

# Stimulation of Poly(A) Tail Elongation by the VP39 Subunit of the Vaccinia Virus-encoded Poly(A) Polymerase\*

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The VP55 subunit of the vaccinia virus-encoded poly(A) polymerase can add a maximum of 35 adenylates to the 3'-end of an RNA primer in a rapid and highly processive manner, whereas the VP55-VP39 heterodimer catalyzes the formation of poly(A) tails several hundred nucleotides in length. Here, we describe the overexpression of the VP39 subunit, its purification to near homogeneity, and its ability to associate physically with VP55 and to stimulate polyadenylation. Although VP39 possessed no independent poly(A) polymerase activity, RNA primers with oligo(A) tails greater than 30 adenylates in length could be extended nearly 40-fold more rapidly in the presence of VP39. VP39 enhanced the polyadenylation rate by converting the slow, nonprocessive polyadenylation occurring after the rapid burst in the presence of monomeric VP55, to a rapid, semiprocessive reaction. The effect of VP39 was dramatic when poly(A) primers were used as, 60 mM NaCl, VP39 enhanced the polyadenylation rate 500-fold, and at 90 mM NaCl VP39 was absolutely required. Nevertheless, the VP39-containing polymerase remained selective for polyadenylation of an mRNA 3'-end in the presence of excess poly(A). These data suggest that the role of VP39 in polyadenylation is to increase the affinity of the polymerase for the growing poly(A) tail.

Vaccinia virus is a large DNA virus that replicates in the cytoplasm of the infected cell. Although viral mRNAs possess eukaryotic features, including a 5'-cap and a 3'-poly(A) tail, the entire transcription apparatus appears to be virus-encoded (1). Moreover, the factors and enzymes employed for expression of the early class of genes are packaged within the infectious virus particle and are therefore easily isolated and purified. The virion poly(A) polymerase (PAP)<sup>1</sup> chromatographs and sediments as a heterodimer of the virus-encoded polypeptides VP55 and VP39 (2-4). However, VP39 is present in molar excess over VP55 in extracts of virions (4) or infected cells<sup>2</sup> and so can also be isolated in monomeric form. *In vitro*, the purified PAP heterodimer can catalyze the addition of

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<sup>1</sup> The abbreviations used are: PAP, poly(A) polymerase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; nt, nucleotide; VGF, virus growth factor.

<sup>2</sup> P. D. Gershon, unpublished results.

several hundred adenylates to the 3'-ends of RNA primers of various size and sequence (4-6). Incubation of the heterodimer with immobilized antibody to an N-terminal peptide of VP55 and subsequent washing, led to the anchoring of VP55 and dissociation and elution of VP39 (4). Polyadenylation activity remained associated with the immobilized VP55, although only short poly(A) tails were synthesized. Synthesis of long poly(A) tails was restored, however, by the addition of either the dissociated VP39 or monomeric VP39 isolated from the virion extract (4). It was concluded that VP55 and VP39 are catalytic and stimulatory subunits, respectively. VP39 was also found, either by itself or in association with VP55, to possess an apparently unrelated activity, that of catalyzing cap-specific mRNA (nucleoside-O<sup>2'</sup>-)-methylation (7). Thus, the same enzyme appears to modify both the 5'- and 3'-ends of the mRNA.

The catalytic properties of highly purified, overexpressed monomeric VP55 were determined, using a primer corresponding to the 3'-end of a specific vaccinia virus transcript. Approximately 30-35 adenylates were added by VP55 in a rapid processive burst, after which polyadenylation decelerated abruptly and became nonprocessive (8). By using RNA primers with preformed poly(A) tails of various length, it was shown that the transition between processive and nonprocessive modes of polyadenylation is regulated by the net length of the 3'-oligo(A) tail rather than by the number of adenylate additions catalyzed by VP55. Poly(A) primers are extended by VP55 only slowly and nonprocessively (8) and cannot compete efficiently with a primer corresponding to a nascent vaccinia mRNA 3'-end for the initial burst of polyadenylation. The properties of VP55 would be appropriate for the discrimination of nascent, non-polyadenylated from mature, polyadenylated transcripts.

In the present study, we have further analyzed the role of VP39 in poly(A) tail elongation. To accomplish this, VP39 was individually overexpressed and purified to near homogeneity.

## EXPERIMENTAL PROCEDURES

**Materials**—VP55-C<sub>coli</sub> and VP39-N-pep antisera (4) were used for detection of VP55 and VP39. Poly(A)-Sepharose column fraction 45 (8) provided the highly purified VP55 used for all experiments.

**Overexpression of VP39**—Plasmid pPG170 was made by reversing the orientation of the expression cassette (T7 promoter-EMC leader-polylinker-T7 terminator) of the vector pTM1 (9) with respect to the flanking sequences derived from *Hind*III fragment J of the vaccinia genome. Plasmid pPG175 was made by cloning a PCR copy of vaccinia virus open reading frame J3R in pPG170. The complete DNA sequence of the PCR-generated fragment of pPG175 was verified. Recombinant virus vPG175 was made from pPG175 using standard methods (9). HeLa cells ( $5 \times 10^6$ ) were coinfecte with vPG175 and vTF7-3 (9), each at 10 pfu/cell, and harvested at 18 h.

**Purification of Overexpressed VP39**—Cytoplasmic extracts from  $5 \times 10^9$  infected cells were chromatographed on a 40-ml DEAE-cellulose

(DE52) column and applied to an 80-ml phosphocellulose (P11) column as described (8). After extensive washing with buffer A (50 mM Tris-HCl, pH 8.0, 15% glycerol, 0.1 mM EDTA, 2 mM dithiothreitol) containing 0.2 M NaCl, the column was eluted with a 500-ml linear gradient of 0.2–0.55 M NaCl in buffer A. VP39-containing fractions were identified by immunoblotting (4). These fractions were pooled, diluted to 0.2 M NaCl with buffer A, and applied to a 12-ml Cibacron blue (F3GA)-agarose (Affi-Gel blue, Bio-Rad) column. The column was washed with 0.4 M NaCl in buffer A and eluted with a 240-ml linear gradient of 0.4–1.4 M NaCl in buffer A. VP39-containing fractions were identified by immunoblotting and by assaying for stimulation of the polyadenylation activity of highly purified VP55 (8) in 60-s assays, both before and after a 10-fold dilution of the fractions. Active fractions were pooled, diluted to 0.1 M NaCl with buffer B (buffer A containing 20% glycerol), and applied to a 2-ml heparin-agarose (GIBCO-BRL) column. The column was washed with 0.1 M NaCl in buffer B and eluted with a 60-ml linear gradient of 0.1–0.5 M NaCl in buffer B. VP39-containing fractions, identified by silver-stained SDS-PAGE (polyacrylamide gel electrophoresis) (8) and the polyadenylation-stimulation assay, were pooled, diluted to 0.05 M NaCl with buffer B, and applied to a 3-ml poly(A)-Sepharose (Pharmacia LKB Biotechnology Inc.) column. The column was washed with 0.05 M NaCl in buffer B and eluted with an 84-ml linear gradient of 0.05–0.3 M NaCl in buffer B. Protein in the VP39-containing poly(A)-Sepharose fractions was quantitated (10).

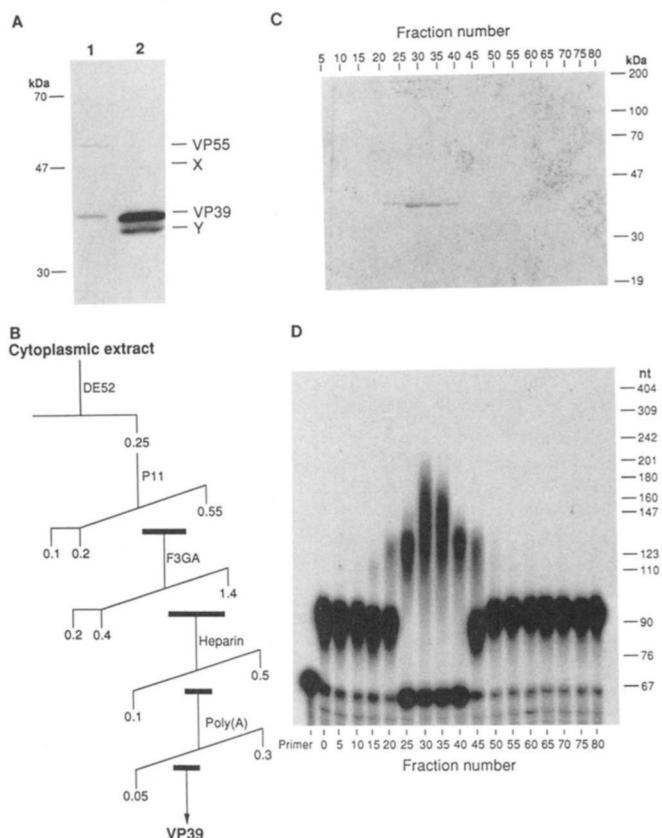
**Sedimentation Analysis**—Immediately prior to sedimentation, samples (which were in column buffer B) were diluted 2-fold with column buffer A lacking glycerol and containing 0.1 M NaCl. The diluted samples (50  $\mu$ l) were sedimented in 0.6-ml gradients of 15–30% glycerol in column buffer A containing 0.2 M NaCl, in an SW 55 rotor spun at 46,000 rpm for 26 h. Fractions (40  $\mu$ l) were harvested from the tops of the gradients and analyzed by immunoblotting as described (4) except for the use of chemiluminescent detection (Amersham Corp.).

**RNA Primers**—Chemical synthesis of the RNA primer  $A_{50}$  has been described (8). The VGF 60-mer RNA, which corresponds to the 60 nucleotides (nt) immediately preceding the poly(A) tail of a transcript of the vaccinia VGF gene (11), and the 60-mer  $A_{42}$  RNA, which corresponds to the VGF 60-mer RNA possessing a 3'-poly(A) tail of 42 adenylates, were synthesized enzymatically. Plasmid pPG191 was made by inserting a PCR copy of the VGF gene into pUC18. Linear, double-stranded DNAs containing the 60-nt sequence were generated by PCR, using pPG191 as the template. The 5'-PCR primer contained the T7 promoter sequence and, for generation of the 60-mer  $A_{42}$  RNA, the 3'-PCR primer contained a tract of 42 thymidylates. T7 transcripts of the linear DNAs were generated by standard methods (12) and, after degradation of the DNA and dephosphorylation of the RNA with calf intestinal phosphatase, were purified by electrophoresis in a 20% polyacrylamide, 7 M urea, Tris/borate/EDTA (TBE) gel. RNAs were 5'-end labeled as described (8). Various size poly(A) primers were 5'-end labeled and isolated as described (8).

**Polyadenylation Assays**—The method used for polyadenylation assays has been described (8). Mg<sup>2+</sup> (1 mM) was the sole divalent cation used throughout this study. Variations in NaCl concentration, type and amount of primer, and VP55/VP39 ratio are described in the legends to figures. Products were resolved in 8% polyacrylamide, 7 M urea gels in TBE buffer.

## RESULTS

**Overexpression, Purification, and Activity of VP39**—The VP39 subunit of vaccinia-encoded poly(A) polymerase was previously isolated in relatively small quantities from vaccinia virions. To obtain larger amounts of VP39, a recombinant vaccinia virus was made containing a second copy of the VP39 gene under the control of a T7 promoter. HeLa cells were infected with this recombinant or with wild-type vaccinia, and coinfecting with a recombinant vaccinia virus expressing T7 RNA polymerase (13). Immunoblots made from cytoplasmic extracts and probed with anti-VP55 and anti-VP39 sera showed that VP39 was specifically overproduced in cells infected with the two recombinant viruses (Fig. 1A). A minor 48-kDa cellular protein cross-reacted with the anti-VP55 serum. The band migrating just ahead of VP39 reacted with the anti-VP39 serum and could also be detected in extracts



**FIG. 1. Overexpression, purification, and activity of VP55.** *Panel A*, immunoblot showing amounts of VP55 and VP39 in unfractionated soluble cytoplasmic extract of HeLa cells infected with recombinant vaccinia virus expressing T7 RNA polymerase in combination with either wild-type virus (*track 1*) or recombinant virus containing a second copy of the VP39 gene under control of a T7 promoter (*track 2*). X, faint cross-reacting cellular protein; Y, minor, additional form of VP39 that can be resolved in some samples. The positions and sizes of marker proteins are shown. *Panel B*, scheme used for the purification of overexpressed VP39 from infected HeLa cell cytoplasmic extract. DE52, P11, F3GA, heparin, and poly(A) denote DEAE-cellulose, phosphocellulose, Cibacron blue-agarose, heparin-agarose, and poly(A)-Sepharose, respectively. Numbers denote NaCl concentration ( $M$ ), and horizontal bars denote pooled fractions. *Panel C*, silver-stained SDS-PAGE of aliquots of fractions from the final column (poly(A)-Sepharose), as also shown in Ref. 7. The positions and sizes of marker proteins are indicated. *Panel D*, aliquots of poly(A)-Sepharose column fractions were assayed for polyadenylation stimulatory activity in the presence of highly purified VP55, the VGF 60-mer primer, and 60 mM NaCl. VP55 and VP39-containing fractions were mixed before the addition of assay buffer, primer, and ATP, and subsequent incubation for 60 s. By protein quantitation, the molar concentration of VP39 in poly(A)-Sepharose column fraction 30 was determined to be 8-fold greater than that of the added VP55. The track labeled *primer* contains primer incubated in the absence of VP55 or VP39. The positions and sizes of DNA markers are shown.

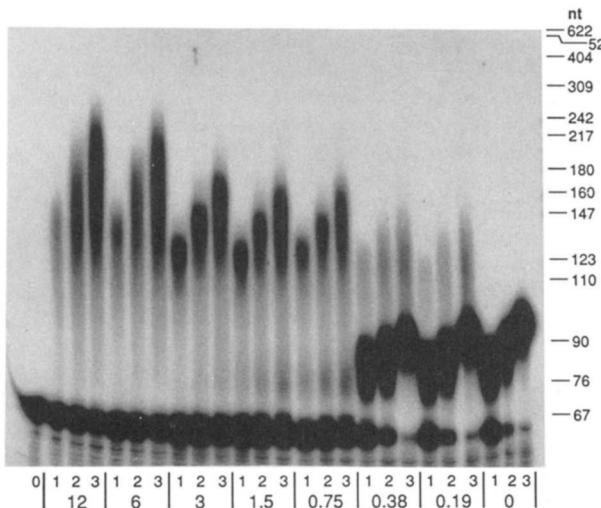
from wild-type virus-infected cells on prolonged autoradiographic exposure. A scheme was developed for the purification of VP39 from the cytoplasmic extracts (Fig. 1B). Fractions were assayed for VP39 and VP55 by immunoblotting. The latter could not be detected after chromatography on phosphocellulose (P11, data not shown). An abundant protein of 39 kDa was detected by silver staining (Fig. 1C) and immunoblotting (not shown) of fractions from the final (poly(A)-Sepharose) column. The minor immunoreactive protein detected in crude cytoplasmic extracts, that migrated ahead of VP39, was still present.

Poly(A)-Sepharose column fractions containing the VP39

peak were assayed for PAP activity using either the VGF 60-mer RNA primer or the VGF 60-mer A<sub>42</sub> primer. As expected, no activity was detected (data not shown). An aliquot of every fifth fraction was then mixed with highly purified VP55 and the mixtures incubated for 60 s with ATP and the VGF 60-mer primer (Fig. 1D). NaCl (60 mM) was included in the assay shown in Fig. 1D, and in other assays (below) employing the VGF 60-mer primer, in order to destabilize nonproductive complexes between VP55 and the RNA.<sup>2</sup> Whereas VP55, either alone or in the presence of fractions containing little or no VP39, was able to add no more than 40 adenylates to the primer during the incubations (Fig. 1D), longer (50–180 nt) poly(A) tails were synthesized in the presence of VP39-containing fractions. This confirms the previously described polyadenylation stimulatory activity of less highly purified VP39 in conjunction with immobilized VP55 (4). Peak fractions from the poly(A)-Sepharose column were also shown to contain the cap-specific RNA methyltransferase activity associated with VP39 (7).

**Association of VP39 and VP55**—By quantitation of protein in the poly(A)-Sepharose column fractions, it was determined that VP39 was in molar excess over VP55 in the assays of fractions 25–40 (Fig. 1D). The effect of VP39/VP55 ratio on polyadenylation of the VGF 60-mer primer was evaluated in further experiments. Poly(A)-Sepharose column fraction 30 was employed for this and all further experiments requiring highly purified VP39. At low ratios, both the shorter, oligoadenylylated products characteristic of monomeric VP55 and the longer VP39-stimulated products were detected (Fig. 2). At ratios of 3 or greater, there was little or no accumulation of short oligoadenylylated RNAs. The rate of polyadenylation, however, continued to increase with increasing VP39/VP55 molar ratios. These data suggested a functional interaction between VP55 and VP39.

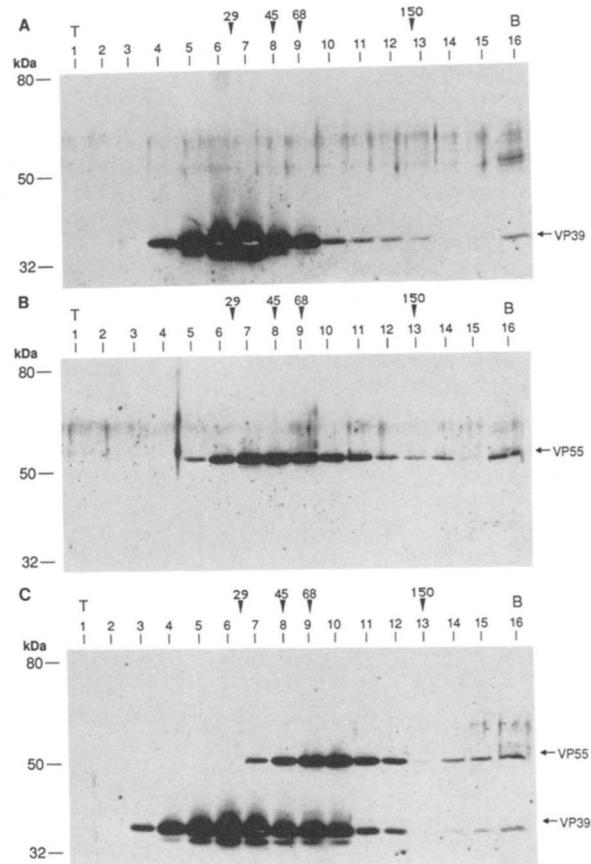
A physical association of VP39 and VP55 was demonstrated by glycerol gradient sedimentation. In parallel gradients, purified VP39 and VP55 were sedimented individually and together after mixing VP55 with a 3-fold molar excess of VP39. Gradient fractions were analyzed by immunoblotting. The sedimentation rates of the individual PAP subunits were



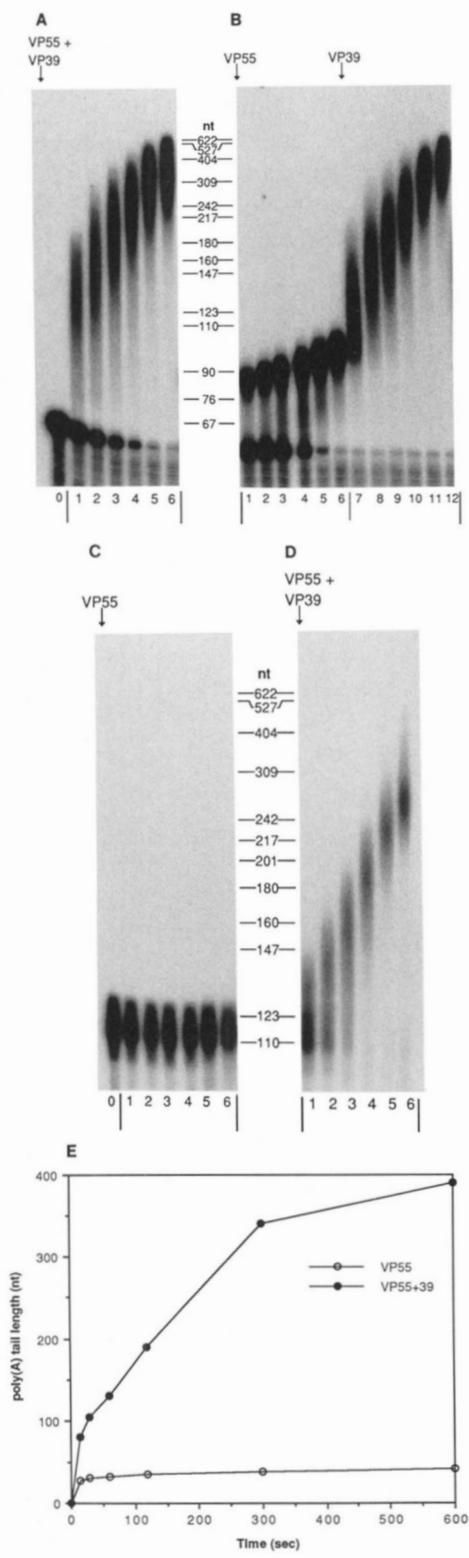
**FIG. 2. Effect of VP39/VP55 molar ratio on polyadenylation of the VGF 60-mer primer.** Lower set of numbers (12, 6, 3, 1.5, 0.75, 0.38, 0.19, and 0) denotes VP39/VP55 molar ratio, which was altered by dilution of VP39. 0 denotes VP55 alone. Tracks 0–3 contain products sampled at 0 (before the addition of enzyme), 15, 40, and 120 s, respectively. The primer/VP55 molar ratio was 2 and the concentration of NaCl was 60 mM. The positions and sizes of DNA markers are shown.

consistent with their molecular weights (Fig. 3, A and B). However, after mixing VP55 and VP39, VP55 sedimented more rapidly (Fig. 3C). As expected, the excess VP39 sedimented as monomer, but there was also a shoulder of more rapidly sedimenting VP39. It was estimated that greater than 90% of the VP55 was converted to the more rapidly sedimenting form. Mixtures of VP55 with excess VP39 will be referred to hereinafter as “VP55+39” to distinguish the resulting heterodimer plus monomeric VP39 from the preparations of heterodimer purified directly from virion extracts.

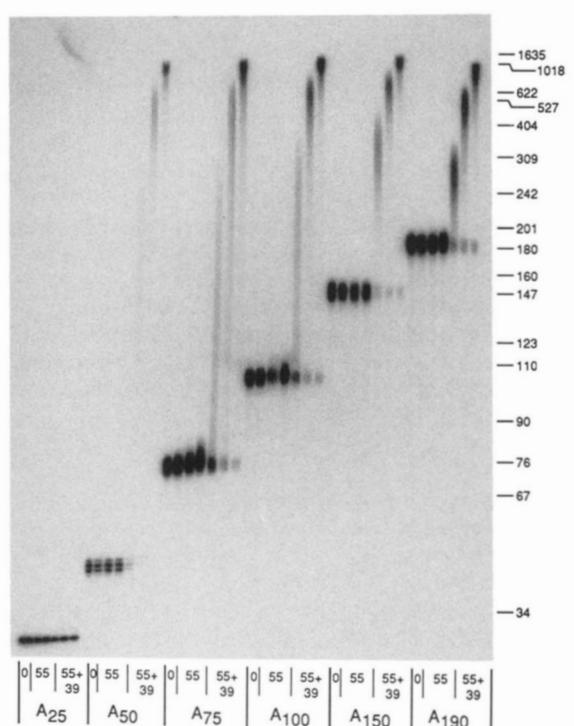
**VP39 Stimulates Elongation of RNAs Possessing a 3'-Oligo(A) Tail**—In time course assays, VP39 was found to stimulate the rate of elongation of VGF 60-mer RNA molecules containing more than 35 3'-adenylates. This was determined in three ways. First, the abrupt deceleration in chain elongation after the addition of 30–35 adenylates to the VGF 60-mer primer (Fig. 4B, tracks 1–6) did not occur if VP55 was premixed with VP39 (Fig. 4A). Second, the slow extension of the VGF 60-mer primer by monomeric VP55 after the initial rapid burst of 3'-oligoadenylation (Fig. 4B, tracks 1–6) was dramatically accelerated upon addition of VP39 to the same reaction (Fig. 4B, tracks 7–12). Third, when premixed VP55 and VP39 were added to an RNA primer comprising the 60-mer RNA possessing a preformed 3'-oligo(A) tail (the VGF 60-mer A<sub>42</sub> primer), rapid polyadenylation occurred (Fig. 4D),



**FIG. 3. VP55 and VP39 can associate in vitro.** The following samples were sedimented in parallel 15–30% glycerol gradients: VP39 (A), VP55 (B), a mixture of VP55 and VP39 (C) in which VP39 was present in 3-fold molar excess over VP55. T and B denote the top and bottom of the gradient, respectively. Arrowheads denote the positions of peaks of carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (68 kDa), and aldolase (150 kDa) after sedimentation in a parallel gradient. The positions and sizes of electrophoresis marker proteins are shown on the left and the positions of VP55 and VP39 on the right.



**FIG. 4. VP39 stimulates the rate of elongation of the VGF 60-mer primer possessing a 3'-oligo(A) tail of greater than 30 nt.** Tracks 0–6 contain products sampled at 0, 15, 30, 60, 120, 300 and 600 s, respectively. *Panel A*, polyadenylation assay in which VP55 and VP39 were mixed prior to the addition of assay buffer, VGF 60-mer primer, and ATP. The VP39/VP55 molar ratio was 12, the primer/VP55 molar ratio was 2, and the concentration of NaCl was 30 mM. *Panel B*, assay in which the VGF 60-mer primer was polyadenylated by VP55 for up to 10 min (tracks 1–6), then the remaining assay mixture was mixed with VP39 such that the VP39/VP55 molar ratio was 12, and products sampled after an additional 15, 30, 60, 120, 300 and 600 s (tracks 7–12). Other conditions were as for *panel A*. The rate of utilization of starting primer by VP55 (tracks 1–6) was



**FIG. 5. Poly(A) is sufficient for efficient VP39-stimulated initiation and rapid elongation.** Poly(A) primers of various lengths between 25 and 190 nt were incubated with either VP55 or VP55+39 in 0–2-min time course assays. 0 denotes products sampled before addition of enzyme. For each assay, successive tracks contain products sampled at 15, 40, and 120 s, respectively. A VP39/VP55 molar ratio of 8 was employed. The positions and lengths in nucleotides of DNA markers are shown.

in contrast to the absence of polyadenylation in the presence of monomeric VP55 (Fig. 4C).

From the data of Fig. 4, *A* and *B*, poly(A) tail length was plotted against time for VP55 and for VP55+39 (Fig. 4E). Between any two time points from 15 to 600 s, the average rate of extension was considerably enhanced in the presence of VP39. Between 120 and 300 s, the average extension rate was increased by a factor of 37.5.

**Poly(A) Is Sufficient for Efficient VP39-stimulated Initiation and Rapid Elongation—**Monomeric VP55 requires nonpoly(A) primer sequences to initiate a rapid burst of polyadenylation. With poly(A) primers, VP55 adds adenylates in only a slow, nonprocessive manner (8). To determine the effect of VP39, various lengths of poly(A) between 25 and 190 nt were incubated with either VP55 or VP55+39, in 0–2 min time course assays (Fig. 5). Whereas the poly(A) primers were either not polyadenylated by VP55 or were polyadenylated very slowly, all were extended rapidly in the presence of VP39. Extension rates were increased greater than 500-fold. Thus, VP39 radically alters the primer specificity of the polymerase. In the presence of excess VP39, a poly(A) sequence is suffi-

lower than that in later assays using the VGF 60-mer, or that by VP55+39 in *panel A* due to the formation of nonproductive complexes between VP55 and the RNA at 30 mM NaCl. *Panel C*, polyadenylation of the VGF 60-mer A<sub>42</sub> primer by VP55. The primer/VP55 molar ratio was 2 and the concentration of NaCl was 60 mM. *Panel D*, polyadenylation of the VGF 60-mer-A<sub>42</sub> primer by VP55+39 in which VP55 and 39 were mixed prior to contact with the assay buffer, ATP, and primer. The VP39/VP55 molar ratio was 12, other conditions were as for *panel C*. *Panel E*, quantitation of the data of *panels A* and *B*. Poly(A) tail length was plotted against time for VP55 and for VP55+39 mixed prior to the initiation of the assay.

cient for efficient initiation and rapid elongation. The change in primer specificity of VP55 presumably results from a direct association with VP39, which can occur quantitatively if VP39 is in molar excess (Fig. 3). It was noted that A<sub>25</sub> was utilized by VP55+39 much less efficiently than the longer poly(A)s, indicating a minimum poly(A) size requirement for efficient initiation by the heterodimer.

**VP39 Does Not Prevent Selective Polyadenylation of the VGF 60-mer Primer in the Presence of Excess Poly(A)**—Since VP39 confers on the polymerase the ability to efficiently utilize poly(A) primers, the effect of excess A<sub>50</sub> primer on polyadenylation of the labeled VGF 60-mer was determined. Although the length of the poly(A) tail was reduced in the presence of excess poly(A), the amount of VGF 60-mer polyadenylated was not greatly affected even in the presence of a 100-fold molar excess of A<sub>50</sub> (Fig. 6). Thus, the presence of VP39 did not compromise the selectivity of the polymerase for a non-polyadenylated mRNA 3'-end.

**VP39-stimulated Polyadenylation Is Semiprogressive**—VP39-stimulated polyadenylation is considerably more processive than that catalyzed by monomeric VP55. This can be deduced by inspection of the amounts of unextended starting primer present in assays of poly(A)-Sepharose column fractions (Fig. 1D). Whereas almost all of the primer was depleted by recycling of monomeric VP55, most of the primer remained unextended in the presence of excess VP39 (fractions 25–40). A similar effect is apparent in Fig. 2. To further analyze the degree of processivity of VP39-stimulated polyadenylation, various PAP/primer molar ratios were assayed. Dilutions of primer were assayed in the presence of fixed amounts of VP55 and VP39, in order not to affect the extent of association of the two subunits. A VP39/VP55 molar ratio of 12 was employed for primer dilution assays, at which virtually all of the VP55 should be associated with VP39 to give a mixture of

heterodimer and monomeric VP39.

Since poly(A) sequences were sufficient for initiation and rapid elongation by the heterodimer, processivity was initially examined using the primer A<sub>50</sub>. Four dilutions of A<sub>50</sub> were assayed in 0–10-min time course assays (Fig. 7A). In a completely processive reaction, the rates of extension of individual primer molecules would be expected to be independent of the primer/PAP molar ratio. In a completely nonprocessive reaction, all primer molecules would be expected to be extended at a uniform rate that is inversely proportional to the primer/PAP molar ratio. The data shown in Fig. 7A are consistent with a semiprogressive reaction in which recycling of the VP55–VP39 heterodimer occurred after processive runs of adenylate additions.

The relatively slow elongation of the poly(A) primer permitted processivity to be assessed using a primer challenge assay. In the experiment shown in Fig. 7B, <sup>32</sup>P-end-labeled A<sub>50</sub> primer was extended in the presence of an approximately equimolar amount of VP55 and a 12-fold molar excess of VP39. After sampling products at 40 s, a 0–10-fold molar excess of unlabeled A<sub>50</sub> was added, and products were sampled at additional times. The average extension rate for the majority of poly(A) molecules decreased after the addition of increasing amounts of challenge primer, although in each assay a fraction (~5%) of the poly(A) molecules was refractory to challenge. This confirmed that polyadenylation of the majority of poly(A) molecules is not completely processive in the presence of VP39.

The processivity of extension of the VGF 60-mer was also examined by assaying polyadenylation at different primer/PAP molar ratios. Four dilutions of the 60-mer were assayed in 0–10-min time course assays (Fig. 7C). As with the primer A<sub>50</sub>, polyadenylation appeared to be semiprogressive, since the average poly(A) size at a given time point decreased with increasing primer/PAP ratio.

The data of Fig. 7 are consistent with the heterodimer mediating a semiprogressive mode of polyadenylation in which bursts of synthesis are followed by dissociation from the poly(A) moiety. In the two assays shown in Fig. 7C in which the VGF 60-mer primer was initially in molar excess over VP55, the polyadenylation products were fairly discrete in size and products with short oligo(A) tails were not detected. This suggests that the heterodimer, like monomeric VP55, initiates on non-poly(A) sequences with a processive burst of oligoadenylation that is independent of the rate at which subsequent extension occurs. By contrast, in the two equivalent assays shown in Fig. 7A, there was a continuous range of product sizes, suggesting that no such burst occurs with a poly(A) primer or, presumably, during the semiprogressive elongation of a poly(A) tail.

**Effect of NaCl on Polyadenylation**—Monomeric VP55 polyadenylates poly(A) primers slowly and nonprocessively. The effect of NaCl on this reaction was examined in 0–5-min time course assays with the primer A<sub>50</sub> (Fig. 8A). Increases in NaCl concentration from 11 to 90 mM caused progressive decreases in the rate of polyadenylation. At NaCl concentrations greater than 90 mM, no polyadenylation was detected in the 5-min assays. The VP39-stimulated reaction was significantly less NaCl sensitive than that catalyzed by monomeric VP55 (Fig. 8B). VP39-stimulated polyadenylation was affected only slightly by raising the NaCl concentration from 30 to 60 mM, although higher concentrations led to a decreased polyadenylation rate.

The effect of NaCl on polyadenylation of the VGF 60-mer primer was also examined (Figs. 8, C and D). With monomeric VP55, polyadenylation was optimal at NaCl concentrations

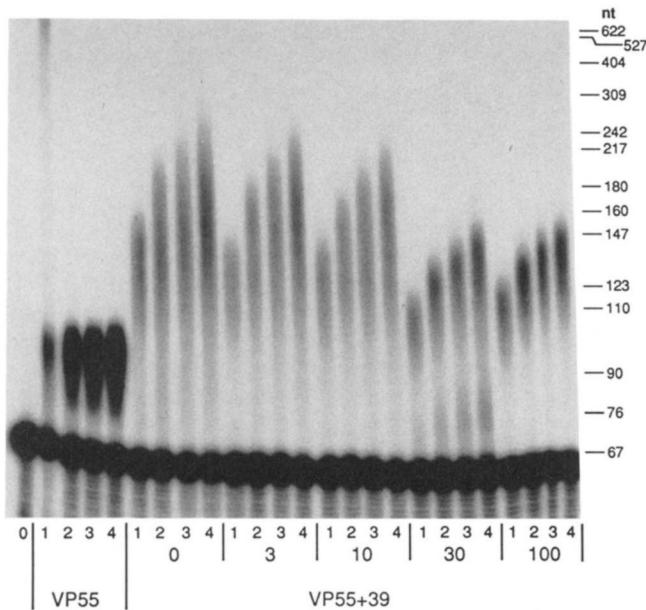
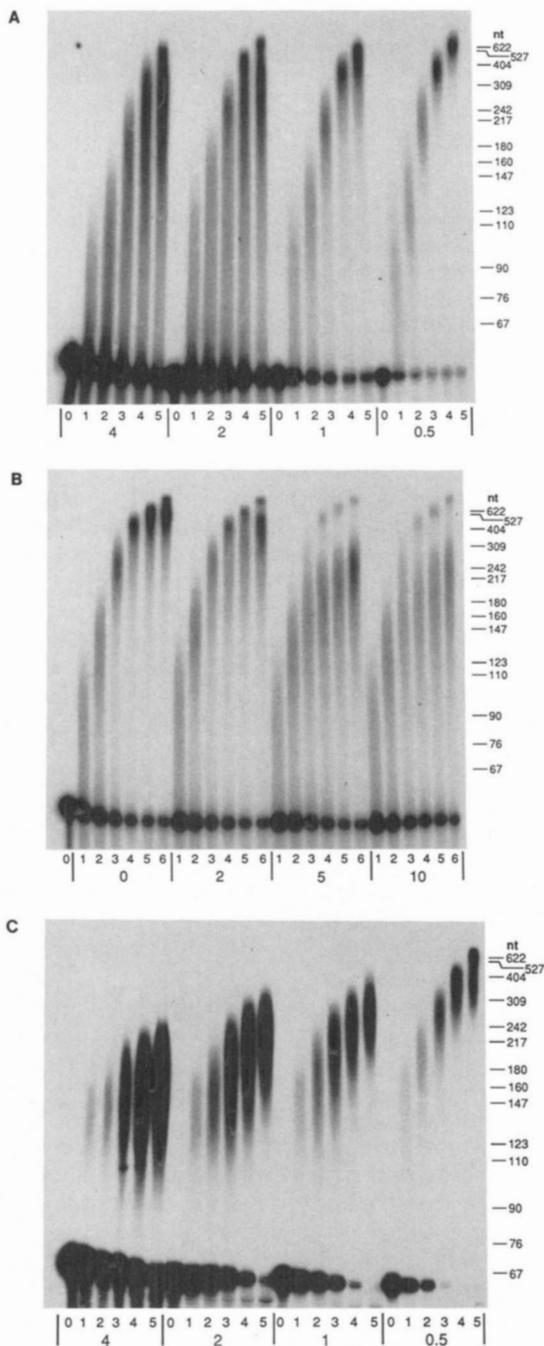
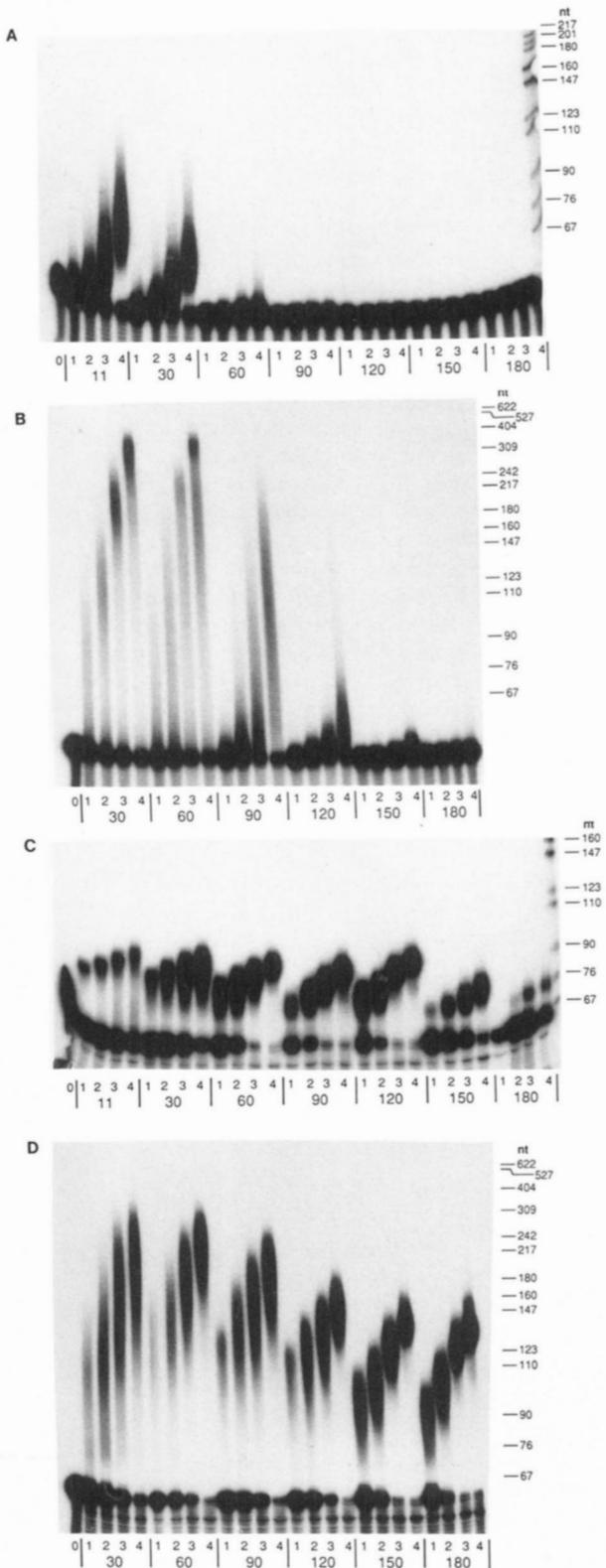


FIG. 6. Excess poly(A) does not prevent the selective initiation of polyadenylation of the VGF 60-mer primer in the presence of VP39. Tracks 0–4 contain products sampled at 0, 10, 15, 20, and 30 s, respectively. VP55, control assay of VP55 in the absence of VP39 or unlabeled A<sub>50</sub>. VP55+39, assays in the presence of VP39 and unlabeled A<sub>50</sub>. Lower set of numbers (0, 3, 10, 30, 100) denotes fold molar excess of unlabeled A<sub>50</sub> over labeled VGF 60-mer primer at time 0. The VP39/VP55 molar ratio was 12, the primer/VP55 molar ratio was 2 and the concentration of NaCl was 60 mM. The positions and sizes of DNA markers are shown.



**FIG. 7. VP39-stimulated polyadenylation is semiprogressive.** Tracks 0–6 contain products sampled at 0, 15, 40, 120, 300, 600, and 1200 s, respectively, and the VP39/VP55 molar ratio was 12. The positions and sizes of DNA markers are shown. *Panel A*, different amounts of the primer  $A_{50}$  were assayed. Lower set of numbers (4, 2, 1, 0.5) denotes primer/VP55 molar ratio. *Panel B*, assays in which polyadenylation of labeled  $A_{50}$  primer was challenged with various amounts of unlabeled  $A_{50}$ . Lower set of numbers (0, 2, 5, 10) denotes fold molar excess of unlabeled challenge primer over labeled assay primer. 0 denotes mock challenge. The challenge was made immediately after taking product sample 2 (at 40 s). The starting primer/VP55 molar ratio was 1. *Panel C*, different amounts of the VGF 60-mer primer were assayed. The concentration of NaCl was 60 mM. Other conditions were described in *panel A*.

between 60 and 120 mM (Fig. 8C). The increased rate of reinitiation at these NaCl concentrations may be due, in part, to the destabilization of nonproductive complexes between VP55 and the primer.<sup>2</sup> With increasing NaCl concentration, the transition from rapid processive burst to slow nonprocess-



**FIG. 8. Effects of NaCl on polyadenylation.** Lower sets of numbers (30, 60, 90, 120, 150, and 180) denote NaCl concentration (millimolar). Tracks 0–4 contain products sampled at 0, 15, 40, 120, and 300 s, respectively. The VP39/VP55 molar ratio was 12 and the primer/VP55 molar ratio was 2. Polyadenylation was catalyzed by: VP55 with the A<sub>50</sub> primer (A); VP55+39 with the A<sub>50</sub> primer (B); VP55 with the VGF 60-mer primer (C); VP55+39 with the VGF 60-mer primer (D).

sive polyadenylation occurred after the addition of fewer adenylates.

The rate of the VP39-stimulated reaction with the VGF 60-mer primer was optimal at ~60 mM NaCl (Fig. 8D). Comparison of Fig. 8, C and D, indicated that at elevated NaCl concentrations (150 and 180 mM), VP39 stimulated both the initiation and elongation phases of polyadenylation. Polyadenylation of the VGF 60-mer primer was much more resistant to elevated NaCl concentrations than that of A<sub>50</sub>. This was so in either the presence or absence of VP39.

#### DISCUSSION

The VP55 subunit of the vaccinia virus-encoded poly(A) polymerase can rapidly and processively add a maximum of 35 adenylates to the 3'-end of an RNA primer, whereas the VP55-VP39 heterodimer can catalyze the formation of poly(A) tails several hundred nucleotides in length. Here, we have analyzed the role of VP39 in poly(A) tail elongation. To accomplish this, VP39 was overexpressed in HeLa cells using recombinant vaccinia viruses and purified with five chromatographic steps. Although the final preparation of VP39 appeared essentially homogeneous after SDS-PAGE and silver staining, small amounts of an additional, minor band with slightly greater electrophoretic mobility reacted with anti-VP39 antibody. Due to the highly purified nature of the VP39 and its immunoreactivity, the minor band was presumed to be closely related to the major form of VP39. The minor form was also detected in crude preparations of overexpressed VP39 and, previously, in crude and purified preparations of VP39 from wild-type virus (Ref. 4, data not shown). Because of the variability in abundance of the minor form and its copurification with the major form during a variety of chromatographic steps, we suspect that it is an artifact of sample preparation for SDS-PAGE.

The model primer used in this study was the VGF 60-mer, which corresponds to the non-polyadenylated 3'-end of a vaccinia virus transcript. This primer is related to the VGF 30-mer primer used previously (8), but is extended at the 5'-end. Although greater polyadenylation rates have been observed in the presence of Mn<sup>2+</sup> (4, 6), Mg<sup>2+</sup> was the sole divalent cation used in this study, since it is physiologically relevant, and increases the nucleoside triphosphate-donor specificity of VP55 for ATP (8).

Although VP39 possessed no independent poly(A) polymerase activity, this subunit was shown to associate with VP55, the polyadenylation-catalytic subunit to form a heterodimer, and to stimulate polyadenylation. Whereas polyadenylation catalyzed by VP55 became slow and nonprocessive after the addition of 30–35 adenylates to the 3'-end of the VGF 60-mer primer, the heterodimer formed after mixing VP55 with VP39 efficiently utilized 3'-oligoadenylated RNAs as substrates. VP39 greatly enhanced the polyadenylation rate by converting the slow, nonprocessive polyadenylation that occurs after the rapid burst in the presence of monomeric VP55, to a rapid, semiprocessive reaction. With a starting primer of either A<sub>50</sub> or the VGF 60-mer, the heterodimer was shown to act semiprocessively. The heterodimer could efficiently initiate upon and rapidly elongate poly(A) primers, which were polyadenylated only slowly and nonprocessively by monomeric VP55. The rate of extension of these primers was enhanced >500-fold when VP39 was added to polyadenylation reactions.

Although polyadenylation of a poly(A) primer decreased in rate with increasing NaCl concentration, the effect was less marked in the presence of VP39. At salt concentrations of 90 mM or greater, VP39 was absolutely required for the polyadenylation of poly(A). The action of VP39 in increasing proc-

essivity and conferring NaCl resistance during polyadenylation of poly(A) suggests that this subunit increases the affinity of the polymerase for the elongating poly(A) tail. Consistent with this hypothesis, VP39 caused poly(A) to be retained on nitrocellulose filters (4), whereas monomeric VP55 did not.<sup>2</sup> Moreover, the combination of VP39 and VP55 enhanced the filter binding of poly(A). The mechanism by which VP39 stimulates elongation of the poly(A) tail is presumably related to its ability to bind both poly(A) and VP55 (8). VP39 may anchor VP55 to the growing poly(A) tail for rounds of processive synthesis comparable to the initial burst catalyzed by monomeric VP55, followed by dissociation and reinitiation. Alternatively, VP39 may permit the polymerase to translocate with respect to the growing tail. In the latter case, the semiprocessive nature of elongation could result from the weak association of the two PAP subunits. These mechanistic questions await further analysis.

Vaccinia virus PAP presumably exhibits specificity for nascent non-polyadenylated mRNA *in vivo*. The 5'-cap structure, the localization of the PAP, or the primer selectivity of VP55 (8) provide possible means by which specificity could be established. Since VP39 is a cap-dependent 2'-O-methyltransferase, it presumably interacts with the 5'-cap structure. However, polyadenylation is a cap-independent process under a variety of *in vitro* conditions, and in *in vitro* comparisons of 5'-phosphorylated and cap-labeled VGF 110-mer (4) RNA no qualitative differences in polyadenylation were detected.<sup>2</sup> For these reasons, a role for the cap in targeting mRNA for polyadenylation is considered unlikely. In the early phase of infection, specificity for nascent mRNA could be conferred by localization of the vaccinia PAP within the infecting virus particle and the rapid translocation of mature transcripts to the external milieu. Since such compartmentalization may not occur at later stages of infection, an association of PAP with the transcribing vaccinia RNA polymerase would provide an alternative means of localizing the former for the selective polyadenylation of nascent mRNA. However, under conditions in which a stable interaction between the vaccinia-capping enzyme and RNA polymerase has been demonstrated (14, 15), an interaction between vaccinia PAP and RNA polymerase could not be detected in either virion (Ref. 14)<sup>2</sup> or cytoplasmic<sup>2</sup> extracts. The possibility that specificity for non-polyadenylated mRNA *in vivo* results from the properties of VP55 is considered the most likely since, in the presence of excess poly(A), monomeric VP55 is indeed selective for a non-polyadenylated mRNA 3'-end (8). Although monomeric VP55 was not detected in virion extracts, the ready dissociability of the heterodimer might provide a mechanism for initiation by monomeric VP55. However, selectivity was shown to be maintained in the presence of excess VP39 *in vitro*, suggesting that VP55 may not be required in monomeric form to confer specificity for non-polyadenylated mRNA 3'-ends. Indeed, the stimulatory effect of VP39 on initiation with the VGF 60-mer primer at physiological NaCl concentrations suggests that initiation by the heterodimer can occur.

Although poly(A) tails of up to 600 nt were synthesized *in vitro*, these lengths may not be inconsistent with physiological sized poly(A) tails, particularly if generated within a non-cytoplasmic compartment such as the infecting virion. In the mouse oocyte, large changes in 3'-poly(A) size can occur rapidly after nuclear cytoplasmic translocation (16). Indeed, the RNA signal that specifies cytoplasmic changes in poly(A) size (17, 18) closely resembles the vaccinia early transcriptional termination signal (19, 20), an RNA element close to the 3'-ends of vaccinia early mRNAs. Although the generation of long poly(A) tails by vaccinia PAP *in vivo* may be compat-

ible with subsequent poly(A) metabolism and function, *in vivo* steady-state tail lengths could actually be shorter than the lengths generated *in vitro* for a number of reasons. Rates of polyadenylation are inversely related to salt concentration, being considerably reduced at 150 mM NaCl. In addition, since tail elongation is not highly processive, extension rates and terminal lengths pertaining *in vivo* may be affected by the relative and absolute amounts of substrate, product, VP55, and VP39. Furthermore, if translocation of early mRNAs out of the infecting virus particle is a relatively rapid process, and starts before 3'-polyadenylation is complete, then an absence of full processivity in polyadenylation may promote the export of transcripts with shorter poly(A) tails than those generated in *in vitro* extracts.

Biphasic polyadenylation of a "precleaved" mRNA 3'-end in a HeLa cell nuclear extract has been described. In the first phase, up to nine adenylates were added in a sequence-specific manner. The second phase required an RNA possessing a short 3'-oligo(A) tail and was entered upon addition of the tenth adenylate (21). The second phase has been accomplished *in vitro* using purified bovine PAP in combination with a bovine protein factor (22). The presence of this factor can increase the rate of polyadenylation by bovine poly(A) polymerase, of RNAs possessing short 3'-oligo(A) tails or of poly(A) alone. Like VP39, the bovine factor is a poly(A)-binding protein. Whether VP39 and the bovine factor are functionally homologous may be ascertained only after further studies of both and determination of the sequence of the latter.

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