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The Positive Transcription Elongation Factor Activity of the Vaccinia Virus J3 Protein Is Independent from Its (Nucleoside-2′-O-) Methyltransferase and Poly(A) Polymerase Stimulatory Functions

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Previous genetic and biochemical experiments have shown that the vaccinia virus J3 protein has three different roles in mRNA synthesis and modification. First, J3 is a (nucleoside-2′-O-)methyltransferase which methylates the 2′ position of the first transcribed nucleotide, thus converting a cap-0 to a cap-1 structure at the 5′ ends of mRNAs. Second, J3 is a processivity factor for the virus coded poly(A) polymerase. Third, J3 has recently been shown to have intermediate and late gene positive transcription elongation factor activity *in vivo*. Previous experiments have shown that the poly(A) polymerase stimulatory activity and the (nucleoside-2′-O-)methyltransferase activity are two independent functions of the protein that can be genetically separated through site-directed mutagenesis. In this article, the relationship between the J3-mediated transcription elongation activity and the two other functions of the protein was investigated by constructing several site-directed mutant viruses that contain specific defects in either methyltransferase or poly(A) polymerase processivity functions. The results demonstrate that the J3 positive transcription elongation factor activity is a third independent function of the protein that is genetically separable from its two other functions in mRNA modification. The results also show that neither the poly(A) polymerase stimulatory nor the methyltransferase activities of the J3 protein is essential for virus growth in cell culture.

Key Words: vaccinia; poxvirus; transcription; elongation; termination; J3; VP39; methyltransferase; E1; poly(A) polymerase.

INTRODUCTION

Vaccinia, the prototypic orthopoxvirus, has a 192-kb double-stranded DNA genome which encodes approximately 200 genes and replicates strictly in the cytoplasm of host cells. Due to the location of its replication cycle, vaccinia must encode its own multi-subunit RNA polymerase and transcription factors that are necessary for carrying out distinct early, intermediate, and late stages of gene expression (Moss, 2001). Thus, vaccinia has served as a good model for studying the fundamental mechanisms that regulate transcription.

Intermediate and late (together termed "postreplicative") vaccinia gene transcription is distinct from the transcription of vaccinia early genes specifically with respect to the process of termination. Early gene transcription termination occurs in response to a *cis*-acting termination signal that is recognized as the mRNA sequence U_5NU (Yuen and Moss, 1987; Shuman and Moss, 1988). Upon transcribing this signal, the polymerase terminates 30–50 nucleotides downstream in a manner that is dependent upon three virus coded factors: the heterodimeric capping enzyme (VTF) (Shuman *et al.,* 1987), a DNA-dependent ATPase (NPH-I) (Christen *et al.,* 1998; Deng and Shuman, 1998), and the early gene-specific RNA polymerase subunit H4 (Mohamed and Niles, 2000). As a result of this termination mechanism, early gene transcripts are characteristically homogeneous in length. In contrast, a polymerase that transcribes an intermediate or late gene reads through U_5NU early termination sequences and terminates in a seemingly random fashion to produce a population of transcripts that is very heterogeneous in length (Mahr and Roberts, 1984). To date, no *cis*-acting termination signal has been identified for postreplicative genes and if one exists, it must be both inefficient and degenerate in sequence. However, previous genetic and biochemical experiments have shown that the vaccinia gene products A18, G2, and J3 influence postreplicative gene transcription elongation and termination in opposing and complementary ways

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(Black and Condit, 1996; Xiang *et al.,* 1998, 2000; Latner *et al.,* 2000; Lackner and Condit, 2000).

It was recently shown using an *in vitro* transcription assay that the 56-kDa A18 protein is a transcript release factor required for dissociation of nascent RNA from a ternary transcription elongation complex (Lackner and Condit, 2000). In addition, it has been previously shown that A18 is a DNA-dependent ATPase and a 3′-5′ DNA helicase (Bayliss and Condit, 1995; Simpson and Condit, 1995). Consistent with these *in vitro* biochemical properties, viruses with mutations in A18 produce abnormally long transcripts *in vivo* that, when transcribed from opposing promoters, can hybridize to one another and form excessive amounts of double-stranded RNA. This process induces the cellular 2–5A pathway, leading ultimately to RNase L-catalyzed RNA degradation and abortion of the infection (Pacha and Condit, 1985; Bayliss and Condit, 1993; Xiang *et al.,* 1998; Lackner and Condit, 2000).

Mutations in the G2 and J3 genes have been identified by two independent genetic selections (Condit *et al.,* 1996; Latner *et al.,* 2000). First, mutations in both genes have been discovered as extragenic suppressors of a temperature-sensitive mutation in the A18 transcript release factor. Second, mutations in both genes have been identified from a selection for viruses that are dependent for growth on the transcription elongation enhancing drug isatin-β-thiosemicarbazone (IBT). Although the mechanism of action of IBT is unknown, treatment of an infection with IBT has the same effect as mutations in A18, that is, IBT causes the production of excessively long transcripts leading to dsRNA formation and abortion of the infection (Pacha and Condit, 1985; Bayliss and Condit, 1993). In direct contrast to A18 mutants, viruses containing IBT-dependent mutations in either the G2 or the J3 gene produce postreplicative gene transcripts that are shorter than normal and that are specifically truncated from the 3′ end. As a result of these defects in transcription, G2 and J3 mutants produce reduced quantities of large proteins at late times during infection (Black and Condit, 1996; Xiang *et al.,* 2000). The suppression of both G2 and J3 mutants by A18 and their dependence on IBT for growth make sense: both mutation of A18 and treatment with IBT enhance transcription elongation, thus compensating for the truncation of transcripts resulting from mutation of G2 or J3. In summary the *in vivo* genetic evidence suggests that G2 and J3 have similar roles as positive transcription elongation factors for intermediate and late genes.

Although the G2 protein has no previously described function and shares no significant sequence homology to any known proteins with the exception of other poxvirus homologues, the J3 protein has two previously described mRNA modification activities. First, J3 is a (nucleoside-2′-O-)methyltransferase that converts a cap-0 to a cap-1 structure at the 5′ end of mRNAs by using S-adenosylmethionine to methylate the 2′ position of the first transcribed nucleotide (Barbosa and Moss, 1978a,b; Schnierle *et al.,* 1992). Methylation at the 2′ position on the penultimate nucleotide of transcripts is a common modification of higher eukaryotic mRNA, although its purpose remains a mystery (Wei and Moss, 1975). Second, J3 is the processivity factor subunit of the heterodimeric viral poly(A) polymerase (Gershon *et al.,* 1991; Gershon and Moss, 1993). The large catalytic subunit of the poly(A) polymerase, E1, will by itself rapidly catalyze the addition of approximately 35 A residues on the 3′ end of mRNA at a rate of about 120 residues per minute *in vitro*. After the poly(A) tail grows to about 35 bases in length, E1 abruptly ceases rapid elongation of the tail, then very slowly continues to add A residues at a rate of 0.25–1 per minute (Gershon and Moss, 1992; Schnierle *et al.,* 1992). Upon addition of J3 to the *in vitro* reaction, E1 returns to a rapid processive mode of elongation until the total length of the poly(A) tail reaches about 200 nucleotides (Gershon and Moss, 1993). Thus, in addition to its activity as a positive transcription elongation factor for postreplicative genes, J3 is involved in modifying both the 5′ and the 3′ ends of mRNAs.

Published site-directed mutagenesis experiments have shown that the (nucleoside-2′-O-)methyltransferase and E1 stimulatory activities are two independent functions of J3 that are genetically separable (Gershon *et al.,* 1998). More specifically, these experiments have shown that certain mutations in the J3 protein can precisely abrogate either the (nucleoside-2′-O-)methyltransferase activity or the E1 stimulatory activity as measured *in vitro* with mutant J3 proteins that have been overexpressed and purified from bacteria (Schnierle *et al.,* 1994; Shi *et al.,* 1997). These experiments in combination with the more recent observation that J3 is a positive transcription elongation factor (Latner *et al.,* 2000; Xiang *et al.,* 2000) have invited the following question: Is the J3 positive transcription elongation factor activity somehow functionally linked to one or both of its two other RNA modifying activities, or is the role of J3 as a transcription factor a third independent function of the protein? An *in vitro* assay to measure the transcription stimulatory activity of J3 does not currently exist. Therefore, an answer to this question required the construction and *in vivo* characterization of several viruses which contain site-directed mutations that have been shown *in vitro* to specifically disrupt either E1 poly(A) polymerase stimulatory activity or (nucleotide-2′-O-)methyltransferase activity (Schnierle *et al.,* 1994; Shi *et al.,* 1996, 1997). The results described below demonstrate that J3 mediated transcription elongation activity is a third, independent, genetically separable activity of the protein that is otherwise not related to either of the two other RNA-modifying activities. In addition, several viruses were constructed that contain previously described charge-to-alanine scanning mutations located at or near the surface of the

TABLE 1

Summary of J3 Site-Directed Mutants

Virus	Mutation[a]	*In vitro*[b]		*In vivo*[b]			
		MTase	E1-S	MTase	E1-S	Elongation	IBT
wt	NA	+	+	+	+	+	S
J3x	G96D	−	+	+	+	−	D
J3-7	*fs* 49; 58aa	NA	NA	−	−	−	D
CF3^{c-}	H56R, I58S	+	−	+	−	+	S
K175R	K175R	−	+	−	+	+	S
AS-4	R79, D80-A	+	+	+	+	+	S
AS-5	R97, H98, H99-A	+	+	+	+	+/−[c]	R
AS-6	R107, D108-A	+	+	+	+	+	S
AS-8	R122, K125, K126-A	+	+	+	+	+/−[c]	R
AS-9	H129, K132-A	+	+	+	+/−	+	S
AS-15	R220, R223-A	+	+	+	+	+/−[c]	R

[a] *fs* indicates the *J3-7* mutant contains a single nucleotide deletion in codon 49 that produces a frameshift truncation of the protein 58 amino acids from the N-terminus. Each of the remaining missense mutations are indicated as the wt amino acid, location of the codon which is mutated, and the mutant residue.

[b] +, normal wt function; −, defective or mutant function. S, IBT sensitivity; R, IBT resistance; D, IBT dependence. MTase, (nucleoside-2′-O)methyltransferase activity; E1-S, E1 poly(A) polymerase stimulatory activity. Elongation indicates length of intermediate and late gene transcripts relative to wt.

[c] +/−, IBT-resistant mutants AS-5, AS-8, and AS-15 produce normal length transcripts in the absence of IBT-like wild-type, but they have an altered transcription phenotype in the presence of IBT that is different from the wild-type virus. See text for details.

protein in an attempt to determine which regions of J3 are important for mediating its effects on postreplicative gene transcription. Three of the J3 charge-to-alanine scanning mutants are resistant to IBT, preliminarily suggesting a surface area of the protein that may be important for mediating the effects of J3 on transcription.

RESULTS

Construction of site-directed J3 mutants

To test the hypothesis that the J3 positive transcription elongation factor activity is a third independent activity of the protein, eight J3 site-directed mutant viruses were constructed by transient dominant selection as described under Materials and Methods (Falkner and Moss, 1990; Hassett and Condit, 1994; Hassett *et al.,* 1997). The specific J3 mutations that were inserted into the viruses are listed in Table 1. Seven of the eight mutant alleles that were inserted into the virus have been previously described by *in vitro* experiments (Schnierle *et al.,* 1994; Shi *et al.,* 1997). The remaining previously undescribed allele is called K175R. The eight J3 mutant viruses fall into two groups. The first group consists of two viruses, K175R and CF3^{c-}, that contain point mutations which cause the purified recombinant J3 mutant proteins to be specifically defective *in vitro* for either methyltransferase or poly(A) polymerase stimulation activity, respectively. The K175R virus contains a missense mutation that converts a catalytic lysine at codon 175 in the methyltransferase active site of the J3 protein to an arginine. Purified recombinant protein containing the K175R mutation lacks methyltransferase activity but retains poly(A) stimulatory activity *in vitro* (data not shown). The CF3^{c-} virus contains two missense mutations that convert the histidine at codon 56 to an arginine (H56R) and the isoleucine at position 58 to a serine (I58S) (Shi *et al.,* 1997). These mutations are located on a surface region of the protein that is important for interacting with the E1 poly(A) polymerase, and the purified recombinant protein is thus specifically defective for poly(A) tail synthesis *in vitro* (Shi *et al.,* 1997). Of note, the CF3^{-} virus described here lacks two mutations that were originally described in the initial characterization of the purified recombinant protein. Specifically, the previously described recombinant CF3^{c-} protein contains two additional missense mutations that convert cysteine 178 and cysteine 272 to serine residues and were shown not to affect the assayable properties of the protein (Shi *et al.,* 1997). It was hypothesized that if J3 elongation factor activity is a third independent function of the protein, then viruses containing the K175R or CF3^{c-} mutations would produce normal length transcripts even if they maintain their methyltransferase and E1 stimulation defects *in vivo*. Thus, the K175R and CF3^{c-} viruses serve as two critical controls. The remaining viruses fall into a second group which contains charge-to-alanine scanning (AS) mutations (Schnierle *et al.,* 1994) that are located at or near the surface of the protein and were selected for use in the current study based on the J3 crystal structure observations (Hodel *et al.,* 1996). It has been shown previously that the purified recombinant J3 proteins containing each of these mutations are normal

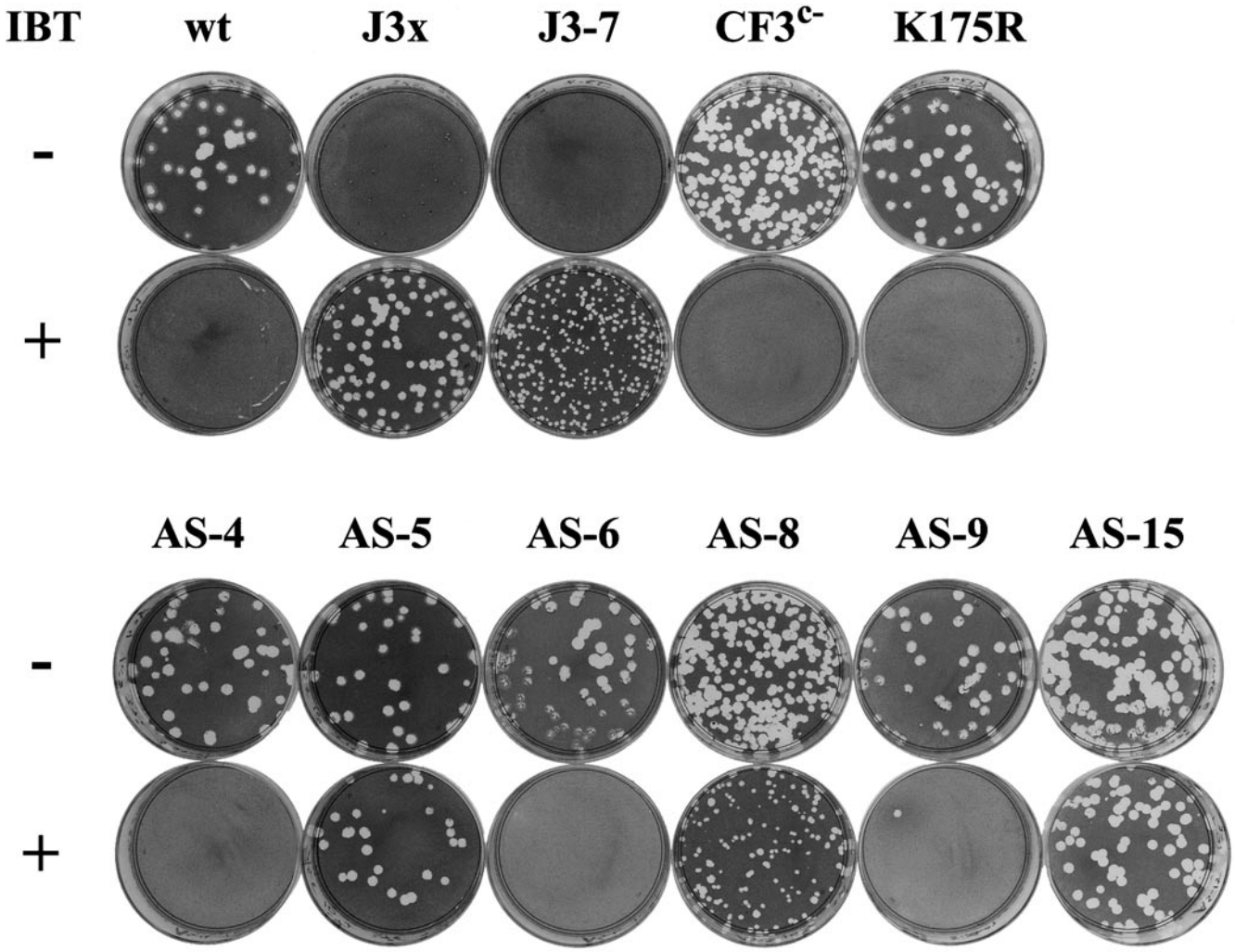


FIG. 1. Plaque phenotypes of mutant viruses. Confluent monolayers of BSC40 cells in 60-mm dishes were infected with an appropriate dilution of virus and incubated at 37°C in the presence or absence of IBT under an agar overlay for 4 days. Dishes were stained overnight with a second neutral red containing agar overlay. Agar was then removed and cells were stained with crystal violet. The mutant used for infection is indicated at the top of each column. The presence or absence of IBT during the infection is indicated at the left of each row.

for methyltransferase and E1 stimulation activities *in vitro* (Schnierle *et al.,* 1994). It was hypothesized that some of these mutations might disrupt a region of J3 that is important for binding to additional proteins in a transcription elongation complex and thus produce a specific defect in transcription elongation while maintaining normal methyltransferase and E1 stimulation activities. Therefore, construction of these viruses could potentially map on the surface of J3 a region that might be responsible for stimulating transcription. The charge-to-alanine scanning mutant viruses that were constructed are called AS-4, AS-5, AS-6, AS-8, AS-9, and AS-15.

Plaque phenotypes of site-directed mutants

Previous experience with G2 and J3 mutants has shown that the response of a virus to IBT is a good indicator of the mRNA synthesis phenotype of the virus (Hassett and Condit, 1994; Black and Condit, 1996; Condit *et al.,* 1996; Latner *et al.,* 2000; Xiang *et al.,* 2000). For example, wild-type virus is IBT sensitive (IBT^s) and makes normal length transcripts, while IBT-dependent (IBT^d) G2 and J3 mutants make short 3′ truncated transcripts in the absence of drug. Interestingly, mutation of G2 can also result in IBT resistance (IBT^r). Although none of the previously isolated G2 IBT^r mutants have been analyzed directly for their transcription elongation phenotype, it has been suggested that IBT resistance may represent a phenotype intermediate between sensitivity and dependence resulting from a partial defect in G2 elongation activity. Therefore, each of the site-directed mutants were examined in a plaque assay for their response to IBT. Figure 1 shows that as controls, the wt virus is IBT sensitive, while the previously characterized *J3x* and *J3-7* viruses are IBT dependent. The *J3x* virus is

Infection	Yield (pfu/cell) 0 hr	72 hr
wt -	0.21	77
wt +	0.23	0.04
J3x -	0.14	0.07
J3x +	0.15	0.83
J3-7 -	0.35	0.07
J3-7 +	0.55	0.31
AS-5 -	0.27	18
AS-5 +	0.24	13
AS-8 -	0.18	6.1
AS-8 +	0.09	3.5
AS-15 -	0.07	32
AS-15 +	0.05	23
$CF3^{c-}$ -	0.16	32
$CF3^{c-}$ +	0.10	0.02
K175R -	0.61	107
K175R +	0.64	0.15
AS-4 -	0.46	125
AS-4 +	0.47	0.41
AS-6 -	0.31	123
AS-6 +	0.37	0.06
AS-9 -	0.45	115
AS-9 +	0.37	0.03

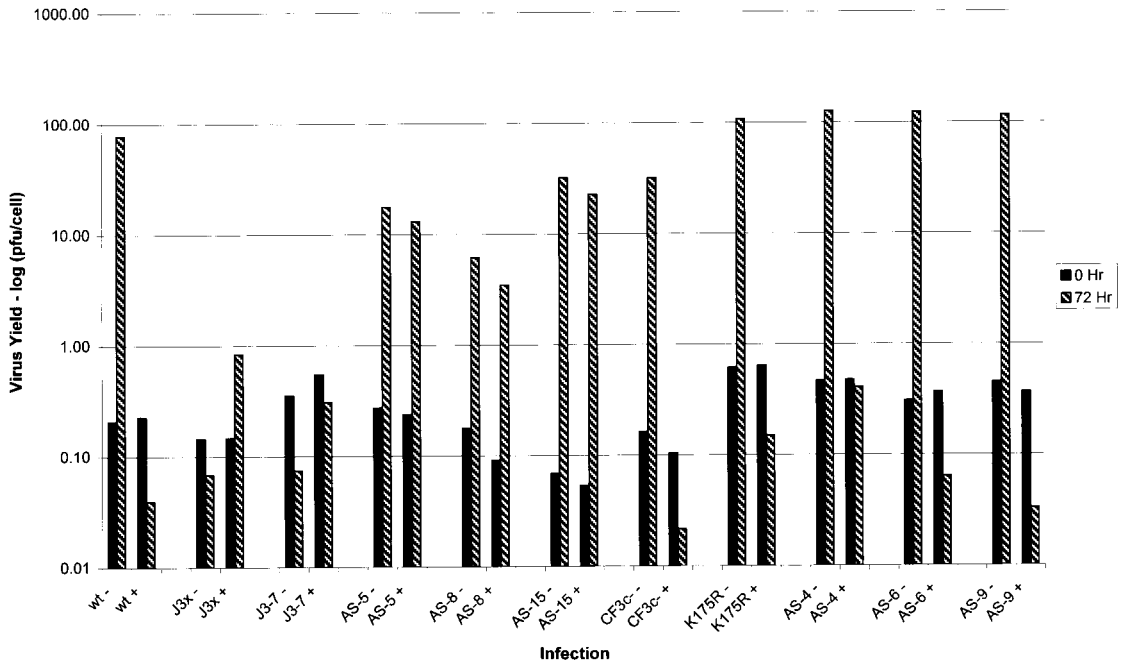


FIG. 2. One-step growth analysis of J3 site-directed mutants. Confluent monolayers of BSC40 cells in 60-mm dishes were infected with the indicated viruses at an m.o.i. = 3.3. Infections were incubated in the presence or absence of IBT at 37°C and were harvested at 0 and 72 h postinfection. Lysates were then titered under permissive conditions for each virus. Specifically, all lysates with the exception of *J3x* and *J3-7* were titered at 37°C in the absence of IBT. The *J3x* and *J3-7* lysates were titered in the presence of IBT at 37°C. The numerical data presented at the left is the same data presented in the graph. Note that virus yield in the graph is shown as PFU/cell on a logarithmic scale. Solid bars indicate lysates that were harvested at 0 h postinfection. Striped bars represent lysates that were harvested at 72 h postinfection.

somewhat leaky at 37°C as attested by the presence of very small, barely visible plaques in the absence of drug, consistent with previously described results. *J3-7*, as previously described (Latner *et al.,* 2000), has a tight IBT^{d} phenotype but forms very small plaques, consistent with the fact that it is difficult to grow to high titer. Importantly, the $CF3^{c-}$ and K175R viruses are both IBT-sensitive similar to wt, suggesting that they may have normal transcription phenotypes. The $CF3^{c-}$ virus consistently produces slightly smaller-than-wt plaques, suggesting that it may be somewhat defective for growth. The AS-4, AS-6, and AS-9 viruses are all IBT sensitive, suggesting that that they also have no transcription defects. Interestingly, the AS-5, AS-8, and AS-15 viruses are all IBT resistant.

One-step growth analysis

To support the plaque phenotype data, one-step growth analysis was performed on each mutant (Fig. 2). By 72 h postinfection, wt virus produced approximately 77 PFU/cell, significantly higher than the T_0 background, in the absence of IBT and was inhibited for growth in the presence of IBT. *J3x* produced only background levels of progeny in the absence of IBT and approximately 0.8 PFU/cell in the presence of IBT, 5.5-fold higher than background. This confirms that the *J3x* virus is IBT dependent and is difficult to grow to high titer. *J3-7* produced background levels of progeny in the absence of IBT and slightly more in the presence of drug, demonstrating that it is significantly crippled and very difficult to grow to a high titer. AS-5 produced between 13 and 17 PFU/cell both in the presence and in the absence of IBT, demonstrating that it is clearly resistant. AS-8 produced 6 PFU/cell in the absence of drug and 3 PFU/cell in the presence, showing that while it is also IBT resistant, it may be weakly inhibited by the drug. This one-step growth phenotype for AS-8 is consistent with the fact that, in contrast to other IBT^{r} AS mutants, AS-8 formed smaller plaques in the presence of IBT compared to the absence of IBT (Fig. 1). AS-15 produced between 22 and 32 PFU/cell in the presence and absence of IBT, showing that it is strongly IBT resistant. The $CF3^{c-}$ virus produced approximately 31 PFU/cell in the absence of IBT and background levels of progeny in the presence of drug. Thus, $CF3^{c-}$ is IBT sensitive similar to wt, but relative to wt may be somewhat hindered for growth. These data are consistent with the observation that $CF3^{c-}$ reproducibly produces slightly smaller plaques than wt (Fig. 1). More extensive one-step growth experiments revealed that $CF3^{c-}$ grows more slowly than wt virus but eventually reaches a wt yield (data not shown). The K175R, AS-4, AS-6, and AS-9 viruses all produced between 106 and 125 PFU/cell in the absence of IBT and only 0.02–0.41 PFU/cell in the presence of IBT. Therefore, they are all IBT sensitive and grow to wt titer. In summary, with the

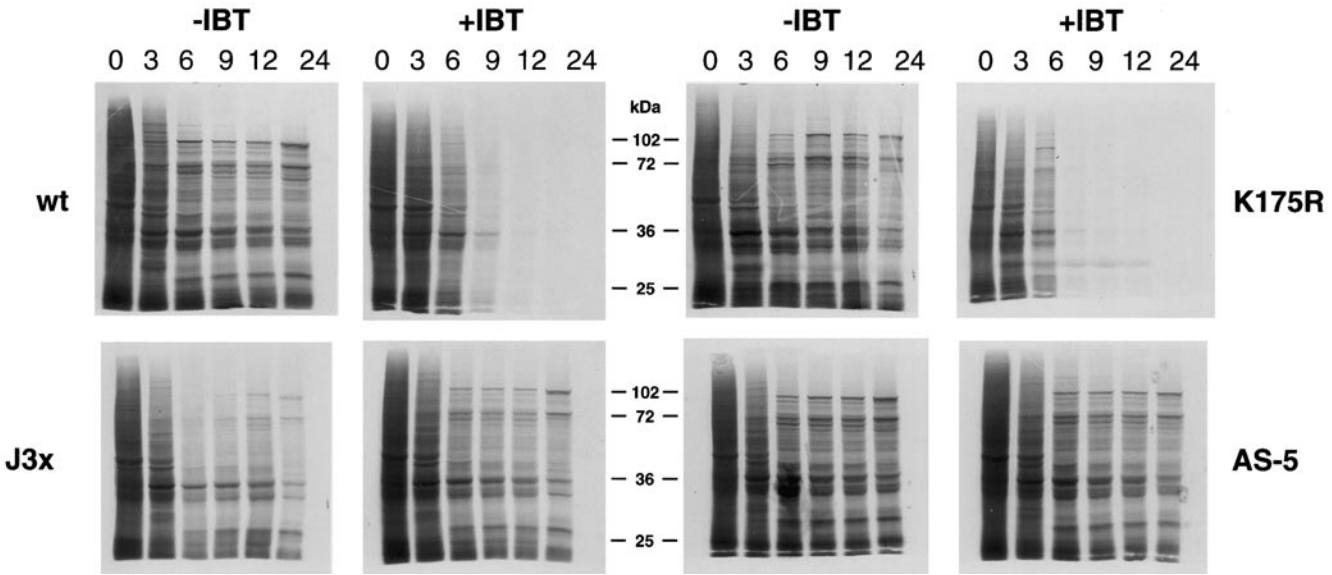


FIG. 3. Protein synthesis in wt and mutant-infected cells. Confluent monolayers of BSC40 cells in 35-mm dishes were infected at m.o.i. = 10 with all viruses except for *J3-7*, which was infected at an m.o.i. = 6.6 due stock titer constraints. Infected cells were incubated at 37°C in the presence or absence of IBT for 0, 3, 6, 9, 12, or 24 h postinfection at which time they were pulse labeled for 15 min with [^{35}S]methionine. Infected cells were then lysed in SDS–PAGE sample buffer and solubilized proteins were analyzed by SDS–PAGE. The gels were stained with Coomassie blue, dried, and autoradiographed. The migration of molecular size standards is shown in kilodaltons. The time postinfection (in hours) at which each sample was harvested and whether IBT was present is indicated at the top of the gels.

exception of J3-7, these results support the plaque phenotypes shown in Fig. 1 and show that the CF3^{c-} and K175R control viruses and three charge-to-alanine scanning mutants are IBT sensitive. In addition, the one-step growth data confirm that the AS-5, AS-8, and AS-15 viruses are all IBT resistant.

Viral protein synthesis

As an initial test of the prediction that the J3 site-directed mutant viruses would have a transcription phenotype that is consistent with their responses to IBT, their patterns of viral gene expression were examined by metabolically labeling proteins in infected cells with [^{35}S]methionine at various times postinfection. The total infected cell protein was then analyzed by SDS–PAGE and autoradiography (Fig. 3). The results show that the protein synthesis profile of each virus can be placed into one of three different phenotypic categories that directly correlate with the responses to IBT. Shown in Fig. 3 are autoradiograms that are representative examples of each phenotype. The first phenotypic category is composed of the IBT-sensitive viruses and is illustrated by the wt and K175R viruses. In the absence of IBT, the wt virus shuts off host protein synthesis between 3 and 6 h postinfection, early viral protein synthesis begins between 0 and 3 h and subsequently decays, and synthesis of intermediate and late virus proteins begins between 3 and 6 h and is maintained throughout the remainder of the infection. In the presence of IBT, wt early protein synthesis and shutoff is normal, late protein synthesis begins on schedule, but all protein synthesis decays by 9 h postinfection. This observation is consistent with previously published results (Cooper *et al.,* 1979; Pacha and Condit, 1985; Black and Condit, 1996) and is largely attributed to induction of the 2-5A RNA degradation pathway in IBT-treated wt infections (Cohrs *et al.,* 1989; Pacha *et al.,* 1990; Meis and Condit, 1991). All of the IBT-sensitive viruses, which include CF3^{c-}, K175R, AS-4, AS-6, and AS-9, show a protein synthesis profile that is indistinguishable from wt virus.

The second phenotypic category is composed of the IBT-dependent viruses and is represented by *J3x*. When the *J3x* mutant is grown in the absence of IBT, it displays normal shutoff of host protein synthesis by 6 h postinfection and has normal early protein synthesis that decays between 3 and 6 h postinfection (Xiang *et al.,* 2000). Beginning at 6 h and maintained throughout infection, *J3x* produces normal amounts of small intermediate and late proteins, but produces reduced quantities of large intermediate and late proteins. As previously described, this specific defect in protein synthesis is due to the fact that the *J3x* postreplicative transcripts are 3′ truncated and are too short to encode full-length large proteins (Xiang *et al.,* 2000). In the presence of IBT, *J3x* shows normal shutoff of host protein synthesis, early protein synthesis begins and decays normally, and equivalent amounts of both large and small proteins are produced at late times during infection, showing that the defect in *J3x* transcription is corrected by the addition of drug. Thus, the profile of *J3x* protein synthesis in the presence of IBT is com-

parable to the wt profile in the absence of IBT in terms of the kinetics of synthesis and the size and quantity of proteins produced.

The third phenotypic category is composed of the IBT-resistant viruses AS-5, AS-8, and AS-15 and is represented in Fig. 3 by AS-5. Both in the presence and in the absence of IBT, the pattern of AS-5 protein synthesis appears identical to the pattern of protein synthesis demonstrated by the wt virus growing in the absence of drug. Thus regardless of IBT treatment, AS-5 has normal shutoff of host synthesis, normal early protein synthesis and shut-off, and normal intermediate and late protein synthesis that persists throughout infection.

Viral mRNA synthesis

A crucial step in determining if the J3 transcription elongation factor activity is linked to the other two functions of the protein was to examine the mRNAs synthesized by each site-directed mutant. Thus mRNAs produced by each virus were examined by Northern blot analysis. Briefly, total RNA was harvested from infected cells at various times postinfection, electrophoresed on formaldehyde agarose gels, transferred to nylon membranes, and hybridized with standard early (gene C11), intermediate (gene G8), and late (gene F17) gene riboprobes. Figure 4A shows RNA harvested at 3 h postinfection and probed with an early gene riboprobe. As previously described (Xiang *et al.,* 2000), the early wt transcripts are characteristically homogeneous in length due to sequence-specific transcription termination. Importantly, all of the site-directed mutant viruses produce early transcripts that are similar in length to those produced by wt, indicting they have no dramatic defects early in mRNA synthesis. When the infections are performed in the presence of IBT (Fig. 4B), the early transcripts still appear normal in all cases, indicating that IBT has no gross effect on early transcription. Close inspection of the data in Figs. 4A and 4B suggests that the early transcripts produced in infections by J3-7 and CF3^{c-} may be slightly shorter than transcripts produced by the other viruses. This small decrease in size would be consistent with a decreased poly(A) tail length (see below) and indicates that in both of these mutants the poly(A) stimulation activity is defective in the context of the virus core where early transcription occurs.

When RNA harvested at 9 h postinfection from cells infected in the absence of IBT is probed with the intermediate gene riboprobe (Fig. 4C), a characteristic heterogeneous population of transcripts is detected in all cases. Consistent with previous results, the wt virus produces a normal distribution of intermediate transcripts and the IBT-dependent mutants, *J3x* and *J3-7*, produce a population of transcripts that is on average shorter than those produced by the wt virus. Importantly, the CF3^{c-} and K175R viruses both produce intermediate transcripts that are of wt length, indicating that they do not have a defect in intermediate gene transcription. The remaining IBT-sensitive viruses, AS-4, AS-6, and AS-9, also produce intermediate transcripts that are of a normal length. Interestingly, the intermediate transcripts produced by the IBT-resistant mutants AS-5, AS-8, and AS-15 are similar to the transcripts produced by wt and the other IBT-sensitive viruses.

When RNA harvested at 9 h postinfection from cells infected in the presence of IBT is probed with the intermediate gene riboprobe, some interesting results are obtained (Fig. 4D). In the presence of IBT, wt virus produces smaller-than-normal intermediate RNAs, consistent with RNA degradation, as described previously (Pacha and Condit, 1985; Bayliss and Condit, 1993). The same smaller-than-normal distribution is observed from each IBT-sensitive virus sample, including CF3^{c-}, K175R, AS-4, AS-6, and AS-9, demonstrating that they, similar to wt, experience RNA degradation in the presence of IBT. For all of these IBT^{s} viruses, IBT treatment also caused rRNA degradation, revealed by ethidium bromide staining of gels prior to transfer (data not shown). In contrast, in the presence of IBT the IBT-dependent viruses *J3x* and *J3-7* both produce transcripts that are longer than RNAs produced in the absence of drug, resembling the transcripts produced from the wt virus in the absence of IBT, between 1.4 and 7 kb in length. This suggests that IBT is compensating for the elongation defects that these IBT^{d} viruses have in transcription. Interestingly, in the presence of IBT, the IBT-resistant viruses AS-5, AS-8, and AS-15 all appear to produce intermediate transcripts that are of similar length to those of the IBT-dependent viruses *J3x* and *J3-7*.

Figure 4E shows RNA harvested at 12 h postinfection and probed with a late gene riboprobe. Once again, wt virus produces a normal heterogeneous distribution of transcripts with a strong homogeneous band of 1.4 kb in length that is consistent with previous observations. In the absence of drug, the *J3x* and *J3-7* IBT-dependent viruses produce transcripts that are, on average, shorter than those produced by the wt virus. The remaining IBT-sensitive and IBT-resistant viruses produce transcripts in the absence of drug that are similar to the wt transcripts. Figure 4F shows RNA harvested at 12 h postinfection from cells infected in the presence of IBT and probed with a late gene probe. By 12 h postinfection, it appears that wt and the other IBT-sensitive viruses have reduced production of late transcripts relative to the amount of transcripts produced in the absence of IBT. This observation is probably a result of the degradation of intermediate transcripts, which encode late transcription factors. The *J3x* and *J3-7* IBT^{d} viruses produce late transcripts that, similar to intermediate transcripts produced in the presence of drug, are slightly longer than the transcripts produced in the absence of drug. This once again suggests that IBT is compensating for the

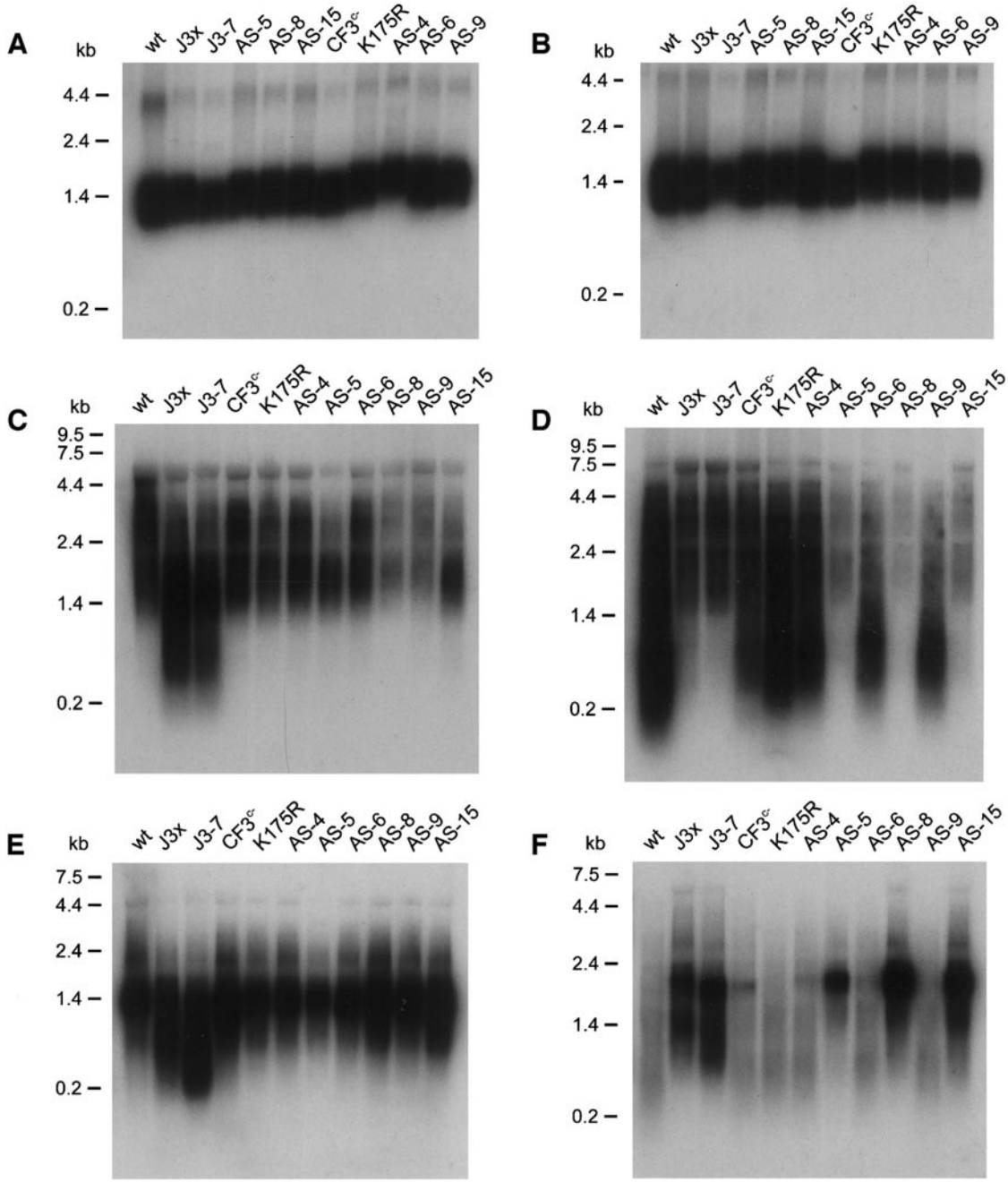


FIG. 4. Northern analysis of RNA from wt and mutant infected cells. Confluent monolayers of BSC40 cells in 100-mm dishes were infected at an m.o.i. = 10 with each virus except for *J3-7*, which was infected at an m.o.i. = 6 due to stock titer constraints. Infected cells were incubated at 37°C in the presence or absence of IBT for 3, 9, or 12 h postinfection; then total cellular RNA was purified. RNAs (1.5 μg each) were electrophoresed on formaldehyde agarose gels and were transferred to nylon. The 3, 9, and 12 h RNAs, respectively, were probed with standard early (gene C11), intermediate (gene G8), or late (gene F17) radiolabeled riboprobes. The autoradiograms are shown as follows: A: 3 h RNA, early C11 probe, -IBT; B: 3 h RNA, early C11 riboprobe, +IBT; C: 9 h RNA, intermediate G8 probe, -IBT; D: 9 h RNA, intermediate G8 probe, +IBT; E: 12 h RNA, late F17 probe, -IBT; F: 12 h RNA, late F17 probe, +IBT. The migration of molecular weight markers are shown at the left of each panel in kilobase pairs.

transcription defects in these viruses, allowing them to synthesize normal length transcripts. The IBT-resistant viruses AS-5, AS-8, and AS-15, unlike the sensitive viruses, produce late transcripts in the presence of drug. In addition, these transcripts produced in the presence of drug are essentially of the same length as those produced in the absence of drug.

In vivo poly(A) polymerase stimulatory activity

After determining that the CF3^{c-} and K175R viruses are wt for RNA synthesis, the next step in determining if the J3 transcription elongation activity is linked to the other two RNA modifying functions was to examine each of the viruses for *in vivo* poly(A) polymerase stimulatory activity.

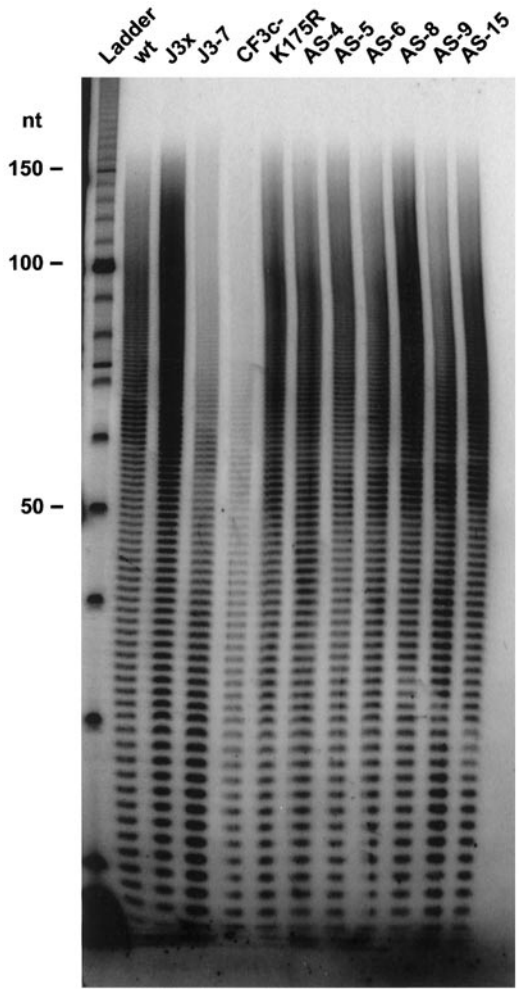


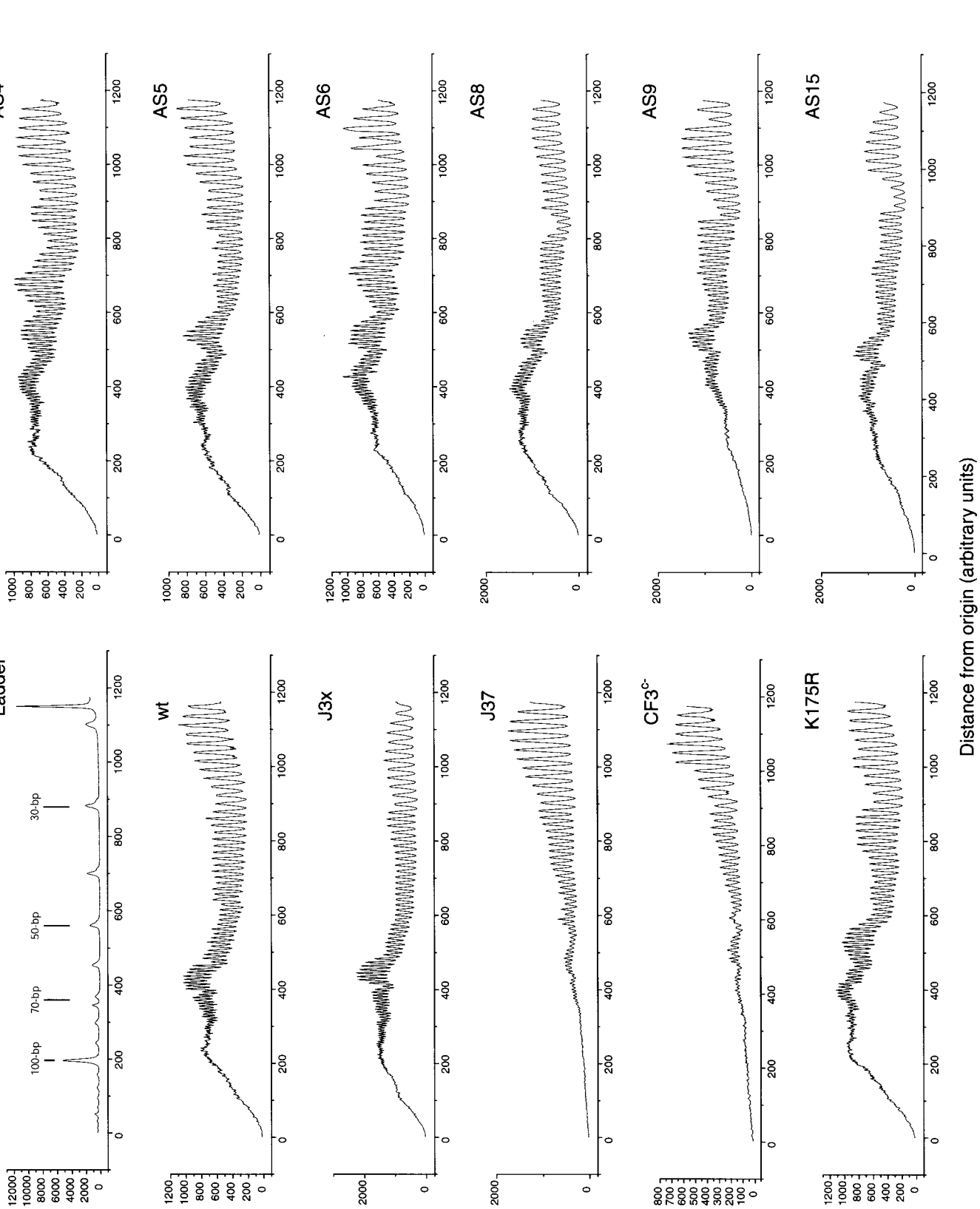
FIG. 5. Analysis of poly(A) tail lengths in wt and mutant viruses. Confluent monolayers of BSC40 cells in 60-mm dishes were infected at an m.o.i. = 10 with each virus except for *J3-7*, which was infected at an m.o.i. = 6.6 due to titer constraints. Infected cells were incubated at 37°C for 12 h; then total cellular RNA was harvested. One microgram of total RNA was then 3′ end labeled with 5 μCi of [^{32}P]pCp (3000 Ci/mmol), digested with RNases A and T_1, and the labeled; RNase-resistant poly(A) tails were electrophoresed on a 10% polyacrylamide 8 M urea sequencing gel. The gel was dried and subjected to autoradiography. The migration of size markers, in nucleotides, is shown at left.

Briefly, cells were infected with each virus in the absence of IBT and total RNA was harvested at 12 h postinfection. The RNAs were then labeled at the 3′ end with [^{32}P]pCp (cytidine 3′, 5′-bisphosphate) by T_4 RNA ligase and were digested with RNases A and T_1. The RNase-resistant labeled poly(A) tails were then analyzed on a denaturing polyacrylamide gel and the autoradiogram is shown in Fig. 5. In addition, the autoradiogram shown in Fig. 5 was quantified on a Phosphorimager and a graphical distribution of the poly(A) tails synthesized by each virus is shown in Fig. 6. Previous experiments have shown that the poly(A) tails present at 12 h postinfection are essentially all viral in origin and do not represent host mRNA poly(A) tails (Xiang *et al.,* 2000). Consistent with previous results (Xiang *et al.,* 2000), the wt virus synthesizes poly(A) tails up to 150 nucleotides in length, with a peak in distribution between 60 and 100 nucleotides in length. The *J3x*, K175R, AS-4, AS-5, AS-6, AS-8, and AS-15 viruses all synthesize poly(A) tails that are similar to those produced by the wt virus, indicating that they have normal poly(A) polymerase stimulatory activity. In contrast, the *J3-7* IBT^{d} null mutant is defective for poly(A) tail synthesis, as previously described (Xiang *et al.,* 2000). Most of the poly(A) tails synthesized by *J3-7* are less than 50 nucleotides in length, which is consistent with this virus lacking the poly(A) polymerase stimulatory function. The distribution of poly(A) tails in a CF3^{c-} infection is reproducibly indistinguishable from a *J3-7* infection and clearly distinguishable from wt virus and other mutant virus infections. This result shows that the CF3^{c-} virus, similar to *J3-7*, is also defective for poly(A) tail synthesis *in vivo*, consistent with previously described *in vitro* results (Shi *et al.,* 1997). There also appears to be a reduction in the overall abundance of poly(A) tails synthesized by both *J3-7* and CF3^{c-} relative to the other viruses; however, the reasons for the difference in abundance is not clear. The apparent reduction in the amount of poly(A) tails produced by both viruses could be attributable either to a generalized inefficiency in poly(A) tail synthesis by each virus *in vivo* or to technical difficulties in recovering small poly(A) tails in the labeling procedure. Importantly however, CF3^{c-} clearly is defective for poly(A) tail synthesis both *in vitro* and *in vivo* but otherwise retains a normal transcription phenotype. Interestingly, the AS-9 virus consistently appeared to have a very slight defect in E1 stimulatory function also, with most of the tails being less than 100 bases in length as detected in several independent experiments. However, the slight defect in AS-9 is difficult to visualize from the experiment shown in Fig. 5.

In vivo 2′-O-methyltransferase activity

The final step in determining if the J3 transcription elongation activity is linked to the other two J3 functions was to examine the *in vivo* 2′-O-methyltransferase activity of each virus. Briefly, BSC40 cells were infected with each virus, incubated at 37°C for 10 h, and then labeled

FIG. 6. Distribution of poly(A) tail lengths in wt, *J3-7*, and CF3^{c-} samples. The distribution of poly(A) tails from the wt, *J3-7*, and CF3^{c-} samples analyzed on the gel shown in Fig. 5 was further examined by Phosphorimager analysis. The relative amounts of poly(A) tails for each sample are plotted as a function of the distance they traveled from the top of the gel. The *x*-axis indicates distance from the top of the gel in arbitrary units, and all graphs, including the ladder (top left), are plotted on the same scale. The *y*-axis indicates relative amounts of radioactivity in arbitrary units.


Ladder
100-bp
70-bp
50-bp
30-bp
wt
J3x
J37
CF3c-
K175R
AS4
AS5
AS6
AS8
AS9
AS15
Intensity (arbitrary units)
Distance from origin (arbitrary units)

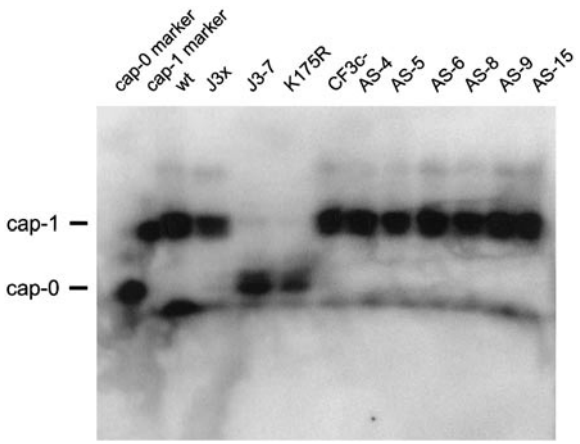


FIG. 7. *In vivo* 2′-O-methyltransferase assay. Confluent monolayers of BSC40 cells in 100-mm dishes were infected at an m.o.i. = 10 with each virus except for *J3-7*, which was infected at an m.o.i. = 6 due to titer constraints. Infections were incubated at 37°C for 10 h; then the infected cells were washed with 6 ml of phosphate-free, serum-free 1× DME. [^{32}P]orthophosphoric acid (3.33 mCi) diluted in 4 ml of phosphate-free, serum-free 1× DME was applied to the cells which were then incubated at 37°C for two additional hours. Poly(A)$^+$-RNA was harvested from the infected, labeled cells at 12 h postinfection. The RNA was then digested with RNases A, T_1, T_2, and calf intestinal alkaline phosphatase. The digestion products were then electrophoresed on a 20% polyacrylamide 8 M urea sequencing gel. The gel was then dried and subjected to Phosphorimager analysis.

with [^{32}P]orthophosphoric acid for 2 h. Poly(A)$^+$-RNA was then purified and digested with RNases A, T_1, T_2, and calf intestinal alkaline phosphatase. The dinucleotide cap structures on the 5′ ends of mRNAs are resistant to ribonucleolytic digestion due to the 5′-5′ nature of the linkage between the 7-methyl G residue and the first transcribed nucleotide. If the first transcribed nucleotide is methylated at the 2′ position, the phosphodiester bond between the first and second transcribed nucleotides is also protected from ribonucleolytic cleavage. After digestion, an mRNA is thus degraded to nucleosides, phosphates, and either a dinucleotide or a trinucleotide that represents the cap-0 or cap-1 structure, respectively. The digestion products were electrophoresed on a 20% denaturing polyacrylamide gel which was dried and subjected to Phosphorimager analysis (Fig. 7). The results show that wt viral transcripts have a cap-1 structure as predicted. In contrast, both the *J3-7* null mutant and the K175R virus, which contains a point mutation in the methyltransferase catalytic site, produce transcripts that have cap-0 structures, demonstrating that both viruses lack the J3 2′-O-methyltransferase activity *in vivo*. Interestingly, the *J3x* mutant transcripts have a cap-1 structure *in vivo*. This result was surprising because the purified recombinant *J3x* protein has been shown to be defective for 2′-O-methyltransferase activity in an *in vitro* assay (Xiang *et al.,* 2000). The remaining viruses, CF3^{c-}, AS-4, AS-5, AS-6, AS-8, AS-9, and AS-15 all produce cap-1 *in vivo*.

DISCUSSION

In light of the fact that the vaccinia virus J3 protein has two previously characterized biochemically independent roles in modifying both the 5′ and the 3′ ends of mRNA (Gershon, 1998), the recent realization that J3 also has a positive transcription elongation factor activity raised the following question: Is the J3 transcription factor activity related to the two other mRNA modification activities or is it a third independent function of the protein? To directly address this question, several site-directed J3 mutant viruses were constructed in an attempt to create viruses that are specifically defective for each of the three J3 functions. Rather than simply assaying the previously described recombinant-purified mutant proteins, actual viruses containing the site-directed mutations were constructed because an *in vitro* assay for the J3 transcription elongation factor activity does not currently exist. Eight site-directed J3 mutant viruses were constructed by transient dominant selection. Two of the viruses, called K175R and CF3^{c-}, contained mutations that were known to disrupt either the methyltransferase or the poly(A) polymerase stimulatory functions, respectively (as assayed *in vitro*) (Shi *et al.,* 1997). The six remaining viruses contained charge-to-alanine scanning (AS) mutations in which the target side chains were known from the crystal structure to be located at or near the solvent-accessible surface of the J3 protein (Hodel *et al.,* 1996). These mutants were constructed in attempt to create viruses that are specifically defective for transcription elongation and thus map onto the surface of J3 an elongation domain. The resulting mutant viruses were biochemically assayed *in vivo* for the three J3 activities. The location of each J3 mutation and the results of the biochemical characterization are summarized in Table 1.

The results provide several lines of evidence showing that the J3 transcription elongation factor activity is a third independent function of the protein that is not related to the 2′-O-methyltransferase or E1 poly(A) polymerase stimulatory activities. First, based on Northern analysis and supported by the protein synthesis profiles, IBT response, and plaque phenotypes, it appears that the CF3^{c-} and K175R viruses have a normal transcription phenotype throughout infection that is indistinguishable from the wt virus. It was also shown that the CF3^{c-} virus is specifically defective for *in vivo* poly(A) tail synthesis and the K175R virus is specifically defective for *in vivo* 2′-O-methyltransferase activity. Thus, the sole defect in CF3^{c-} is for E1 poly(A) polymerase stimulation and the sole defect in K175R is for 2′-O-methyltransferase activity. Second, it was shown that the only defect in the *J3x* point mutant is for transcription elongation activity. Although previous evidence has demonstrated that the recombinant-purified *J3x* protein does not have 2′-O-methyltransferase activity *in vitro*, the 2′-O-methyltrans-

ferase activity is clearly present *in vivo*. (Differences between *in vivo* and *in vitro* phenotype are not uncommon and are probably attributable to the vast differences in assay environment. For example, a temperature-sensitive mutant in the vaccinia mRNA capping enzyme is apparently normal for mRNA capping *in vivo* and defective *in vitro* (Hassett *et al.,* 1997).) Therefore, three different viruses, CF3^{c-}, K175R, and *J3x*, each contain a different defect in one of the three known J3 functions and together demonstrate that the positive transcription elongation activity of J3 is a third independent function of the protein that can be genetically separated from the other two activities.

The results presented here also demonstrate that long poly(A) tails are not essential for vaccinia virus growth in cell culture. Experiments in yeast and metazoan cells demonstrate that the process of polyadenylation is coupled to several other processes including transcription initiation, transcription termination, mRNA capping, and mRNA splicing, and that poly(A) tails themselves play roles in initiation of translation, mRNA stability, and export of mRNA from the nucleus (Minvielle-Sebastia and Keller, 1999; Wahle and Ruegsegger, 1999; Proudfoot, 2000). Consistent with the coupling of transcription and mRNA processing in the eukaryotic host, the evolution of mRNA capping, transcription elongation, and polyadenylation functions within a single protein molecule, the J3 gene product, suggests that these could be concerted reactions in vaccinia. Nevertheless, our results show that the poly(A) polymerase stimulatory function of the J3 protein is not strongly coupled to any other facet of mRNA synthesis, processing, or function, since the profiles of RNA and protein synthesis are identical in wt and CF3^{c-}-infected cells. Similarly, our results show that a mutation that decreases the poly(A) tail length from 100–150 to 30–50 nt in length does not have a dramatic effect on mRNA stability or translation in the vaccinia system. (Nuclear export and splicing are irrelevant to vaccinia mRNA metabolism.) While the absolute requirement for poly(A) tails would probably require study of a mutant in the E1 catalytic subunit of the viral poly(A) polymerase, our results show that in cell culture, the poly(A) polymerase stimulatory function of the viral poly(A) polymerase behaves similar to an accessory function, not absolutely required for normal mRNA metabolism and function.

Additionally, the results presented here demonstrate that cap-1 structures are also not essential for virus growth in tissue culture. The 5′ ends of mRNAs synthesized by most lower eukaryotes and plants contain mainly cap-0, while most higher eukaryotes and their viruses produce mRNAs with cap-0 and cap-1 structures (Banerjee, 1980; Schnierle *et al.,* 1992). Although the importance of the terminal m^{7}G in the stability and translation of mRNA has been well documented (Mitchell and Tollervey, 2001; Pestova *et al.,* 2001), the normal role of additional cap ribose methylation remains unknown. To date, 2′-O-methylated caps are only known to be required for synthesis of influenza virus transcripts (Plotch *et al.,* 1981). Thus, it is perhaps not surprising that 2′-O-methylation of vaccinia transcripts appears to be dispensable.

Some information about the solvent-exposed surface area of the J3 protein that may be important for mediating its effects on transcription can be gleaned by mapping the mutated residues in the IBT^r charge-to-alanine scanning mutants and the IBT^d mutant *J3x* on the published J3 protein crystal structure (Fig. 8). The residues highlighted in green in Fig. 8 indicate the charge-to-alanine scanning mutations that are present in the IBT-resistant viruses AS-5, AS-8, and AS-15. It was initially hypothesized that the resistant mutants would have a transcription phenotype intermediate between that of wt and IBT^d virus. Specifically IBT^r viruses would produce transcripts in the absence of IBT that were only slightly 3′ truncated. The slight truncation would be insufficient to affect protein synthesis but sufficient to compensate for the overstimulation of transcription elongation normally induced by IBT, hence the IBT resistance phenotype. The Northern blot analysis presented here does not reveal any obvious differences in transcript length comparing IBT^r and IBT^s viruses; however, it is possible that the hypothesized elongation defect in IBT^r mutants is too subtle to be detected by this analysis. The most likely alternative explanation for the IBT^r phenotype would be that IBT exerts its action by binding to the J3 protein, and the IBT^r J3 mutations abrogate IBT binding. However, the facts that null mutation of either genes J3 or G2 yield an IBT^d phenotype (Meis and Condit, 1991; Latner *et al.,* 2000), that some mutations of G2 yield an IBT^r phenotype (Hassett and Condit, 1994), and that one additional IBT^r mutation maps to the second largest subunit of the viral RNA polymerase (Condit *et al.,* 1991) all argue against J3 being the binding target for IBT. We favor the interpretation that the primary target of IBT is neither J3 nor G2 but some other protein, perhaps the RNA polymerase, and that mutation of J3 or G2 can modulate elongation in a fashion which compensates for the elongation stimulation normally caused by IBT. Thus we believe that an intermediate elongation defect still seems the most plausible explanation for the IBT^r phenotype of the J3 mutants. In this context, the J3 surface region affected by the IBT^r mutants, shaded green and best visualized in the bottom view of Fig. 8, could represent the outline of an "elongation domain" of the J3 protein. Mechanistically, the elongation domain may represent a surface that interacts with other transcription factors or RNA polymerase in an elongation complex. The elongation domain would also include the glycine residue at codon 96 (highlighted in purple in Fig. 8) that is mutated to an aspartate (G96D) in the *J3x* IBT-dependent point-mutant. The wild-type glycine is close to the surface of the molecule and normally provides a kink in the alpha carbon backbone

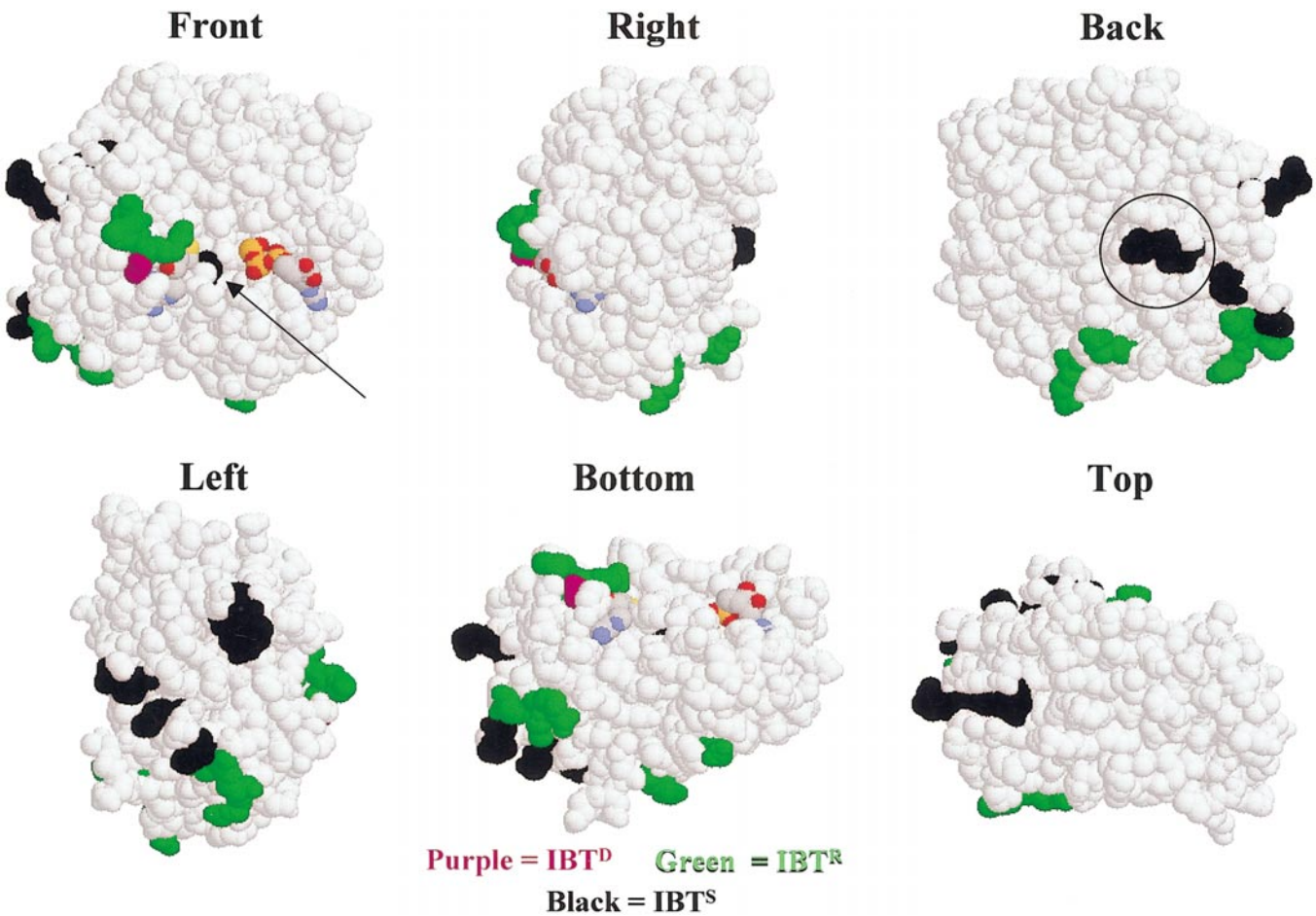


FIG. 8. J3 crystal structure. The published J3 crystal structure [Protein Data Bank (PDB) identification number 1V39 is available through the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov and is shown as a space-filling model]. The panels are labeled according to their rotational relationship to "front" view. Specifically, the right, left, bottom, and top views are rotated 90° relative to the front view. The back view is rotated 180° relative to the front. The arrow indicates the methyltransferase active site. S-adenosylhomocysteine, the product of the reaction between S-adenosylmethionine (SAM) and the 2′ position of the ribose on the penultimate nucleotide, and 7-methyl G-triphosphate are to the left and right of the arrow, respectively. The residue highlighted in purple is glycine 96, which is mutated to aspartate in the IBT-dependent *J3x* point mutant. The residues highlighted in green are the residues that are mutated to alanine in the IBT-resistant mutants (AS-5, AS-8, and AS-15). The residues highlighted in black are those that have no effect on transcription when mutated to alanine (mutants AS-4, AS-6, and AS-8). The circle in the back view indicates the residues that are mutated in the CF3^{c-} poly(A) polymerase stimulatory mutant. These residues are important for binding to the poly(A) polymerase. The arrow points to the K175R residue (in black) that is disrupts methyltransferase activity by inhibiting SAM binding.

between two charged residues, aspartate 95 and arginine 97. Replacement of glycine 96 with a charged residue may significantly alter the local surface charge and structure of the protein such that, by analogy with the IBT-resistant mutants, its ability to bind additional transcription factors may be compromised.

When mutated, the residues in Fig. 8 that are highlighted in black have no effect on transcription elongation. Interestingly, however, the residues indicated by the circle in the "back" view are the mutations in the CF3^{c-} virus and they have provided additional information about the mechanism by which J3 influences transcription. Specifically, the mutations in CF3^{c-} have been shown to inhibit the interaction between J3 and E1 *in vitro*. Noting that the CF3^{c-} virus has a normal transcription phenotype and retains its defect in E1 stimulation *in vivo*, it is likely that heterodimer formation between J3 and E1 is not required for J3-mediated transcription elongation activity. Furthermore, E1 probably does not mediate any hypothetical contacts between J3 and the transcription apparatus.

Although the role of J3 in stimulating postreplicative gene transcription elongation is a third independent function of the protein that can be genetically separated from its 2′-O-methyltransferase and E1 poly(A) polymerase stimulatory activities, the mechanism by which it stimulates transcription remains unclear. Typically, eukaryotic or bacterial transcription factors that regulate postinitiation events fall into one of two broad categories: (1) those that enhance the rate of elongation or (2) those that induce or inhibit termination. Currently, there is not enough information to distinguish whether J3 is acting at

the elongation or termination stage. The transcription defects observed in the J3 null or IBT-dependent mutants could be explained if the normal function of J3 is to stimulate the rate of elongation or to suppress pausing either through modification of the polymerase structure or by stimulating the intrinsic transcript cleavage activity of the polymerase. Alternatively, J3 could prevent termination through direct interaction with release factors such as A18, as supported by preliminary evidence which suggests that J3 directly binds to A18 in a pull-down experiment *in vitro* (E. Niles, personal communication). It is also possible that J3, similar to the pol II elongation factors ELL and CSB, may stimulate the rate of elongation through an alternate mechanism that has not yet been defined (Conaway *et al.,* 2000). Finally, it is also conceivable that J3 does not have a direct role in either elongation or termination, but rather simply contributes to the overall stability of the transcription complex while binding to perform its 2′-O-methyltransferase and poly(A) polymerase stimulatory functions. Establishing the precise mechanism by which J3 enhances transcription will probably require an *in vitro* assay to measure its activity at each stage of transcript synthesis.

MATERIALS AND METHODS

Cell culture, plaque assay, and one-step growth

The conditions for culturing BSC40 African green monkey kidney cells and methods for vaccinia virus cultivation, infection, plaque assay, and one-step growth have been previously described (Condit and Motyczka, 1981; Condit *et al.,* 1983). The wild-type vaccinia strain WR and the IBT-dependent mutants *J3-7* and *J3x* have been previously described (Condit and Motyczka, 1981; Latner *et al.,* 2000; Xiang *et al.,* 2000). IBT was prepared fresh before each use and applied at a final concentration of 45 μM as previously described (Pacha and Condit, 1985).

Plasmids and transient dominant selection

The pGEX/KG plasmids used to express the CF3^{c-}, AS-4, AS-5, AS-6, AS-8, AS-9, and AS-15 mutant copies of the J3 protein for *in vitro* experiments have been previously described (Schnierle *et al.,* 1994; Shi *et al.,* 1997). The K175R missense mutation, constructed in exactly the same fashion as the AS mutations, results in the exchange of a lysine for an arginine at codon 175 of the J3 open reading frame. To prepare the pGEX/KG plasmids for transient dominant selection, the *Eco*RI fragment from pBSgpt4, which contains the vaccinia 7.5K promoter driving expression of the *Escherichia coli* gpt cassette (Hassett *et al.,* 1997), was cloned into the *Eco*RI site of the pGEX/KG plasmids. The sequences of the mutant J3 genes on the recombinant plasmids were verified by sequence analysis prior to transfection.

The method for performing transient dominant selection has been previously described (Falkner and Moss, 1990; Guan and Dixon, 1991; Hassett and Condit, 1994). Briefly, a 60-mm dish of CV-1 cells was pretreated with mycophenolic acid (MPA), infected at a multiplicity of infection (m.o.i.) = 0.5 with wild-type vaccinia virus (strain WR), transfected with 2 μg of plasmid DNA and 30 μl of lipofectin (Life Technologies), and incubated at 37°C until the cytopathic effect was complete. Infected cell lysates were harvested and titered on CV-1 cells pretreated for 12 h with MPA, xanthine, and hypoxanthine and on CV-1 cells growing in the absence of drugs. Plaques were stained with nutrient agar containing 0.005% neutral red 4 days postinfection (Condit *et al.,* 1983). Plaques growing in the presence of MPA were picked and titered in the presence and absence of 45 μM IBT at 31, 37, and 40°C. The MPA-resistant plaques that were isolated from cells transfected with the plasmids containing the AS-4, AS-6, AS-9, K175R, and CF3^{c-} alleles were all sensitive to IBT and grew at all three temperatures. The MPA-resistant plaques that were isolated from cells transfected with the plasmids containing the AS-5, AS-8, and AS-15 alleles were able to grow in both the presence and the absence of IBT at all three temperatures. AS-4, AS-6, AS-9, K175R, and CF3^{c-} candidate mutant plaques that grew at 37°C in the absence of IBT were picked and grown under the same conditions from which they originated. AS-5, AS-8, and AS-15 candidate mutant plaques that grew at 37°C in the presence of IBT were picked and grown under the same conditions from which they originated. The mutations inserted into each virus generated various altered restriction sites within the J3 sequence. Thus, the mutant candidates were screened for the J3 mutations by RFLP analysis of PCR fragments. Candidate J3 mutant viruses were then plaque purified and the entire J3 gene coding region was sequenced from each virus to confirm the presence of the mutations.

Protein pulse-labeling

Pulse-labeling of proteins in virus infected cells was performed as previously described (Condit and Motyczka, 1981). Briefly, confluent 35-mm dishes of BSC40 cells were infected with *wt* or mutant virus at a high m.o.i. in the absence or presence of 45 μM IBT and incubated at 37°C as indicated in the figure legends. At various times postinfection, cells were metabolically labeled with 10 μCi [^{35}S]methionine (1175 Ci/mmol) for 15 min. Cells were lysed on the dishes by addition of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer, and solubilized proteins were analyzed by SDS–PAGE on 8% gels. The gels were stained with Coomassie blue, dried, and autoradiographed.

Isolation of total cellular RNA

Dishes (100 mm) of confluent BSC40 cells were infected with wt or mutant virus at a high m.o.i. in the absence or presence of 45 μM IBT at 37°C as indicated in the figure legend. At various times after infection, total cellular RNA was purified using RNeasy Total RNA purification columns essentially as described by the manufacturer (Qiagen, Inc., Chatsworth, CA) except that the infected cell lysates from each 100-mm dish were applied to two RNeasy columns and the total RNA was eluted in a total volume of 200 μl of RNase-free water.

Northern analysis

Northern analysis was performed essentially as previously described (Xiang *et al.,* 2000) with the following exceptions: 1.5 μg of purified total RNA from infected cells was adjusted to a final concentration of 0.4 × MOPS buffer (1× MOPS buffer = 20 mM MOPS, 8 mM sodium acetate, 1 mM EDTA, pH 7.0), 1.8 M formaldehyde, and 40% formamide in a final volume of 24.5 μl. The samples were heated to 65°C for 15 min and then chilled on ice. One microliter of 1 mg/ml ethidium bromide and 2 μl of RNA loading buffer (50% glycerol, 1 mM EDTA, 0.0 5% bromphenol blue, 0.0 5% xylene cyanole) were added to each sample prior to loading on the gel. The samples were electrophoresed at 80 V through 1.2% agarose gels containing a final concentration of 1 × MOPS buffer and 2.2 M formaldehyde in an electrophoresis buffer containing 1 × MOPS. Riboprobes were synthesized, hybridized, and washed from the blots as previously described and as described in the Promega Protocols and Applications Guide.

In vivo poly(A) tail length measurements

Poly(A) tail lengths were measured using a protocol modified from Minvielle-Sebastia *et al.* (1998). Total RNA (1.5 μg) harvested from cells at 12 h postinfection was labeled in a 30 μl reaction containing 50 mM HEPES pH 7.5, 5 μM ATP, 10 mM $MgCl_2$, 3.3 mM DTT, 10% DMSO, 300 μg/ml BSA, 20 μCi 3′-5′[5′-^{32}P]pCp (3000 Ci/mmol), and 20 units of T_4 RNA ligase (NEB). Reactions were incubated on ice for 18 h and then were digested at 37°C for 4.5 h by the addition of 50 μl of a digest mix containing 40 μg tRNA, 100 U RNase T_1, 10 μg RNase A, 10 mM Tris–HCl pH 7.5, and 300 mM NaCl. Twenty microliters of a mixture containing 2 mg/ml proteinase K, 130 mM EDTA, and 2.5% SDS was added and the reactions were incubated at 37°C for an additional 30 min. Reactions were then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous layer was precipitated by the addition of 60 μg glycogen and 3 vol of 100% ethanol followed by centrifugation for 30 min at 16,100 RCF. Pellets were resuspended in 10 μl of a solution containing 95% formamide, 20 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanole. Five microliters of each sample was electrophoresed on 8% polyacrylamide 8 M urea sequencing gels in 1× TBE at 1700 V, 72 mA, and 35 W with constant power. Gels were fixed in 10% methanol and 10% acetic acid and then were dried and subjected to autoradiography.

Cap marker synthesis

Poly(A) RNA was synthesized essentially as previously described (Shuman and Moss, 1990) in a 100 μl reaction containing 8 μg M13 single-stranded DNA, 50 mM Tris–HCl pH 7.8, 2.5 mM DTT, 3 mM $MnCl_2$, 1.5 mM ATP, 20 μM cordycepin triphosphate, 10 μCi α^{32}P-ATP (3000 Ci/mmol), 10 u *E. coli* RNA polymerase (USB), and 40 u RNasein (Promega). The reaction was incubated at 37°C for 20 h; then poly(A) RNA was purified by batchwise oligo(dT) selection. Briefly, the reaction was made 50 mM with respect to EDTA and then was incubated at 70°C for 10 min. The reaction was combined with an equal volume of 2× binding buffer [1 M NaCl, 20 mM Tris–HCl pH 7.8, 2 mM EDTA, 1% SDS] and was incubated with 10 mg of oligo(dT) cellulose (NEB) for 30 min. The cellulose-bound poly(A) RNA was pelleted by centrifugation (1 min at 6000 RCF) and then was washed three times with 1× binding buffer. Poly(A) RNA was eluted twice with 600 μl of elution buffer [10 mM Tris–HCl pH 7.8, 1 mM EDTA, 0.2% SDS] and was precipitated with 300 mM sodium acetate, 40 μg glycogen, and an equal volume of isopropanol. The poly(A) RNA 5′ ends were converted to the cap-0 structure essentially as previously described (Shuman and Moss, 1990) in a 15 μl reaction containing [50 mM Tris–HCl pH 7.8, 6 mM KCl, 1.2 mM $MgCl_2$, 20 μCi α^{32}P-GTP, 10 pmol poly(A) RNA substrate, 150 nM vaccinia virus capping enzyme (generously provided by Ed Niles), 1 mM S-adenosylmethionine, 40 u RNasein (Promega)]. For conversion to cap-1, 280 ng wild-type vaccinia virus J3 protein, purified as previously described (Xiang *et al.,* 2000), was included in the reaction. Capping reactions were incubated for 19 h at 37°C. Capped poly(A) was once again selected over oligo(dT) cellulose and precipitated as described above. The cap-labeled RNA was digested in a reaction containing 10 mM Tris–HCl pH 7.8, 50 mM sodium acetate, 2 mM EDTA, 1 μg RNase A (Sigma), 100 u RNase T_1 (Boehringer Mannheim), 25 u RNase T_2 (BRL), and 1 u calf alkaline phosphatase (BRL).

In vivo cap labeling

One 100-mm dish of BSC40 cells was infected at an m.o.i. = 10 with each virus (except J3-7 which was at m.o.i. = 4.8 due to titer constraints). Infected cells were incubated with 1× DME (BRL) and 10% fetal calf serum at 37°C for 10 h. The medium was removed and the infected cells were washed with 4 ml of phosphate-free, serum-free 1× DME. Cells were then incubated in the

presence of 3.33 mCi of [^{32}P]orthophosphoric acid (285.5Ci/mg) diluted in 4 ml of phosphate-free 1× DME for 2 h at 37°C. Cells were washed with 4 ml PBS and then total RNA was extracted with an RNeasy kit (Qiagen) according to the manufacturer's instructions except that approximately 10^7 cells were lysed with 1.2 ml of RLT buffer + 1% β-mercaptoethanol, and the lysate was spun over two separate RNeasy columns. The total RNA from each 100-mm dish of infected cells was eluted in a total volume of 200 μl water and made 50 mM with respect to EDTA. Samples were heated to 70°C for 5 min and then poly(A)$^+$ RNA was selected over 10 mg oligo(dT) resin as described above.

Cap analysis

Analysis of labeled caps was adapted from a procedure described by Kuge *et al.* (1998). Isopropanol-precipitated RNA, labeled either *in vitro* or *in vivo*, was resuspended in 10 μl of digestion mix containing 10 mM Tris–HCl pH 7.8, 50 mM sodium acetate, 2 mM EDTA, 1 μg RNase A (Sigma), 100 u RNase T_1 (Boehringer Mannheim), 25 u RNase T_2 (BRL), and 1 u calf alkaline phosphatase (BRL) and incubated for 19 h at 37°C. To dilute the cap markers while normalizing the salt concentrations relative to *in vivo* samples, approximately 5000 Cherenkov cpm of RNase treated, *in vitro* labeled RNA was added to another 10 μl of digestion mix before further processing. Digested samples were then concentrated to approximately 1 μl in a speed vacuum and then resuspended in 10 μl of [95% formamide, 20 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanole]. Samples were electrophoresed on a 20% polyacrylamide, 8 M urea sequencing gel in 1× TBE at 1700 V, 72 mA, 33 W with constant power. The excess free-labeled phosphate from this analysis was distributed between the bottom of the gel and the lower buffer reservoir. The gel was fixed briefly in a solution of 20% methanol and 20% acetic acid, dried, and then subjected to Phosphorimager analysis.

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REFERENCES

Banerjee, A. K. (1980). 5′-terminal cap structure in eucaryotic messenger ribonucleic acids. *Microbiol. Rev.* **44,** 175–205.

Barbosa, E., and Moss, B. (1978a). mRNA(nucleoside-2′-)-methyltransferase from vaccinia virus. Characteristics and substrate specificity. *J. Biol. Chem.* **253,** 7698–7702.

Barbosa, E., and Moss, B. (1978b). mRNA(nucleoside-2′-)-methyltransferase from vaccinia virus. Purification and physical properties. *J. Biol. Chem.* **253,** 7692–7697.

Bayliss, C. D., and Condit, R. C. (1993). Temperature-sensitive mutants in the vaccinia virus A18R gene increase double-stranded RNA synthesis as a result of aberrant viral transcription. *Virology* **194,** 254–262.

Bayliss, C. D., and Condit, R. C. (1995). The vaccinia virus A18R gene product is a DNA-dependent ATPase. *J. Biol. Chem.* **270,** 1550–1556.

Black, E. P., and Condit, R. C. (1996). Phenotypic characterization of mutants in vaccinia virus gene G2R, a putative transcription elongation factor. *J. Virol.* **70,** 47–54.

Christen, L. M., Sanders, M., Wiler, C., and Niles, E. G. (1998). Vaccinia virus nucleoside triphosphate phosphohydrolase I is an essential viral early gene transcription termination factor. *Virology* **245,** 360–371.

Cohrs, R. J., Condit, R. C., Pacha, R. F., Thompson, C. L., and Sharma, O. K. (1989). Modulation of ppp(A2′p)nA-dependent RNase by a temperature-sensitive mutant of vaccinia virus. *J. Virol.* **63,** 948–951.

Conaway, J. W., Shilatifard, A., Dvir, A., and Conaway, R. C. (2000). Control of elongation by RNA polymerase II. *Trends Biochem. Sci.* **25,** 375–380.

Condit, R. C., Easterly, R., Pacha, R. F., Fathi, Z., and Meis, R. J. (1991). A vaccinia virus isatin-beta-thiosemicarbazone resistance mutation maps in the viral gene encoding the 132-kDa subunit of RNA polymerase. *Virology* **185,** 857–861.

Condit, R. C., and Motyczka, A. (1981). Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. *Virology* **113,** 224–241.

Condit, R. C., Motyczka, A., and Spizz, G. (1983). Isolation, characterization, and physical mapping of temperature-sensitive mutants of vaccinia virus. *Virology* **128,** 429–443.

Condit, R. C., Xiang, Y., and Lewis, J. I. (1996). Mutation of vaccinia virus gene G2R causes suppression of gene A18R ts mutants: Implications for control of transcription. *Virology* **220,** 10–19.

Cooper, J. A., Moss, B., and Katz, E. (1979). Inhibition of vaccinia virus late protein synthesis by isatin-beta-thiosemicarbazone: Characterization and in vitro translation of viral mRNA. *Virology* **96,** 381–392.

Deng, L., and Shuman, S. (1998). Vaccinia NPH-I, a DExH-box ATPase, is the energy coupling factor for mRNA transcription termination. *Genes Dev.* **12,** 538–546.

Falkner, F. G., and Moss, B. (1990). Transient dominant selection of recombinant vaccinia viruses. *J. Virol.* **64,** 3108–3111.

Gershon, P. D. (1998). mRNA 3′ end formation by vaccinia virus: Mechanism of action of a heterodimeric poly(A) polymerase. *Sem. Virol.* **8,** 343–350.

Gershon, P. D., Ahn, B. Y., Garfield, M., and Moss, B. (1991). Poly(A) polymerase and a dissociable polyadenylation stimulatory factor encoded by vaccinia virus. *Cell* **66,** 1269–1278.

Gershon, P. D., and Moss, B. (1992). Transition from rapid processive to slow nonprocessive polyadenylation by vaccinia virus poly(A) polymerase catalytic subunit is regulated by the net length of the poly(A) tail. *Genes Dev.* **6,** 1575–1586.

Gershon, P. D., and Moss, B. (1993). Stimulation of poly(A) tail elongation by the VP39 subunit of the vaccinia virus-encoded poly(A) polymerase. *J. Biol. Chem.* **268,** 2203–2210.

Gershon, P. D., Shi, X., and Hodel, A. E. (1998). Evidence that the RNA methylation and poly(A) polymerase stimulatory activities of vaccinia virus protein VP39 do not impinge upon one another. *Virology* **246,** 253–265.

Guan, K. L., and Dixon, J. E. (1991). Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192,** 262–267.

Hassett, D. E., and Condit, R. C. (1994). Targeted construction of temperature-sensitive mutations in vaccinia virus by replacing clustered charged residues with alanine. *Proc. Natl. Acad. Sci. USA* **91,** 4554–4558.

Hassett, D. E., Lewis, J. I., Xing, X., DeLange, L., and Condit, R. C. (1997). Analysis of a temperature-sensitive vaccinia virus mutant in the viral mRNA capping enzyme isolated by clustered charge-to-alanine mutagenesis and transient dominant selection. *Virology* **238,** 391–409.

Hodel, A. E., Gershon, P. D., Shi, X., and Quiocho, F. A. (1996). The 1.85

A structure of vaccinia protein VP39: A bifunctional enzyme that participates in the modification of both mRNA ends. *Cell* **85,** 247–256.

Kuge, H., Brownlee, G. G., Gershon, P. D., and Richter, J. D. (1998). Cap ribose methylation of c-mos mRNA stimulates translation and oocyte maturation in *Xenopus laevis*. *Nucleic Acids Res.* **26,** 3208–3214.

Lackner, C. A., and Condit, R. C. (2000). Vaccinia virus gene A18R DNA helicase is a transcript release factor. *J. Biol. Chem.* **275,** 1485–1494.

Latner, D. R., Xiang, Y., Lewis, J. I., Condit, J., and Condit, R. C. (2000). The vaccinia virus bifunctional gene J3 (nucleoside-2′-O-)-methyltransferase and poly(A) polymerase stimulatory factor is implicated as a positive transcription elongation factor by two genetic approaches. *Virology* **269,** 345–355.

Mahr, A., and Roberts, B. E. (1984). Arrangement of late RNAs transcribed from a 7.1-kilobase *Eco*RI vaccinia virus DNA fragment. *J. Virol.* **49,** 510–520.

Meis, R. J., and Condit, R. C. (1991). Genetic and molecular biological characterization of a vaccinia virus gene which renders the virus dependent on isatin-beta-thiosemicarbazone (IBT). *Virology* **182,** 442–454.

Minvielle-Sebastia, L., Beyer, K., Krecic, A. M., Hector, R. E., Swanson, M. S., and Keller, W. (1998). Control of cleavage site selection during mRNA 3′ end formation by a yeast hnRNP. *EMBO J.* **17,** 7454–7468.

Minvielle-Sebastia, L., and Keller, W. (1999). mRNA polyadenylation and its coupling to other RNA processing reactions and to transcription. *Curr. Opin. Cell Biol.* **11,** 352–357.

Mitchell, P., and Tollervey, D. (2001). mRNA turnover. *Curr. Opin. Cell Biol.* **13,** 320–325.

Mohamed, M. R., and Niles, E. G. (2000). Interaction between nucleoside triphosphate phosphohydrolase I and the H4L subunit of the viral RNA polymerase is required for vaccinia virus early gene transcript release. *J. Biol. Chem.* **275,** 25798–25804.

Moss, B. (2001). Poxviridae: The viruses and their replication. *In* "Fields Virology" (D. M. Knipe and P. M. Howley, Eds.), pp. 2849–2884. Lippincott Williams and Wilkins, Philadelphia.

Pacha, R. F., and Condit, R. C. (1985). Characterization of a temperature-sensitive mutant of vaccinia virus reveals a novel function that prevents virus-induced breakdown of RNA. *J. Virol.* **56,** 395–403.

Pacha, R. F., Meis, R. J., and Condit, R. C. (1990). Structure and expression of the vaccinia virus gene which prevents virus-induced breakdown of RNA. *J. Virol.* **64,** 3853–3863.

Pestova, T. V., Kolupaeva, V. G., Lomakin, I. B., Pilipenko, E. V., Shatsky, I. N., Agol, V. I., and Hellen, C. U. (2001). Molecular mechanisms of translation initiation in eukaryotes. *Proc. Natl. Acad. Sci. USA* **98,** 7029–7036.

Plotch, S. J., Bouloy, M., Ulmanen, I., and Krug, R. M. (1981). A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell* **23,** 847–858.

Proudfoot, N. (2000). Connecting transcription to messenger RNA processing. *Trends Biochem. Sci.* **25,** 290–293.

Schnierle, B. S., Gershon, P. D., and Moss, B. (1992). Cap-specific mRNA (nucleoside-O2′-)-methyltransferase and poly(A) polymerase stimulatory activities of vaccinia virus are mediated by a single protein. *Proc. Natl. Acad. Sci. USA* **89,** 2897–2901.

Schnierle, B. S., Gershon, P. D., and Moss, B. (1994). Mutational analysis of a multifunctional protein, with mRNA 5′ cap-specific (nucleoside-2′-O-)-methyltransferase and 3′-adenylyltransferase stimulatory activities, encoded by vaccinia virus. *J. Biol. Chem.* **269,** 20700–20706.

Shi, X., Bernhardt, T. G., Wang, S. M., and Gershon, P. D. (1997). The surface region of the bifunctional vaccinia RNA modifying protein VP39 that interfaces with poly(A) polymerase is remote from the RNA binding cleft used for its mRNA 5′ cap methylation function. *J. Biol. Chem.* **272,** 23292–23302.

Shi, X., Yao, P., Jose, T., and Gershon, P. (1996). Methyltransferase-specific domains within VP-39, a bifunctional protein that participates in the modification of both mRNA ends. *RNA* **2,** 88–101.

Shuman, S., Broyles, S. S., and Moss, B. (1987). Purification and characterization of a transcription termination factor from vaccinia virions. *J. Biol. Chem.* **262,** 12372–12380.

Shuman, S., and Moss, B. (1988). Factor-dependent transcription termination by vaccinia virus RNA polymerase. Evidence that the cis-acting termination signal is in nascent RNA. *J. Biol. Chem.* **263,** 6220–6225.

Shuman, S., and Moss, B. (1990). Purification and use of vaccinia virus messenger RNA capping enzyme. *Methods Enzymol.* **181,** 170–180.

Simpson, D. A., and Condit, R. C. (1995). Vaccinia virus gene A18R encodes an essential DNA helicase. *J. Virol.* **69,** 6131–6139.

Wahle, E., and Ruegsegger, U. (1999). 3′-End processing of pre-mRNA in eukaryotes. *FEMS Microbiol. Rev.* **23,** 277–295.

Wei, C. M., and Moss, B. (1975). Methylated nucleotides block 5′-terminus of vaccinia virus messenger RNA. *Proc. Natl. Acad. Sci. USA* **72,** 318–322.

Xiang, Y., Latner, D. R., Niles, E. G., and Condit, R. C. (2000). Transcription elongation activity of the vaccinia virus J3 protein in vivo is independent of poly(A) polymerase stimulation. *Virology* **269,** 356–369.

Xiang, Y., Simpson, D. A., Spiegel, J., Zhou, A., Silverman, R. H., and Condit, R. C. (1998). The vaccinia virus A18R DNA helicase is a postreplicative negative transcription elongation factor. *J. Virol.* **72,** 7012–7023.

Yuen, L., and Moss, B. (1987). Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. *Proc. Natl. Acad. Sci. USA* **84,** 6417–6421.