tagged H5 protein. ^{32}P-Labeled F17 transcript (430 nucleotides) was incubated at 30 °C for 10 min with fractions obtained at individual fractionation steps. The reaction was stopped with proteinase K and fractionated on a 6% polyacrylamide-urea gel. The peak of cleavage activity is in gel filtration fractions 9–12. *D,* immunoblot analysis of gel filtration fractions. Samples (40 μl) were electrophoresed through a 0.1% SDS, 12% polyacrylamide gel, transferred to nitrocellulose membrane overnight, and immunoblotted with anti-H5 antibody. Molecular weight markers are indicated on the *left*. The peak of H5 protein is in gel filtration fractions 9–12.

pool fraction was adjusted to 1 M NaCl and then applied to the nickel-affinity column. In addition, the nickel-affinity column was developed with a linear elution gradient between 150 and 300 mM imidazole. H5 protein-containing fractions from this purification were then pooled and applied to a Superose 300 gel filtration column. Cleavage activity and H5 protein co-eluted from this column with an apparent molecular mass of 463 kDa, similar to that observed in the conventional purification (440 kDa) (Fig. 3, *B–D*).

The protein composition of the gel filtration-purified, cleavage-active fractions from the his-H5 purification were analyzed by SDS-gel electrophoresis followed by mass spectrometry of individual protein containing gel slices. A comparison of peak fractions from both H5 purifications (conventional preparation and the his-H5 overexpression preparation) and the A18 purification, analyzed in duplicate by SDS-PAGE, stained with silver or Coomassie Brilliant Blue, is shown in Fig. 4. H5 was the most abundant and the only viral protein identified in the his-H5 preparation by mass spectrometry. At least three host proteins were identified to co-elute with purified his-H5 as follows: a heterotetrameric splicing factor PSF/p54^{nrb}, comprised of two polypeptides of 100 and 54 kDa, and two chaperone proteins, hsp70 and mortalin, comprising polypeptides of 70 and 74 kDa, respectively (Fig. 4). Based on gel electrophoretic and mass spectrometric analysis, the only protein that is obviously common to both the conventional cleavage factor preparation and the his-H5 preparation and missing from the A18 preparation is H5. However, the possible role of a host protein in catalyzing RNA cleavage has not been ruled out.

Phosphorylation Is Essential for Cleavage Activity—Because H5 and several of the host factors that co-elute with H5 are phosphorylated, we tested the effect of a nonspecific phosphatase, SAP on cleavage activity (Fig. 5). Active fractions from the DEAE column (DEAE Pool) were treated with either 2 or 4 units of SAP during the cleavage reaction (Fig. 5, *4th* and *5th lanes*). As a control, DEAE Pool was treated either with SAP buffer or heat-inactivated SAP under the same conditions (Fig. 5, *2nd* and *3rd lanes*). Under both control treatment conditions, the amount of cleavage activity observed was comparable with that observed with untreated DEAE Pool (Fig. 5, *1st lane*). However, treatment with SAP either severely reduced (*2 units, 4th lane*) or completely abolished (*4 units, 5th lane*) cleavage activity suggesting a role for phosphorylation in cleavage.

Bacterially Expressed H5 Is Inactive in Cleavage Activity—In a further attempt to determine whether post-translational modifications and/or association with host proteins might be required for cleavage activity, his-H5 was expressed in *E. coli*, purified by nickel affinity chromatography, assayed for cleavage activity, and sized by gel filtration. The bacterially expressed protein was inactive in cleavage and eluted from a gel filtration column with an apparent molecular mass of 460 kDa (data not shown).

DISCUSSION

In this study we purified a vaccinia virus-specific mRNA cleavage activity from 48 liters of virus-infected HeLa cells and

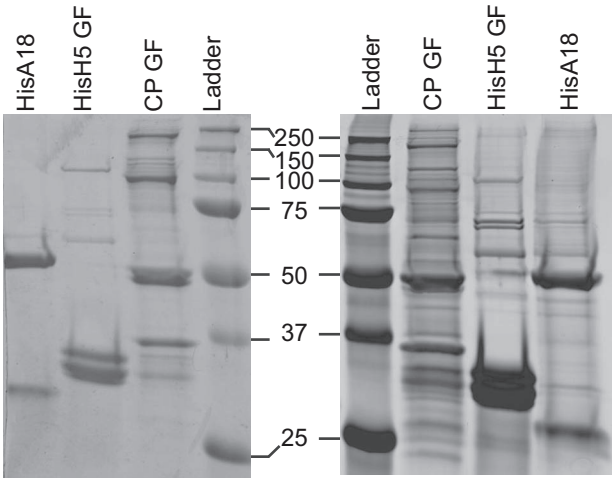

FIGURE 4. **Comparison of the protein composition of peak active fractions from the H5 purifications (conventional and affinity) and affinity-purified A18.** Samples (40 μl) were electrophoresed through 0.1% SDS, 12% polyacrylamide gels in duplicate and subsequently stained with Coomassie Blue (for a quantitative analysis) or silver-stained (for an analytical analysis). Molecular weight markers are indicated between the two gels. *HisA18,* purified His-tagged A18 from infected HeLa cells; *HisH5 GF,* purified His-tagged H5 (Superose fractions 9–12; Fig. 3); *CP GF,* Superdex fraction 20 from conventional preparation (Fig. 1).

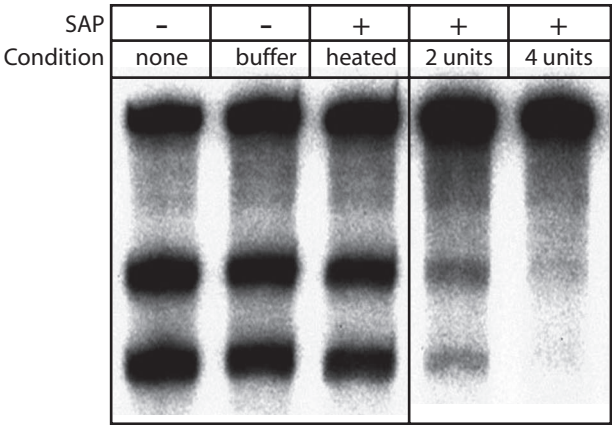

FIGURE 5. **Effect of phosphorylation on cleavage activity.** ^{32}P-Labeled F17 transcript (430 nucleotides) was incubated at 30 °C for 10 min with cleavage-competent DEAE fractions (Control; *1st lane*) and with 1× SAP buffer (Buffer Control, *2nd lane*) or 1× SAP buffer and heat-inactivated shrimp alkaline phosphatase (heated control, 4 units, *3rd lane*), or 1× SAP buffer and 2 units of SAP (*4th lane*) or 1× SAP buffer and 4 units of SAP (*5th lane*). Cleavage activity in *2nd* to *5th lanes* was compared with that obtained in *1st lane*.

found that the vaccinia H5 protein is associated with this activity. We confirmed the specific association of vaccinia H5 with cleavage by demonstrating that the cleavage activity co-purified with a His-tagged H5 protein that was overexpressed in vaccinia virus and purified by nickel affinity chromatography and gel filtration. As described under "Results," the cleavage assay used for the purification is qualitative rather than quantitative. We have considered several possible explanations for the difficulties encountered in devising a quantitative assay. First, it is possible that conformational isomers of the substrate affect the linearity of a substrate titration. A similar observation was made during the purification of a mammalian endoribonuclease specific for α-globin mRNA (37). Second, a dynamic interaction of H5 with the substrate could also limit cleavage. Third, we suspect that the cleavage activity may require association of H5 with host factors (see below), and that the dynamics of this interaction could affect the quantitative nature of the assay. Finally, we have experienced stability issues with purified fractions of the cleavage factor resulting in variation in reproducibility of the cleavage assay.

The *H5R* gene is highly conserved in the chordopoxviruses but is absent from entomopoxviruses. H5 has a predicted molecular mass of 22.3 kDa but migrates anomalously on SDS-polyacrylamide gels at 35 kDa, possibly because of a proline-rich domain in its N terminus and several acidic and basic regions (38–40). H5 is a phosphoprotein that is constitutively expressed in abundance during viral replication and packaged into virions (38). Early during viral infection, H5 is diffusely localized within the cytoplasm; however, following DNA replication, it is localized specifically to virosomes (18, 41, 42). Both single and double strand DNA cellulose chromatography have been used to purify H5 suggesting that it is a DNA-binding protein; however, the nucleic acid binding properties of H5 have not been investigated in detail (18, 39, 43). H5 is phosphorylated by both viral kinases (F10 and B1) and cellular kinases (39, 44). Brown *et al.* (45) have demonstrated that B1 can phosphorylate H5 at least on two threonine residues (positions 84 and 85) *in vitro*; the residues phosphorylated *in vivo* have not been mapped.

Both H5 and cleavage activity elute from gel filtration columns with a high apparent molecular mass (~460 kDa) during both conventional and affinity purifications. Similar results were obtained with fairly crude fractions, DEAE FT as well as DEAE FT treated with micrococcal nuclease (not shown), suggesting that this large molecular mass is not a result of protein-nucleic acid interactions and that cleavage-competent H5 specifically interacts with itself and/or other host proteins. Interestingly, cleavage incompetent H5 from *E. coli* elutes with a similarly high molecular mass (460 kDa) upon gel filtration. This suggests that the large molecular mass of cleavage-competent H5 represents self-association in a large homomultimer (15–20 H5 subunits) rather than association with HeLa cellular proteins. Although we cannot rule out the possibility that the large molecular weight of cleavage-incompetent H5 purified from *E. coli* is as a result of interaction with other bacterial proteins, no other proteins were observed during SDS-PAGE analysis of the gel filtration fractions of bacterially purified H5.

H5 has been implicated in several steps in viral replication, including DNA synthesis, post-replicative gene transcription, and virion morphogenesis. Recently, our laboratory has identified a temperature-sensitive vaccinia mutant (Dts57) that maps to the H5 gene and that has a DNA-negative phenotype, strongly suggesting a central role for H5 in viral DNA replication (46).[3] In addition, H5 interacts with A20, the viral DNA replication processivity factor, and B1, a viral protein kinase required for viral DNA replication (48, 49). H5 has been shown to stimulate late transcription initiation *in vitro* (18), and H5 interacts with itself, with the late transcription initiation factors A2 and G8 and with the post-replicative elongation factor G2 (19, 20, 49). Most importantly, an H5 mutant has recently been

[3] S. M. D'Costa, T. W. Bainbridge, S. E. Kato, C. Prins, and R. C. Condit, manuscript in preparation.

isolated, which results in resistance to the transcription elongation enhancing drug, IBT (21). These data implicate H5 in control of post-replicative transcription, perhaps as an elongation factor. Another temperature-sensitive mutant engineered in H5 by alanine scanning mutagenesis (tsH5-4) has been shown to be unaffected in viral DNA replication and gene expression but defective for early virion morphogenesis (36). This implies that H5 also plays a role in virus assembly; however, it is possible that this mutant may have a subtle defect in transcription that could have a pleiotropic effect on morphogenesis. Our observation that H5 is associated with post-replicative mRNA cleavage is consistent with a role for H5 in post-replicative transcription, and furthermore links post-replicative mRNA cleavage with post-replicative gene transcription elongation.

Although H5 is present throughout virus infection and packaged in virions, mRNA cleavage activity is present only at late times post-infection; cleavage is absent at early times post-infection, and virion extracts do not contain cleavage activity (29). Furthermore, H5 expressed in *E. coli* is inactive in cleavage. These results imply that during the transition from the early to the late phase of infection H5 must either undergo some modification or it must associate with other viral or host proteins to be activated for cleavage. We have shown that phosphatase treatment of crude extracts inactivates cleavage, implicating phosphorylation in cleavage activity. The two viral protein kinases that phosphorylate H5 are active in the infected cell at different times post-infection (B1 is active early and F10 is active late); therefore, the phosphorylation status of H5 may change during infection. In addition, cellular kinases have also been implicated in the phosphorylation of H5. We hypothesize that this intricate and differential phosphorylation of H5 contributes to mRNA cleavage late during infection.

Vaccinia post-replicative transcription termination bears provocative similarities to pol II transcription termination in eukaryotes, and interestingly, a precedent exists for a requirement for co-transcriptional cleavage during pol II transcription termination. Mature pol II transcripts are formed by co-transcriptional cleavage of the nascent mRNA 20–30 nucleotides downstream of a *cis*-acting sequence AAUAAA in a GU-rich region. The cleavage reaction is followed by polyadenylation of the upstream mature transcript by the poly(A) polymerase. Cleavage and polyadenylation require several *trans*-acting factors that make up a cleavage/polyadenylation complex that is tethered to the pol II C-terminal domain. pol II does not terminate at the polyadenylation site but rather continues to transcribe, ultimately terminating inefficiently at numerous sites downstream of the polyadenylation site, reminiscent of the termination process in vaccinia virus. RNA cleavage plays a role in pol II termination in three ways. First, the efficiency of the cleavage/polyadenylation reaction itself can impact on the efficiency of downstream transcription termination possibly through the tethering of the cleavage/polyadenylation complex to the C-terminal domain. Second, the Rat1/Xrn2 5′–3′-exonuclease has been shown to influence termination of some pol II transcripts, inspiring the "torpedo model" of transcription termination. The torpedo model states that the 5′ end of the downstream nascent transcript resulting from the cleavage/polyadenylation reaction serves as an entry site for the Rat1/Xrn2 5′–3′-exonuclease, which then moves downstream toward the transcribing/paused RNA polymerase as it degrades the nascent RNA, ultimately resulting in destabilization of the elongation complex (reviewed in Refs. 50, 51). However, exonucleolytic degradation of the downstream transcript alone may not be sufficient to cause termination. Another factor, for example transcription termination factor 2 (TTF-2), may ultimately catalyze the actual dissociation of the elongation complex. Third, in certain exceptional cases, for example the β-globin gene, a second cleavage site, termed co-transcriptional cleavage, exists 825–1630 nucleotides downstream of the poly(A) site (52). Proudfoot and co-workers (53) have shown that the RNA at this site undergoes an autocatalytic cleavage reaction providing an additional entry site for the exonuclease Rat1/Xrn2.

It is unclear whether H5 alone acts as the cleavage factor or whether other host and/or viral proteins are required for cleavage. Although some host proteins were found in the active fractions following both a conventional purification and the purification of affinity-tagged H5, mass spectrometric data identified a different set of host proteins in each of the purifications. It is possible that some of the same host proteins were present in both preparations, but their identity was masked by other more ionizable peptides. It is interesting to note that a heterotetrameric splicing factor PSF/p54^{nrb} specifically co-eluted with H5 in the affinity purification. This factor is known to be involved in DNA replication and repair as well as RNA transcription, splicing, and turnover (54). Interestingly, this multifunctional protein has recently been shown to recruit the Rat1/Xrn2 "torpedo" exonuclease to the site of 3′ end formation and thereby facilitate pol II-driven termination (47). This raises the possibility that vaccinia actually uses elements of the host torpedo mechanism to effect post-replicative transcription termination. Experiments are currently underway to address the role of PSF/p54^{nrb} in cleavage as well as vaccinia replication.

It is unclear whether the vaccinia cleavage reaction is confined to just a few late transcripts such as the ATI and F17R transcripts, or whether it plays a global role in synthesis of all post-replicative RNAs. If cleavage is a rare event that affects only a few late transcripts, then it may be that cleavage is a vestigial function of a host protein (*e.g.* a splicing factor) that is recruited by the virus for other functions in viral transcription. If the cleavage reaction is global, then we propose that inefficient and degenerate *cis*-acting cleavage sequences must be present in most post-replicative RNAs resulting in the formation of heterogeneous 3′ ends; in certain exceptional cases, including the ATI and F17 transcripts, hypersensitive cleavage sites may exist resulting in homogeneous transcripts. We propose further that, as has been proposed for pol II transcription, RNA cleavage is linked to transcription termination. A18, the vaccinia late transcript release factor, along with the help of an as yet unidentified host factor are implicated in post-replicative vaccinia transcription termination (12). However, A18 and the host factor alone may not be able to effectively cause vaccinia termination; a cleavage event may be required to destabilize the ternary complex and thereby allow for efficient termination. Thus A18 and H5 may act in concert to bring about 3′ end formation. In support of this hypothesis, previous experiments

have shown that A18 and H5 co-immunoprecipitate from vaccinia-infected cells.

In summary, our experiments suggest that co-transcriptional or post-transcriptional endoribonucleolytic cleavage plays a role in 3′ end formation during vaccinia virus late transcription, and that the viral H5 protein is a critical component of the cleavage activity. The presence of PSF/p54^{nrb} in the affinity-purified complex raises the interesting possibility that vaccinia co-opts elements of the host pol II transcription termination system to bring about termination of post-replicative transcripts. The H5 cleavage factor could play a role similar to the co-transcriptional cleavage in the β-globin gene, generating a 5′ end for entry of host exonucleases, leading ultimately to a destabilization of the transcription elongation complex and leaving it susceptible to the action of a viral transcription termination factor, probably A18. Ultimately, determination of the role of the vaccinia ATI/F17R cleavage factor in viral mRNA metabolism will require elaboration of the biochemical mechanism of cleavage, the role of phosphorylation of H5 in cleavage activity, and identification of additional host proteins that could be involved in the cleavage reaction.

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