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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

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Vaccinia virus genes A18 and G2 affect the elongation and termination of postreplicative viral gene transcription in opposite ways. Viruses with mutations in gene A18 produce abnormally long transcripts, indicating that A18 is a negative transcription elongation factor. Viruses containing mutations in gene G2 produce transcripts that are abnormally short, truncated specifically from their 3′ ends, indicating that G2 is a positive transcription elongation factor. Despite the fact that both A18 and G2 are essential genes, A18-G2 double-mutant viruses are viable, presumably because the effects of the mutations are mutually compensatory. In addition, the anti-poxviral drug isatin-β-thiosemicarbazone (IBT) seems to enhance elongation during a vaccinia infection: IBT treatment of a wildtype vaccinia infection induces a phenotype identical to an A18 mutant infection, and G2 mutant viruses are dependent on IBT for growth, presumably because IBT restores the G2 mutant truncated transcripts to a normal length. These observations inspire two independent genetic selections that have now been used to identify an additional vaccinia gene, J3, that regulates postreplicative transcription elongation. In the first selection, a single virus that contains an extragenic suppressor of the A18 temperature-sensitive mutant, *Cts23,* was isolated. In the second selection, several spontaneous IBT-dependent (IBT^d) mutant viruses were isolated and characterized genetically. Marker rescue mapping and DNA sequence analysis show that the extragenic suppressor of *Cts23* contains a point mutation in the J3 gene, while each of seven new IBT^d mutants contains null mutations in the J3 gene. The J3 protein has previously been identified as a (nucleoside-2′-*O*-)-methyltransferase and as a processivity subunit for the heterodimeric viral poly(A) polymerase. The nature of the two independent selections used to isolate the J3 mutants strongly suggests that the J3 protein serves as a positive postreplicative transcription elongation factor during a normal virus infection.

INTRODUCTION

Vaccinia is a member of the orthopox virus family. It encodes approximately 200 genes on a 192-kb double-stranded DNA genome that replicates strictly in the cytoplasm of host cells. Due to the cytoplasmic location of its replication cycle, vaccinia must encode its own multisubunit RNA polymerase and almost all of the transcription factors required to carry out distinct early, intermediate, and late stages of gene expression (Moss, 1996). Historically, vaccinia has served as an excellent model for studying the essentials of eukaryotic transcription (Traktman, 1990).

Intermediate and late (together termed "postreplicative") vaccinia gene transcription is notably different from the transcription of early genes specifically with respect to the process of termination. Early termination is catalyzed by the virus coded capping enzyme (VTF) (Shuman *et al.,* 1987), along with a viral DNA-dependent ATPase (NPH I) (Christen *et al.,* 1998; Deng and Shuman, 1998), in response to a *cis*-acting termination sequence, U_5NU, located 30–50 nucleotides upstream from the 3′ end of the nascent early mRNA (Shuman and Moss, 1988; Yuen and Moss, 1987). Through this termination mechanism, the polymerase produces from early genes a population of transcripts that is characteristically homogeneous in length. By contrast, the polymerase transcribing 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). No *cis*-acting termination sequences have yet been identified for intermediate or late genes.

Previous genetic and biochemical evidence has suggested that two different vaccinia genes, A18 and G2, affect the termination and elongation of postreplicative gene transcripts in opposite ways. The 56-kDa A18 protein has been characterized as a DNA-dependent ATPase (Bayliss and Condit, 1995) and a 3′-5′ DNA helicase (Simpson and Condit, 1995). Viruses with mutations in A18 produce abnormally long transcripts that

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(Xiang *et al.,* 1998), when transcribed from opposing promoters, can hybridize to one another, forming double-stranded RNA, which in turn induces the cellular 2-5A pathway, leading ultimately to RNase L catalyzed RNA degradation and abortion of the infection (Bayliss and Condit, 1993; Cohrs *et al.,* 1989; Pacha and Condit, 1985). Consistent with this *in vivo* phenotype, it has been recently demonstrated using an *in vitro* transcription assay that the A18 protein is required for release of nascent transcripts from an elongation complex, showing that A18 serves as a transcript release factor (Lackner and Condit, 2000). In contrast to A18 mutants, viruses containing mutations in the gene G2 produce intermediate and late transcripts that are shorter than wildtype transcripts and that are specifically truncated from their 3′ ends (Black and Condit, 1996). As a result, G2 mutants produce reduced quantities of large proteins at late times during infection. These findings suggest that the 26-kDa G2 protein, which has no significant homology to any known protein, serves as a postreplicative positive transcription elongation factor.

Viruses with G2 mutations have a phenotype that is selectable by two distinct methods. First, G2 null mutants are extragenic suppressors of temperature-sensitive mutations in gene A18 (Condit *et al.,* 1996). The suppression follows logically from the complementary nature of the individual mutant phenotypes: 3′ truncation of RNAs resulting from a G2 mutation should compensate for the read-through transcription caused by mutation of the A18 transcript release factor. Second, G2 mutants are dependent upon the anti-poxviral drug isatin-β-thiosemicarbazone (IBT) for growth (Meis and Condit, 1991). Although the exact mechanism of action of IBT is unknown, treatment of wildtype (wt) virus infections with IBT induces a phenotype identical to an A18 mutant infection, indicating that IBT promotes read-through transcription in a wt virus infection (Bayliss and Condit, 1993; Pacha and Condit, 1985). Theoretically, IBT compensates for a G2 mutation by causing elongation of the 3′ truncated G2 mutant RNAs, hence the IBT dependence of G2 mutants. Theoretically, selection of new extragenic suppressors of A18 temperature-sensitive mutants or new IBT-dependent (IBT^d) mutants should reveal any viral genes in addition to G2 that might affect viral transcription elongation.

In this report, we describe isolation of one new extragenic suppressor of gene A18 and nine new IBT^d mutants. Marker rescue mapping and DNA sequence analysis show that the novel extragenic suppressor of *Cts23* contains a point mutation in the J3 gene, while each of the seven new IBT^d mutants contains null mutations in the J3 gene. Interestingly, the J3 protein has previously been identified as a (nucleoside-2′-*O*-)-methyltransferase and as a processivity subunit for the heterodimeric viral poly(A) polymerase (Schnierle *et al.,* 1992). These results provide genetic evidence to suggest that the vaccinia J3 gene product, like G2, also serves as a positive transcription elongation factor. The accompanying article presents experiments in support of this conclusion (Xiang *et al.,* 2000).

RESULTS

Mutant isolation

Isolation of a novel A18-Cts23 extragenic suppressor. Two published observations show that mutations in the vaccinia gene G2 suppress temperature-sensitive mutations in gene A18 (Condit *et al.,* 1996). First, a double-mutant virus (*x41*), constructed by crossing an IBT^d G2 deletion mutant (*G2A*) with an A18 ts mutant (*Cts23*), is viable. Second, selection for phenotypic revertants (ts+) of A18 ts mutants (*Cts23* or *Cts22*) resulted in isolation of extragenic suppressor mutations that mapped to gene G2. When segregated from the ts A18 allele by a test-cross with wildtype virus, the suppressing G2 alleles display an IBT^d phenotype. Based on these results, we hypothesized that mutations in other genes that influence transcription elongation might be isolated by selecting for novel extragenic suppressors of *Cts23.*

To isolate new extragenic suppressors of the A18 temperature-sensitive mutant *Cts23,* the mutant, which normally grows at 31°C and is sensitive to IBT, was plated at the nonpermissive temperature (40°C) in the absence of IBT. Several spontaneous revertant viruses formed plaques at 40°C. Twenty of these plaques were picked and tested directly for plaque formation at both 31 and 40°C in the presence and in the absence of IBT. In this test, one of these viruses, *r51,* displayed a plaque phenotype distinct from either wildtype or *Cts23,* suggesting that *r51* might be an extragenic suppressor rather than a true revertant. Specifically, *r51* formed smaller than wildtype plaques at both 31 and 40°C and was IBT sensitive. *r51* was replaque-purified and grown for further analysis.

Based on previous experience with G2 suppressors of *Cts23,* we hypothesized that the putative suppressor mutation in *r51* might result in IBT dependence when separated from the *Cts23* mutation. To test this hypothesis, *r51* was crossed with wt virus to generate recombinants that contained either the *Cts23* allele or the putative suppressing allele, but not both. Virus containing the putative suppressor alone should be readily detectable in a plaque assay of recombinant progeny done in the presence of IBT since both wt and *r51* are IBT sensitive. To generate recombinants, BSC40 cells were coinfected with wt virus and *r51* at a multiplicity of infection (m.o.i.) of 6 each and infections were incubated at 40°C without IBT for 48 h. Wildtype and *r51* single infections were done in parallel at an m.o.i. of 12 as controls. The lysates were then titered in the presence and in the absence of drug at both 31 and 40°C to detect the suspected IBT^d phenotype; plaque assays of stocks of

TABLE 1

Test-Cross of r51

	Titer[a]			
	31°C		40°C	
Virus infection[b]	−IBT	+IBT	−IBT	+IBT
wt	4.0×10^6	$<1.0 \times 10^2$	4.0×10^6	1.0×10^3
r51	2.0×10^6	$<1.0 \times 10^1$	2.0×10^6	$<1.0 \times 10^1$
wt + r51	7.0×10^6	8.0×10^5	7.0×10^6	1.6×10^6
G2A	$<1.0 \times 10^3$	1.0×10^7	2.0×10^3	1.5×10^7
ts23	3.0×10^8	4.0×10^4	3.6×10^5	$<1.0 \times 10^3$

[a] Virus stocks (*G2A* and *Cts23*) or lysates from high m.o.i. infections (all others) were plaque titrated under the indicated conditions.

[b] Rows labeled G2A and Cts23 represent control plaque assays done on stocks of virus grown under appropriate permissive conditions. For all other rows, cells were infected at high m.o.i. with the indicated viruses, and lysates were prepared as described under Materials and Methods.

Cts23 and *G2A* were done in parallel as controls (Table 1). The results of this experiment show that, relative to single infections with wt virus or *r51,* the mixed infection contained a high proportion of virus that formed plaques in the presence of IBT, indicating that an IBT variant had been produced in the cross. To determine the phenotype of these IBT variants, 10 plaques that grew in the presence of IBT at 40°C were picked and titered with and without IBT at both 31 and 40°C. The results showed that all 10 viruses were IBT dependent for growth at both 31 and 40°C. One IBT[d] isolate, named *r51x4,* was replaque-purified, grown, and subjected to further characterization.

The IBT[d] mutation in *r51x4* was mapped by marker rescue with wildtype DNA fragments as previously described (Meis and Condit, 1991; Thompson *et al.,* 1989). Coarse mapping was first accomplished using a two-step rescue protocol. BSC40 cells were infected with *r51x4* at an m.o.i. of 0.01 and transfected individually with cosmid clones containing wildtype vaccinia sequences that span the entire genome in an overlapping fashion. The infection–transfections were incubated at 37°C in the absence of drug until a complete cytopathic effect was observed. The lysates were then harvested and titered in the absence of IBT at 37°C (data not shown). Three overlapping cosmid clones, pWR45-83, pWR67-98, and pWR74-111 (Fig. 1), were able to rescue the IBT-dependent phenotype, thus localizing the IBT[d] mutation in *r51x4* to a region spanning the *Hin*dIII J fragment. Subsequent two-step marker rescue experiments (data not shown) with transfected *Hin*dIII J fragment subclones pJ7 and pJ3R (Fig. 1), the latter of which contains the precise J3 coding sequence, showed that the IBT dependence mutation mapped to the J3 open reading frame. Sequence analysis of the J3 gene revealed that *r51x4* contains two missense mutations (Fig. 1). The upstream mutation exchanges a glycine for an aspartic acid residue at codon 96 (G96D). The second mutation is located in the downstream region of J3 that overlaps the J4 open reading frame. This mutation results in the exchange of an arginine for a lysine at codon 327 (R327K) in the J3 gene and the substitution of an asparagine for aspartic acid at codon 22 (D22N) of the J4 gene. To determine which of the two mutations was responsible for the IBT[d] phenotype, a final one-step marker rescue experiment was performed with the pJ5 and pJ6 subclones that contain sequence surrounding the G96D and R327K mutations, respectively. BSC40 cells were infected with *r51x4* at a low m.o.i., transfected with DNA, incubated for 4 days at 40°C in the absence of drug, and stained directly with crystal violet (Fig. 1). Subclone pJ6 failed to rescue, thereby demonstrating that the R327K mutation played no role in the IBT[d] phenotype. Conversely, the pJ5 subclone rescued IBT dependence in *r51x4,* implicating the G96D mutation as the IBT[d] allele and also suggesting that it is responsible for the suppression of A18-*Cts23.* Additional genetic experiments described below were performed to verify the conclusions drawn from these results.

Creation of the J3x single-mutant virus. A virus containing only the upstream G96D mutation was generated to confirm that this mutation was solely responsible for the IBT-dependent phenotype. In order to segregate the G96D mutation from the downstream R327K mutation, *r51x4* was crossed to wt virus and the progeny were screened by PCR and restriction fragment length polymorphism (RFLP) analysis. Specifically, BSC40 cells were coinfected with *r51x4* at an m.o.i. of 3 and wt at an m.o.i. of 15 and then incubated at 37°C for 72 h in the absence of drug. The lysates were then harvested and titered at 37°C in the presence and in the absence of IBT. Twenty plaques that grew in the presence of IBT were picked and grown, and viral DNA was isolated and subjected to RFLP analysis as follows. Wildtype sequence spanning codon 96 of the J3 gene contains and *Eag*I restriction site that is removed by the G96D mutation. Similarly, wildtype sequence spanning codon 327 of the J3 gene contains a *Bst*YI restriction site that is removed by the R327K mutation. The regions surrounding both mutations were PCR-amplified from the 20 candidate viruses and the products were digested with the appropriate enzyme (data not shown). One of the 20 virus isolates appeared to contain only the G96D mutation according to restriction digestion. This virus was plaque-purified and sequence analysis of the entire J3 gene confirmed that it contained only the G96D mutation. The single mutant was designated *J3x* and was used for further characterization.

Creation of J3x23. The final step in the genetic characterization of the *J3x* virus was to formally prove that the G96D mutation in J3 suppresses the *Cts23* mutation in

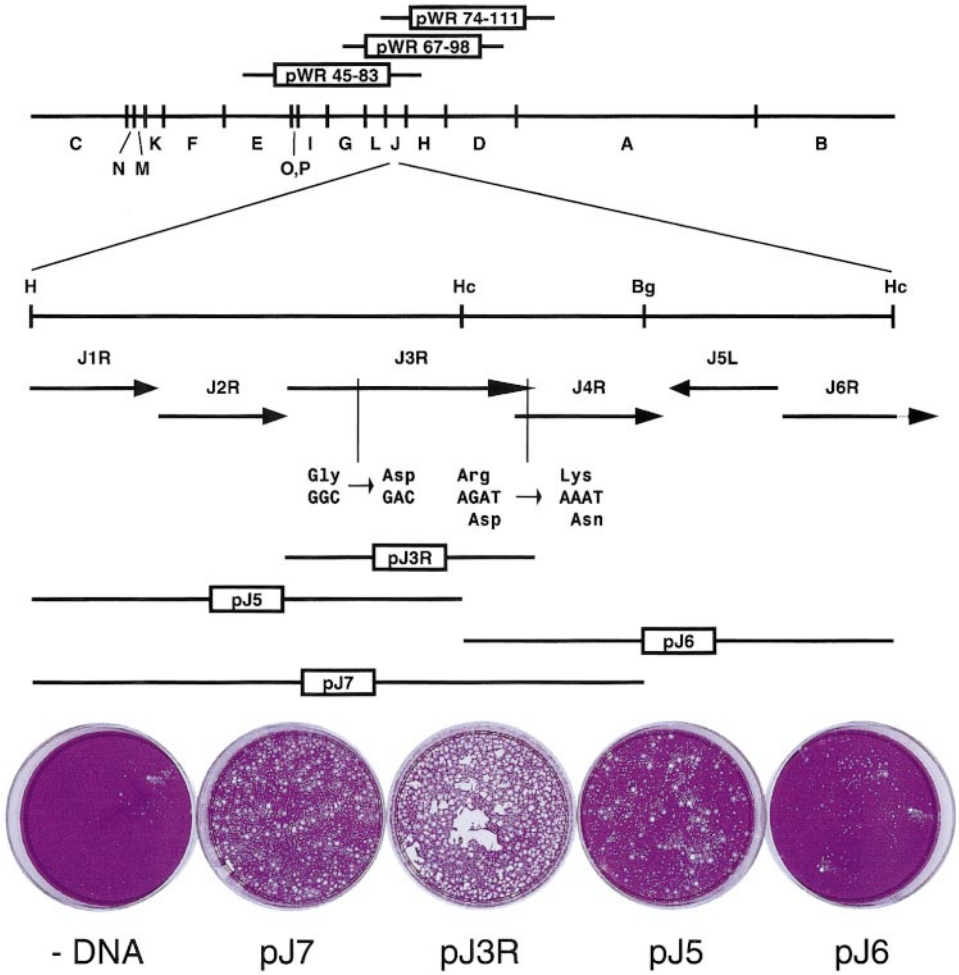


FIG. 1. Marker rescue mapping of the IBT[d] allele in *r51x4*. Cells were infected with *r51x4* at an m.o.i. of 0.01 PFU/cell, transfected with the indicated wt DNA clones, incubated at 40°C in the presence of IBT for 5 days, and then stained with crystal violet. The presence of plaques on a dish, significantly above the -DNA background, indicates marker rescue. The figure shows, from the top down: map positions of three cosmid clones (pWR. . .) that rescue *r51x4;* a *Hin*dIII map of the vaccinia genome; a blow-up restriction map of the *Hin*dIII J fragment (H, *Hin*dIII; Hc, *Hin*cII; Bg, *Bgl*II); *Hin*dIII J fragment genes (J1R–J6R) represented as arrows; *r51x4* mutations represented as vertical lines leading to sequence details; *r51x4* sequence details with wt sequence at the left of each arrow and *r51x4* sequence at the right, J3 sequence above the DNA sequence and J4 sequence below; position of *Hin*dIII J fragment subclones used in the marker rescue, and crystal violet stained dishes from the rescue experiment.

A18 by creating a double-mutant virus in a cross between *Cts23* and *J3x.* BSC40 cells were coinfected with *J3x* and *Cts23* at m.o.i. of 2.5 each and incubated at 31°C for 72 h. The lysate was titered at 31 and 40°C in the presence and in the absence of IBT. Plaques that grew at 40°C in the absence of drug were picked and screened for the presence of G96D by PCR and restriction digestion as described above. A recombinant virus that contained the G96D mutation was shown by sequence analysis to also contain the A18-*Cts23* mutation and was designated *J3x23.* We conclude from these experiments that the G96D mutation in J3 is responsible for the IBT[d] phenotype and that it serves as an extragenic suppressor of A18-*Cts23.*

Isolation of IBT-dependent mutants. Given that previously characterized mutants of the G2 gene are dependent upon IBT for growth, we attempted to identify additional genes encoding transcription elongation factors by selecting for novel spontaneous IBT-dependent mutants. Wildtype virus was plaqued at 37°C in the absence of drug and 10 well-isolated plaques were picked and grown to create working stocks. This step was performed to ensure that any mutants derived from these wt stocks would not be related to one another. The 10 wt lysates were then titered in the presence and in the absence of IBT at 37°C. In each of the 10 wt stocks, several spontaneous mutant viruses that formed plaques in the presence of IBT were observed. For each of the 10 original wt stocks, 10 plaques that grew in the presence of drug were picked, giving a total of 100 viruses capable of growing in the presence of IBT. Next, these 100 viruses were plaqued in the presence and in the absence of drug to determine whether they were resistant to or dependent upon IBT for growth. At least one IBT-dependent

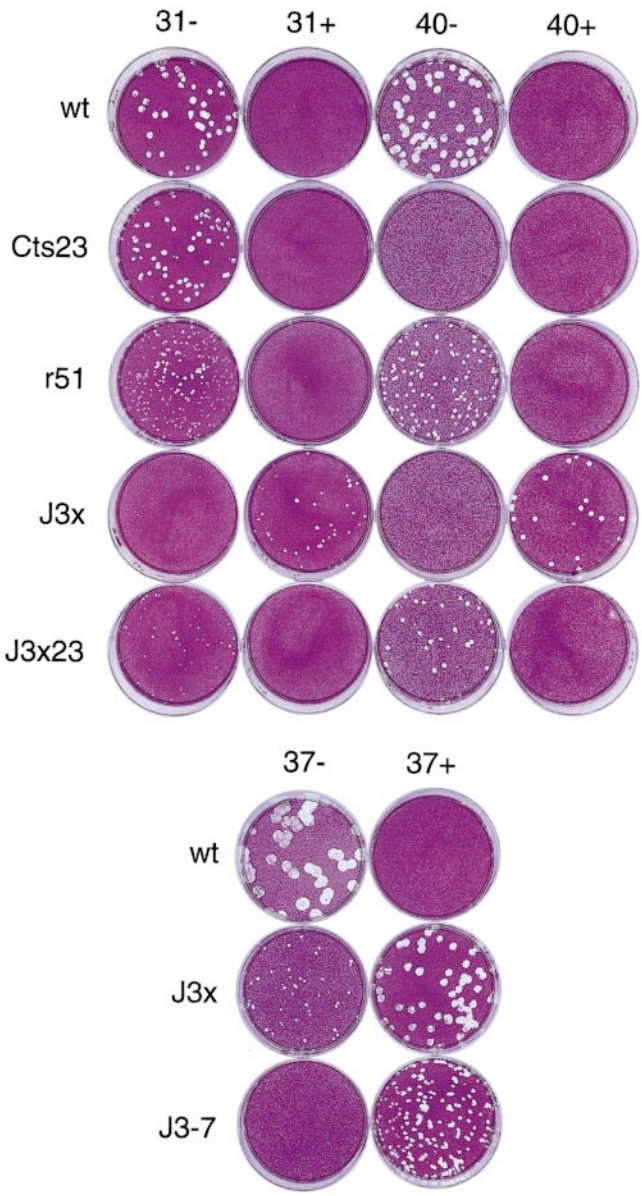


FIG. 4. Plaque phenotype of mutant viruses. Confluent monolayers of BSC40 cells in 60-mm dishes were infected with an appropriate dilution of virus and incubated in the presence or in the absence of IBT under an agar overlay for 6 days. Dishes were stained overnight with a second neutral red containing agar overlay. Agar was then removed and cells were stained with crystal violet. The mutant used for infection is indicated at the left of each row. Each column is labeled at the top to show the temperature of incubation (31, 37, or 40°C) and whether or not IBT was included (+ or −).

virus isolate was identified from 9 of the 10 original wt virus stocks. One IBT-dependent plaque arising from each of these 9 wt stocks was chosen for in-depth study. The mutation in each IBT-dependent virus was mapped by marker rescue in a fashion similar to *J3x* and the region containing the mutation was sequenced. The results, summarized in Tables 2 and 3, show that two of the mutants contain deletions in gene G2 and seven of the

TABLE 2

G2 IBT[d] Mutants

Isolate	Mutation[a]	Protein[b]
Wildtype	wt	wt; 220 aa
G2-5	Deletion (3)	V105Δ; 219 aa
G2-2	Deletion (1)	*fs* 209; 213 aa

[a] The number of nucleotides affected by each mutation is included in parentheses.

[b] *"fs-#"* indicates the codon within which a frameshift occurs. "Δ" indicates deletion.

mutants contain alterations in gene J3. *G2-5* contains a 3-nucleotide in-frame deletion that removes a valine from position 105 of the G2 gene. *G2-2* contains a single base deletion that results in a frameshift at codon 209 and a premature termination, yielding a truncated polypeptide 213 amino acids in length. All of the IBT-dependent J3 mutants contain insertions, deletions, or nonsense mutations that result in truncation of the protein significantly from its wt length of 333 amino acids. Interestingly, each of the J3 insertion and deletion mutations is located in a region of the coding sequence that contains a long stretch of A or T residues (Fig. 2). Presumably, these hot spots for deletion and insertion arise from slipped mispairing during DNA replication (Streisinger *et al.,* 1966). One of the J3 mutant viruses, *J3-7,* was chosen for further characterization. *J3-7* contains a single base deletion at codon 49, resulting in a frameshift and a truncation of the protein to 58 amino acids in length. In summary, these results confirm that, like G2, null mutation of gene J3 results in IBT dependence and by analogy with G2 indicates that the J3 gene plays a role in the regulation of postreplicative transcription elongation during vaccinia virus infection.

TABLE 3

J3 IBT[d] Mutants

Isolate	Mutation[a]	Protein[b]
Wildtype	wt	wt; 333aa
J3-1	Insertion (1)	*fs* 50; 50aa
J3-3	Insertion (1)	*fs* 50; 50aa
J3-4	Nonsense	W183*; 182aa
J3-7	Deletion (1)	*fs* 49; 58aa
J3-8	Insertion (1)	*fs* 34; 50aa
J3-9	Nonsense	S5*; 4aa
J3-10	Deletion (1)	*fs* 235; 246aa

[a] The number of nucleotides affected by each mutation is included in parentheses.

[b] *"fs-#"* indicates the codon within which a frameshift occurs.

* Indicates a stop codon.

Isolate		Sequence Context
wt	135	AGAATTATTTTTT.CTTAGTAA
J3-1	135	AGAATTATTTTTTTCTTAGTAA
J3-3	135	AGAATTATTTTTTTCTTAGTAA
J3-7	135	AGAATTATTTTT..CTTAGTAA
wt	87	GGTCGCAAAAAAA.CTGCCGTA
J3-8	87	GGTCGCAAAAAAAACTGCCGTA
wt	689	TAAATTATGAAAAAAAGATGTA
J3-10	689	TAAATTATGAAAAAA.GATGTA

FIG. 2. Sequence context of J3 deletion and insertion mutations. Sequence of J3 insertion and deletion mutants from three different regions of the J3 gene are compared in each case to wt sequence. The number to the left of the sequence is the number of the first nucleotide in the sequence, setting the A in the J3 ATG initiation condon as +1. Dots have been inserted into either wt or mutant sequence to facilitate alignment as necessary.

Analysis of mutants

Western blot analysis of A18, J3, and G2 proteins in mutant infections. Western blot analysis was performed to confirm predictions derived from sequence data regarding expression of the A18, G2, and J3 proteins in each mutant virus infection (Fig. 3). Infected cell lysates were electrophoresed through 8% SDS polyacrylamide gels and transferred to nitrocellulose. The blots were simultaneously probed with anti-A18, anti-G2, and anti-J3 antibodies and developed by chemiluminescence. All three proteins are readily apparent in wildtype virus lysates. *Cts23* makes all three proteins, but the relative amount of A18 protein is reduced, consistent with previous results (Simpson and Condit, 1994). The *G2A* control virus encodes a frameshift in the G2 gene and, as previously described (Black *et al.,* 1998), fails to produce G2 protein. The *J3x* missense mutant produces detectable J3 protein while the other IBT-dependent J3 truncation mutants, *J3-1, J3-3, J3-4, J3-7, J3-8, J3-9,* and *J3-10,* produce no observable J3 protein. No G2 protein was observed in *G2-2,* which is missing a valine from position 105, or in *G2-5,* which has a frameshift truncation of the C-terminal 11 amino acids of the G2 protein, indicating that these mutant proteins are unstable. A18, J3, and G2 proteins are all detectable in the *J3x23* double-mutant but at very low levels, presumably because this particular virus grows poorly and is therefore present in the lysate at a low concentration. In addition, the relative amount of A18 protein in the *J3x23* lysate is low, consistent with the presence of the *Cts23* mutation in this virus. In summary, the results of the Western blot analysis are consistent with the DNA sequence analysis of these viruses, showing that the J3 protein of the *J3x* mutant is stable and that the new IBT[d] J3 and G2 mutants produce no detectable J3 or G2 protein.

Plaque assay. Plaque phenotypes of the *J3x, J3x23,* and *J3-7* viruses are compared to parental viruses in the assay shown in Fig. 4. Wildtype virus forms large plaques at 37°C and intermediate-sized plaques at 31 and 40°C and is IBT sensitive. *Cts23* is both temperature sensitive and IBT sensitive. *r51,* the phenotypic revertant of *Cts23* and parent of *J3x,* is ts+ and IBT sensitive, but forms smaller than wt plaques at both 31 and 40°C. *J3x* is IBT dependent and phenotypically identical to its parent *r51x4* (data not shown), confirming that the downstream J3 R327K mutation in *r51x4* does not contribute to the *J3x* mutant plaque phenotype. *J3x* forms larger plaques at 37°C relative to 31 and 40°C, but is somewhat leaky with respect to IBT dependence at both 31 and 37°C. *J3x23* is phenotypically identical to *r51,* confirming that the J3 G96D mutation in *J3x* is sufficient to suppress *Cts23* and that the downstream J3 R327K mutation in *r51* does not contribute to the plaque phenotype of *r51. J3-7,* which contains a frameshifting deletion of the J3 gene, has a noticeably different phenotype when compared to the point mutant *J3x* virus. *J3-7* forms smaller plaques than *J3x* at 37°C and is tighter with respect to IBT dependence at 37°C. Since *J3-7* is likely to be a null mutant, this observation suggests that the *J3x* virus may retain some J3 activity that contributes to its growth phenotype.

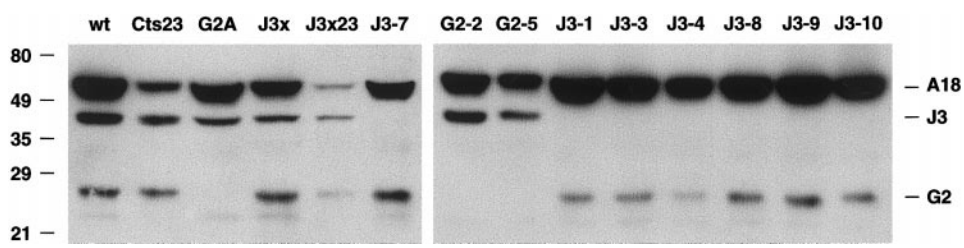
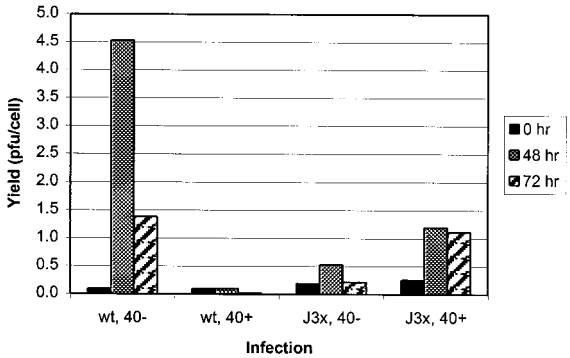


FIG. 3. Western blot analysis of A18, J3, and G2 proteins synthesized during wt and mutant infections. Infected cell lysates were electrophoresed on SDS–PAGE, proteins were transferred to membranes, and membranes were probed simultaneously with anti-A18, anti-J3, and anti-G2 sera and developed by chemiluminescence as described under Materials and Methods. The migration of molecular mass standards, in kDa, is shown at the left. The identity of the mutant virus used for infection is shown at the top. The migration of the specific protein detected is shown at the right.

A

J3x

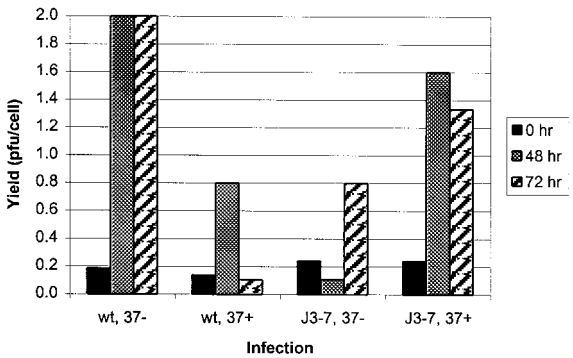
Infection	Yield (pfu/cell) 0 hr	48 hr	72 hr
wt, 40-	0.10	4.53	1.39
wt, 40+	0.09	0.10	0.02
J3x, 40-	0.19	0.53	0.22
J3x, 40+	0.26	1.20	1.12



B

J3-7

Infection	Yield (pfu/cell) 0 hr	48 hr	72 hr
wt, 37-	0.19	184.00	186.67
wt, 37+	0.14	0.80	0.11
J3-7, 37-	0.24	0.11	0.80
J3-7, 37+	0.24	1.60	1.33



C

J3x23

Infection	Yield (pfu/cell) 0 hr	48 hr	72 hr
wt, 31-	0.15	8.00	12.53
wt, 40-	0.10	4.53	1.39
Cts23, 31-	0.27	8.00	4.00
Cts23, 40-	0.27	0.35	0.05
J3x23, 31-	0.05	0.67	0.88
J3x23, 40-	0.04	0.43	0.80

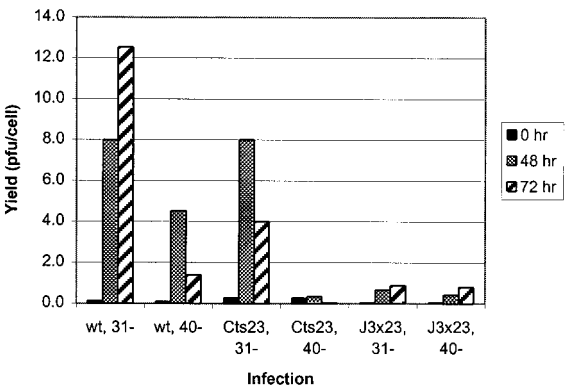


FIG. 5. One-step growth analysis of J3 mutant viruses. Confluent monolayers of BSC40 cells in 35-mm dishes were infected with the viruses indicated at a m.o.i. of 10 for wt, *Cts23, J3x,* and *J3-7* or at a m.o.i. of 5 for *J3x23.* Infections were incubated in liquid medium under varying conditions of temperature (31, 37, or 40°C) in the presence or in the absence of IBT (+ or −) and harvested at 0, 48, and 72 h, as indicated. Lysates were harvested and plaque titrated under permissive conditions appropriate for each virus: wt, 37°C −IBT; *Cts23,* 31°C −IBT, *J3x,* 40°C + IBT; *J3-7,* 37°C + IBT; *J3x23,* 40°C −IBT. In each panel, numerical data are presented at the left, and bar graphs of the same data are shown on the right. (A) *J3x* is compared to wt. (B) *J3-7* is compared to wt. Note in the bar graph in B that the wt data have been truncated at 2 PFU/cell to aid in visualization of the mutant data. (C) *J3x23* is compared to wt and *Cts23.*

One-step growth. To characterize the growth phenotypes of each virus in more detail, one-step growth experiments were performed. BSC40 cells were infected at an m.o.i. of 10 with wt, *Cts23,* or *J3-7* or at an m.o.i. of 5 with *J3x23.* The infections were incubated under permissive and nonpermissive conditions appropriate for each virus, harvested at various times postinfection, and then titered under permissive conditions appropriate for each virus (Fig. 5). Wildtype virus growth was compared to *J3x* growth at 40°C in the presence and in the absence of IBT (Fig. 5A). At 40°C, wildtype virus yielded 1–5 PFU/cell, significantly higher than the T_0 background, and growth was inhibited by IBT. *J3x* produced only background levels of progeny in the absence of IBT and approximately 1 PFU/cell in the presence of drug, slightly higher than background. This result confirms and extends the plaque assay, showing that *J3x* is IBT dependent and difficult to grow to high titer. *J3-7* growth was compared to wildtype at 37°C in the presence and in the absence of IBT (Fig. 5B). Wildtype grew to very high titer in the absence of drug, and growth was inhibited by IBT. *J3-7* yielded less than 1 PFU/cell at 37°C in the absence of drug and slightly greater than 1 PFU/cell in the presence of IBT, indicating that under one-step growth conditions

J3-7 is moderately IBT dependent and confirming that, like *J3x,* it is also difficult to grow. The growth of *J3x23* was compared to wt and *Cts23* at both 31 and 40°C in the absence of IBT. Both wt and *Cts23* produced 4–10 PFU/cell at 31°C, but *Cts23* produced only background levels of virus at 40°C, consistent with plaque assay results. *J3x23* generated 0.5–1 PFU/cell at both temperatures, which is higher than background, but less than wildtype. These results confirm that the *J3x* mutation suppresses *Cts23* and show that *J3x23* grows very poorly. In summary, these experiments show that the one-step growth properties of all three mutant viruses are generally consistent with the plaque assay phenotype and that the mutant viruses all grow poorly under permissive conditions.

DISCUSSION

Based on previous phenotypic analysis of mutants in the vaccinia virus G2 postreplicative positive transcription elongation factor, we have used two independent genetic selections to search for additional vaccinia genes involved in the regulation of viral transcription elongation. In one selection, we isolated nine viruses containing spontaneous mutations that render the viruses dependent upon the anti-poxviral drug IBT for growth. Consistent with previous results (Meis and Condit, 1991), two of these mutants have defects in the G2 elongation factor. Mutations in the other seven IBT[d] selected viruses mapped to the J3 gene. In the second selection an additional J3 gene mutant, *J3x,* was isolated as an extragenic suppressor of the *Cts23* mutation in the A18 transcript release factor. By analogy with previous characterization of the G2 gene (see Introduction), these results suggest that the J3 gene is a postreplicative positive transcription elongation factor.

Interestingly, the vaccinia J3 gene product has been previously characterized as a bifunctional protein containing both poly(A) polymerase stimulatory and (nucleoside-2′-*O*-)-methyltransferase activities (Schnierle *et al.,* 1992). The vaccinia virus coded poly(A) polymerase is a heterodimer, comprising the 55-kDa E1 protein complexed with the 39-kDa J3 protein (Gershon *et al.,* 1991). The E1 subunit contains the adenylyltransferase active site, and E1 by itself can processively add only 30–35 adenylate residues to a nascent mRNA 3′ end (Gershon and Moss, 1993). The J3 protein binds both the E1 protein and poly(A) and stimulates the E1 protein to add additional adenylate residues in a semiprocessive fashion to build poly(A) tails averaging approximately 150 nt in length (Gershon and Moss, 1992). The J3 (nucleoside-2′-*O*-)-methyltransferase activity methylates the ribose of the first transcribed base of the 5′ cap structure on messenger RNAs, converting a cap-0 structure to a cap-1 structure (Barbosa and Moss, 1978a,b). During infection, the J3 protein is expressed in fivefold molar excess relative to the E1 protein, and the (nucleoside-2′-*O*-)-methyltransferase activity is present in both the monomeric and the heterodimeric forms of the protein (Gershon *et al.,* 1991; and E. Niles personal communication). The results reported here suggest a third activity for the J3 protein, specifically a positive postreplicative transcription elongation factor activity, raising the interesting question of whether either of the previously characterized J3 protein functions is related to the putative transcription elongation function.

The nature of the newly isolated mutations in the J3 gene provides clues to the relationship between the putative J3 elongation factor activity and the previously described poly(A) polymerase stimulatory and (nucleoside-2′-*O*-)-methyltransferase activities. Both DNA sequence and Western blot analyses of the seven new J3 mutants selected initially for IBT dependence show that these are null mutants: each contains a chain-terminating nonsense or frameshift mutation, and none synthesizes detectable J3 protein. Thus all of these seven mutants should be lacking in both poly(A) polymerase stimulatory and (nucleoside-2′-*O*-)-methyltransferase activities. By contrast, *J3x,* which was initially isolated as a *Cts23* suppressor but which is also IBT dependent, contains a missense mutation (G96D) that does not affect the steady state levels of J3 protein produced during infection. Under permissive conditions *J3x* forms larger plaques than a representative J3 null mutation (*J3-7*), suggesting that the *J3x* mutant may retain some J3 protein function. Interestingly, the published crystal structure of J3 shows that the *J3x* G96D missense mutation is localized very near the methyltransferase active site of the J3 protein, between a highly conserved aspartic acid at position 95 and an arginine at position 97, both of which form hydrogen bonds with the methyl donor *S*-adenosylmethionine (Hodel *et al.,* 1996). Thus if the *J3x* protein is missing either of the previously identified J3 activities, it seems most likely that it would be defective in methyltransferase and retain poly(A) polymerase stimulatory activity. This hypothesis is tested in the accompanying article (Xiang *et al.,* 2000).

The two different selections described, IBT dependence and *Cts23* suppression, may discriminate the two different types of J3 mutants isolated. Specifically, selection for IBT dependence resulted in isolation entirely of null J3 mutants, while selection of *Cts23* resulted in isolation of the more subtle *J3x* missense mutation, which, as described above, may retain some J3 protein activity. It is noteworthy in this regard that while mutation of J3 suppresses *Cts23* as judged by plaque formation, the resulting double-mutant virus grows extremely poorly, as judged by one-step growth. Since J3 null mutants are more defective in growth than *J3x,* it seems likely that null mutation of *J3,* while it may compensate for the effects of *Cts23,* would leave a double-mutant virus too crippled to grow at all, thus prohibiting isolation

of J3 null mutant suppressors of *Cts23.* Consistent with this idea, we have been unsuccessful in several attempts to construct a *J3-7, Cts23* double-mutation by recombination.

By contrast with the J3 gene, our results show that null mutants of the G2 gene *can* suppress ts mutants in A18, including both *Cts23* and *Cts22.* Specifically, previous results show that a frameshifting deletion in G2 can suppress *Cts23* (Condit *et al.,* 1996). We have also performed Western blot analysis on three previously isolated G2 point mutant suppressors of *Cts23* and *Cts22* (*r41, cs1,* and *cs4*) and determined that none of these mutants produce detectable G2 protein (D. Latner, unpublished observations). These observations could suggest that the G2 gene has a more limited range of function than the J3 gene, such that a knockout of the G2 function can be tolerated in the presence of an A18 gene mutation.

In summary, based on the genetic analysis reported here, we hypothesize that the J3 gene product, a bifunctional (nucleoside-2′-*O*-)-methyltransferase and poly(A) polymerase processivity factor, is also a positive postreplicative transcription elongation factor. Phenotypic analysis of J3 mutant viruses and biochemical analysis of the *J3x* protein presented in the accompanying article (Xiang *et al.,* 2000) provides support for this hypothesis.

MATERIALS AND METHODS

Cells and virus

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 *et al.,* 1983; Condit and Motyczka, 1981). The wildtype vaccinia virus strain WR, the A18 mutant *Cts23,* and the G2 mutant *G2A* have been previously described (Condit *et al.,* 1983; Condit and Motyczka, 1981; Meis and Condit, 1991). In short, *Cts23* was isolated as a temperature-sensitive virus that arose from random nitrosoguanidine mutagenesis of wildtype virus. *G2A* contains an engineered 10-bp deletion in gene G2, resulting in a frameshift at codon 90 and truncation of the 220-amino-acid protein at position 93. IBT was prepared fresh before each use and applied at a final concentration of 45 μM as previously described (Pacha and Condit, 1985).

DNA sequence analysis

Sequence of mutant virus DNA was obtained by sequencing PCR products amplified from genomic DNA that was isolated by one of two different methods: (1) DNA was isolated as described except the amounts were scaled down 20-fold (Esposito *et al.,* 1981). (2) Qiagen DNeasy miniprep spin columns (Qiagen Inc., Santa Clarita, CA) were utilized to purify total infected cell DNA from 200 μl of infected cell lysate following the manufacturer's instructions for isolating DNA from cells in culture.

The complete J3 coding sequence was PCR-amplified with two primers that hybridize just outside of the open reading frame and yield a 1197-bp product. Sequence was obtained from both strands of DNA with the same primers used for amplification and with two additional primers that hybridize in the middle of the coding sequence to give overlapping products. Sequencing was performed by the University of Florida Interdisciplinary Center for Biotechnology Research DNA Sequencing Core Laboratory.

RFLP analysis

The screen for the *J3x* single-mutant virus from the *r51x4* test-cross was performed by restriction fragment length polymorphism analysis of two different PCR products generated from virus genomic DNA. Candidate mutant plaques were picked and grown, and viral genomic DNA was prepared as described above. A 379-bp PCR product that spanned the G96D upstream mutation was amplified with appropriate primers and then digested with *Eag*I. A downstream 522-bp PCR product that spanned the mutation in the J3/J4 overlapping region (J3:R327K/J4:D22N) was amplified with appropriate primers and then digested with *Bst*YI. Restriction fragments were analyzed on 1% MetaPhor agarose (FMC BioProducts, Rockland, ME) in TBE. Both restriction enzymes cleave wildtype sequence but not mutant sequence. A mutant virus was identified that was resistant to *Eag*I in the 379-bp upstream PCR product but sensitive to *Bst*YI in the downstream 522-bp PCR product. Subsequent sequence analysis of the entire J3 open reading frame confirmed the presence of the upstream mutation and absence of the downstream mutation. The single-mutant virus was designated *J3x* and was utilized for further characterization.

DNA clones and marker rescue

Marker rescue was performed as previously described (Meis and Condit, 1991; Thompson and Condit, 1986). The cosmid clones used for initial marker rescue experiments have been previously characterized (Thompson and Condit, 1986). The J fragment subclones pJ5, pJ6, and pJ7 (Fig. 1) were generously supplied by Jerry Weir and have been described (Ensinger *et al.,* 1985). The clone pJ3R, which contains the precise J3 open reading frame cloned in pET14b, was a gift from Ed Niles (SUNY Buffalo).

Western blot analysis

One hundred twenty-five microliters of virus lysate was combined with 250 μl of SDS–PAGE sample buffer. Thirty-five microliters of each sample was electrophoresed on an 8% polyacrylamide–SDS gel and then the proteins were transferred from the gel to nitrocellulose in 25 mM Tris base, 192 mM glycine, 20% methanol for 3 h at 4°C and 80 V. The membrane was blocked with 5% dry milk in PBS for 1.5 h, washed three times for 5 min with 0.05% NP-40 in PBS, and incubated for 1 h with anti-A18, anti-G2, and anti-J3 primary antibodies diluted 1:10,000, 1:1000, and 1:5000, respectively, in PBS plus 5% dry milk. The monoclonal anti-A18 and anti-G2 antibodies have been previously described (Black and Condit, 1996). The rabbit polyclonal anti-J3 antibody was a gift from Ed Niles (SUNY Buffalo). The blot was then washed three times for 5 min with 0.05% NP-40 in PBS and incubated with secondary HRP-conjugated anti-rabbit and anti-mouse antibodies diluted 1:10,000 and 1:5000, respectively, for 1 h. The blot was washed three times for 10 min with PBS plus 0.05% NP-40 and once for 2 min in PBS. Final detection was performed with an ECL Western detection kit (Amersham Pharmacia) according to the manufacturer's instructions.

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REFERENCES

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

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

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.

Black, E. P., Moussatche, N., and Condit, R. C. (1998). Characterization of the interactions among vaccinia virus transcription factors G2R, A18R, and H5R. *Virology* **245,** 313–322.

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.

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.

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.

Ensinger, M. J., Weir, J. P., and Moss, B. (1985). Fine structure marker rescue of temperature-sensitive mutations of vaccinia virus within a central conserved region of the genome. *J. Virol.* **56,** 1027–1029.

Esposito, J., Condit, R., and Obijeski, J. (1981). The preparation of orthopoxvirus DNA. *J. Virol. Methods* **2,** 175–179.

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). Uridylate-containing RNA sequences determine specificity for binding and polyadenylation by the catalytic subunit of vaccinia virus poly(A) polymerase. *EMBO J.* **12,** 4705–4714.

Hodel, A. E., Gershon, P. D., Shi, X., and Quiocho, F. A. (1996). The 1.85 Å structure of vaccinia protein VP39: A bifunctional enzyme that participates in the modification of both mRNA ends. *Cell* **85,** 247–256.

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.

Mahr, A., and Roberts, B. E. (1984). Arrangement of late RNAs transcribed from a 7.1-kilobase EcoRI 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-β-thiosemicarbazone (IBT). *Virology* **182,** 442–454.

Moss, B. (1996). *Poxiviridae:* The viruses and their replication. *In* "Fundamental Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), pp. 1163–1197. Lippincott–Raven, 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.

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.

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.

Simpson, D. A., and Condit, R. C. (1994). The vaccinia virus A18R protein plays a role in viral transcription during both the early and the late phases of infection. *J. Virol.* **68,** 3642–3649.

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

Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E., and Inouye, M. (1966). Frameshift mutations and the genetic code. *Cold Spring Harbor Symp. Quant. Biol.* **31,** 77–84.

Thompson, C. L., and Condit, R. C. (1986). Marker rescue mapping of

vaccinia virus temperature-sensitive mutants using overlapping cosmid clones representing the entire virus genome. *Virology* **150,** 10–20.

Thompson, C. L., Hooda-Dhingra, U., and Condit, R. C. (1989). Fine structure mapping of five temperature-sensitive mutants in the 22- and 147-kilodalton subunits of vaccinia virus DNA-dependent RNA polymerase. *J. Virol.* **63,** 705–713.

Traktman, P. (1990). Poxviruses: An emerging portrait of biological strategy. *Cell* **62,** 621–626.

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.