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Mutation of Vaccinia Virus Gene G2R Causes Suppression of Gene A18R ts Mutants: Implications for Control of Transcription

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This report provides genetic evidence that two vaccinia virus genes, A18R and G2R, both of which affect the fidelity of viral transcription *in vivo,* interact with each other or act on a common biochemical pathway. Previous experiments with the antipoxviral drug isatin-β-thiosemicarbazone suggest that lethal mutation of gene G2R would compensate for mutations in gene A18R. We therefore tested the hypothesis that gene G2R is an extragenic suppressor of A18R mutations. First, we constructed a recombinant which contains both a G2R deletion mutation and an A18R temperature-sensitive mutation and found that this recombinant was viable. Second, we isolated both cold-sensitive and temperature-insensitive phenotypic revertants of A18R temperature-sensitive mutants and found in both cases that the revertants contained G2R mutations. In the case of the cold-sensitive revertants, we were able to prove that the cold-sensitive phenotype mapped to the G2R gene. Combined with the biochemical data on A18R and G2R, these results imply that the A18R and G2R genes interact with each other either directly or indirectly in a fashion which affects the fidelity of intermediate and late viral transcription.

INTRODUCTION

Vaccinia virus, the prototypical orthopoxvirus, possesses a 200-kb double-stranded DNA genome which encodes approximately 200 genes. The virus replicates entirely in the cytoplasm of infected cells and therefore encodes most of the functions required for viral transcription (Moss, 1990b). Vaccinia genes are transcribed by a virus-coded, multisubunit, core RNA polymerase in a cascade comprising three gene classes, early, intermediate, and late. Gene expression is regulated primarily by the ordered synthesis of class-specific viral transcription initiation factors (Moss, 1990a; Moss *et al.,* 1991; Baldick and Moss, 1993).

Termination of early viral transcription differs significantly from termination of intermediate or late transcription. Early transcription termination is catalyzed by a virus-coded termination factor in response to a specific *cis* acting sequence present in the transcribed RNA (Yuen and Moss, 1987; Shuman *et al.,* 1987). Because they have discrete 5′ and 3′ ends, early transcripts are relatively homogeneous in size. By contrast, transcription which is initiated at intermediate or late promoters reads through early transcription termination signals and results in synthesis of a family of transcripts approximately 1 to 4 kb in size possessing homogeneous 5′ ends and extremely heterogeneous 3′ ends (Mahr and Roberts, 1984). Relatively little is known about the mechanism of termination of intermediate or late transcription.

Genetic analysis of vaccinia has identified three genes which affect the fidelity of viral transcription *in vivo,* but whose protein products are apparently not among the minimal set of factors required for specific transcription of vaccinia genes *in vitro* (Bayliss and Condit, 1993; Black and Condit, 1995; Kunzi and Traktman, 1989). This report provides genetic evidence that two of these genes, A18R and G2R, interact with each other or act on a common biochemical pathway.

The A18R gene encodes a 56-kDa protein that possesses both DNA-dependent ATPase and DNA helicase activities (Bayliss and Condit, 1995; Simpson and Condit, 1995). The A18R protein is synthesized both early and late during infection and is packaged in virions (Simpson and Condit, 1994). While *in vitro* analysis of A18R mutant virions suggests that the A18R protein plays a role in early virion-directed transcription (Simpson and Condit, 1994), *in vivo* the phenotype of A18R mutants is manifested at intermediate and late times, following DNA replication. The A18R mutant phenotype *in vivo* is characterized by "promiscuous transcription," that is, transcription late during infection of regions of DNA that are normally transcribed only early during infection (Bayliss and Condit, 1993). The mechanism of promiscuous transcription is currently unknown, but could theoretically represent random, nonspecific transcription initiation, reactivation of early promoters late during infection, or readthrough transcription from upstream intermediate or late promoters.

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The G2R gene encodes a 26-kDa protein which has no recognizable functional motifs, no significant homology with other known proteins, and whose biochemical activity is so far uncharacterized. The G2R gene is transcribed only early during viral infection (Meis and Condit, 1991). During infection with G2R mutant viruses, early viral transcription appears normal, intermediate and late viral promoters appear to function normally, but intermediate and late viral mRNAs are reduced in size, truncated from the mRNA 3′ ends (Black and Condit, 1995). These results suggest that the G2R protein acts to regulate the elongation potential of the viral RNA polymerase late during a vaccinia virus infection.

Experiments with the antipoxviral drug isatin-β-thiosemicarbazone (IBT) suggest a relationship between the A18R and G2R genes. Treatment of wild-type virus infections with IBT results in a phenotype which is indistinguishable from A18R mutant infections, characterized by promiscuous transcription (Bayliss and Condit, 1993; Pacha and Condit, 1985), suggesting that IBT may directly or indirectly to compromise A18R function. One IBT-resistant mutant of vaccinia has been mapped to the second largest subunit of the viral RNA polymerase, supporting the perspective that the primary target of IBT is the viral transcription apparatus (Condit *et al.,* 1991). Interestingly, lethal mutation of the G2R gene results in IBT dependence (Meis and Condit, 1991). Specifically, deletions of G2R grow only in the presence of IBT, and growth of temperature-sensitive G2R mutants at the nonpermissive temperature is rescued by IBT. Since IBT treatment of wild-type infections induces promiscuous transcription, these observations suggest that lethal mutation of the G2R gene can counteract or compensate for the effects of promiscuous transcription. Since mutation of the A18R gene also causes promiscuous transcription, we hypothesize that lethal mutation of gene G2R should compensate for mutations in gene A18R.

In this report, we demonstrate that gene G2R is an extragenic suppressor of A18R mutations. Combined with the biochemical data on A18R and G2R, our results imply that the A18R and G2R genes interact with each other either directly or indirectly in a fashion which affects the fidelity of intermediate and late viral transcription. We propose a model in which the A18R and G2R proteins interact with each other and with the RNA polymerase to modulate elongation and termination of intermediate and late viral transcription.

MATERIALS AND METHODS

Cells and virus

The continuous African green monkey kidney cell line BSC40 and conditions for cell culture have been previously described (Condit and Motyczka, 1981; Condit *et al.,* 1983). Wild-type vaccinia virus strain WR, gene A18R ts mutants Cts23 and Cts22, and gene G2R deletion mutant G2A have been previously described (Condit and Motyczka, 1981; Condit *et al.,* 1983; Meis and Condit, 1991; Pacha *et al.,* 1990). Briefly, Cts22 and Cts23 are temperature-sensitive mutants of vaccinia virus strain WR that were isolated by random nitrosoguanidine mutagenesis of wild-type virus. Cts22 and Cts23 are noncomplementing, they have both been mapped to the A18 gene by marker rescue, and they have identical biochemical phenotypes *in vivo.* The gene G2R mutant G2A is an engineered 10-bp deletion mutant which has its 5′ endpoint in codon 90 of the 220-codon gene. The mutation causes a frameshift starting at codon 90 and premature termination at codon 93 of the mutant sequence. Conditions for virus growth, infection, and plaque assay have been previously described (Condit and Motyczka, 1981; Condit *et al.,* 1983). Stock solutions of IBT were made fresh for each use as previously described, and IBT was routinely used at a final concentration of 45 μM (Pacha and Condit, 1985).

Marker rescue

Marker rescue was done as previously described (Thompson and Condit, 1986), except that DNA was transfected into infected cells using lipofectin (Life Technologies, Gaithersberg MD) following the manufacturer's instructions.

DNA sequence analysis

DNA sequence of A18R and G2R mutations was obtained by direct sequencing of PCR products amplified from genomic viral DNA. Genomic viral DNA was obtained by any of three methods: (1) Large-scale preparations of viral DNA were purified from viral cores isolated from cytoplasmic extracts of infected cells as previously described (Esposito *et al.,* 1981; Condit *et al.,* 1983). (2) Small-scale preparations of total cytoplasmic nucleic acid containing viral DNA were made using a modification of the large-scale procedure as follows. A single confluent 60-mm dish of BSC40 cells was infected with virus at a m.o.i. of approximately 1 and incubated under appropriate conditions until a complete cytopathic effect was observed. Cells were harvested, washed with isotonic buffer, resuspended in 250 μl of hypotonic buffer, and frozen and thawed once (Esposito *et al.,* 1981). Cells were lysed by addition of 25 μl of 10% Triton X-100 and 1 μl β-mercaptoethanol and incubation on ice for 60 min. Nuclei were removed by low-speed centrifugation, and the cytoplasmic supernatant was removed, adjusted to 1% SDS, 100 μg/ml proteinase K, 65 m*M* NaCl, and incubated at 45°C for 6 hr. Nucleic acids were then purified from the cytoplasmic extract by phenol extraction and ethanol precipitation. The final pellet was resuspended in 50 μl TE, and 1 μl was used in PCR reactions. (3) Infected cell lysates prepared by standard methods (Con-

dit and Motyczka, 1981; Condit *et al.,* 1983) were used directly for PCR. Lysates contain 1×10^7 infected cells per milliliter in PBS and have been frozen and thawed at least twice. Five microliters of lysate was added to 95 μl of TE, boiled for 5 min, quick cooled, and 70 μl of this preparation was used as a source of template in a 100-μl PCR reaction.

For DNA sequence analysis, the 1482-nt A18R coding sequence was amplified using five different primer pairs which produced overlapping double-stranded PCR products ranging in size from 394 to 533 bp. The 663-nt G2R coding sequence was amplified using three different primer pairs which produced overlapping double-stranded PCR products ranging in size from 337 to 389 bp. In each case, the upstream primer contained at the 5′ end the 18-nt M13 reverse universal primer sequence and was 5′ biotinylated. DNA products from 100-μl PCR reactions were purified using Wizard PCR preps (Promega Corp., Madison WI) columns and eluted in a final volume of 50 μl water. Strand separation and DNA sequence analysis were done by the University of Florida BEECS Genetic Analysis Core and the ICBR DNA Sequencing Core Laboratory. Briefly, the PCR products were mixed with streptavidin-conjugated paramagnetic beads. The beads were retained with a magnet and then the nonbiotinylated strand was released by treatment with alkali while biotinylated DNA was retained on the beads. This procedure yields alkali-released single-stranded template DNA which has at its 3′ end the complement of the M13 reverse universal primer sequence. DNA was sequenced using dye-labeled primers and an Applied Biosystems Model 373A DNA sequencer. Mutations were confirmed and ambiguities were resolved by re-sequencing the same strand or by sequencing isolated portions of the complementary strand using a double-stranded PCR product as DNA template, appropriate DNA sequencing primers, and dye-terminated DNA sequencing reactions.

Oligonucleotide primers were synthesized by the University of Florida ICBR DNA Core Laboratory.

DNA clones

Cosmid clones of wt vaccinia virus (Thompson and Condit, 1986) and the wt *Hin*dIII G fragment clone (Belle Isle *et al.,* 1981) have been described previously. pgG2Rap contains the precise coding region of wild-type vaccinia virus strain WR gene G2R, cloned in the plasmid vector pGEM3ZF−. It was constructed by PCR amplification of the G2R coding sequence from genomic vial DNA using primers which positioned at the 5′ end a synthetic *Nde*I site at the initiating ATG downstream from a synthetic *Xba*I site, and at the 3′ end a synthetic *Bam*HI site immediately following the G2R translation termination codon. The PCR-amplified G2R DNA was trimmed with *Xba*I and *Bam*HI and cloned into *Xba*I, *Bam*HI cleaved pGEM3ZF−. The sequence of the insert was confirmed by DNA sequence analysis.

TABLE 1

Plaque Assay of a Cts23, G2A Mixed Infection

Virus infection[a]	Titer[b] 31° −IBT	31° +IBT	40° −IBT	40° +IBT
Cts23	2.0×10^7	1.6×10^{4c}	2.0×10^4	$<1.0 \times 10^2$
G2A	$<1.0 \times 10^2$	2.0×10^5	$<1.0 \times 10^2$	2.0×10^5
Cts23 + G2A	2.0×10^7	2.6×10^6	4.0×10^6	2.0×10^6

[a] Cells were infected at high m.o.i. with the indicated viruses, and lysates were prepared as described under Materials and Methods.

[b] Lysates from each high m.o.i. infection were plaque titrated under the indicated conditions.

[c] These plaques were small and heterogeneous in size and may represent breakthrough of the IBT inhibition at 31° rather than true genetic resistance.

RESULTS

Mutant isolation

Construction of a viable A18R, G2R double mutant. When mutated individually, the A18R and G2R genes of vaccinia virus behave like essential genes. However, based on previous results (see Introduction) we hypothesized that lethal mutation of *both* genes of would yield a viable virus. As an initial test of this hypothesis, we attempted to construct a recombinant between a ts mutant, Cts23, in gene A18R and an IBT-dependent (IBT^d) deletion mutant, G2A, in gene G2R. BSC40 cells were infected with a mixture of Cts23 and G2A at a m.o.i. of 5 for each virus. Control infections were done in which cells were infected with each mutant alone at a m.o.i. of 10. Infections were incubated at 31° for 72 hr in drug-free growth medium and then titered at 31° and 40° in the absence of drug or in the presence of IBT. The plaque assay results from this cross are shown in Table 1. Single infection with Cts23 results in growth of virus which is temperature sensitive and sensitive to IBT. Because the initial conditions of infection are nonpermissive for growth of the IBT^d mutant G2A, single infection with this mutant results in a low yield of virus which forms plaques only in the presence of IBT. Relative to the single infections, the mixed infection results in an increase in the amount of virus which forms plaques at 40° in the absence of IBT or at either temperature in the presence of IBT. The increase in virus titer in the presence of IBT probably represents complementation of G2A by Cts23 in the initial infection. Most of the virus which forms plaques at 40° in the absence of IBT is recombinant. Five plaques were picked from plaque assays incubated at 40° in the absence of IBT and retested by plaque assay

at 31 and 40° + or − IBT. Of these five isolates, two appeared wt in plaque phenotype, one appeared to contain a mixture of Cts23 and G2A viruses, and two formed smaller than wt plaques at both 31 and 40° in the absence of IBT. One of these latter two isolates, named x41, was grown and used for further study.

Isolation of cold sensitive phenotypic revertants of Cts22. Our hypothesis suggests that it should be possible to isolate extragenic suppressors of ts mutants in gene A18R and that some of these mutants should map to gene G2R. We tested this hypothesis by attempting to isolate extragenic suppressors of another gene A18R ts mutant, Cts22. To streamline the isolation, we sought phenotypic revertants of Cts22 which were recognizably different that true wild-type revertants. Knowing that cold sensitivity is a frequent consequence of extragenic suppression in other systems, we sought cold-sensitive (cs) phenotypic revertants of Cts22. Because the stock of Cts22 we used contained no detectable spontaneous revertants, we induced reversion by chemical mutagenesis (Condit and Motyczka, 1981). BSC40 cells were infected with Cts22 at a m.o.i. of 10 and incubated at 31° in growth medium containing 10 μg/ml nitrosoguanidine. At 10 hr postinfection, the medium was replaced with fresh growth medium lacking mutagen. The virus was harvested at 48 hr postinfection. Relative to a control infection lacking mutagen, the mutagenesis reduced the virus yield by 85%, consistent with previous results (Condit and Motyczka, 1981). Mutagenized virus was diluted to a concentration of 1 x 10^6 PFU/ml, seeded on 25 100-mm dishes at a m.o.i. of 0.16, overlayed with nutrient agar, incubated at 40° for 4 days, and stained overnight with neutral red. On Day 5, a total of 12 plaques were observed, all of which were isolated and tested directly for plaque formation at 31 and 40°. Of these 12 plaques, 1 contained no detectable virus, 1 had an ambiguous phenotype, 3 were ts, 5 grew at both temperatures (ts^+), and 2 were cs. Interestingly, all of the ts^+ isolates formed smaller than wt plaques at both temperatures. The cs viruses, named cs1 and cs4, were grown and used for further study.

Isolation of ts^+ phenotypic revertants of Cts23. We also attempted isolation of extragenic suppressors of Cts23. A stock of Cts23 containing approximately 10^{-3} revertants was plated at an appropriate dilution at 40°, and 20 plaques growing at 40° were isolated and tested for plaque formation at 31 and 40° + and − IBT. Two of these isolates contained no detectable virus, 16 were indistinguishable from wt virus, and 2 formed smaller than wt plaques at both temperatures. No cs isolates were detected in this experiment. One of the small-plaque, ts^+ viruses, named r41, was grown and used for further study.

In summary, we have isolated five new putative double mutant viruses for further study. These consist of a Cts23, G2A recombinant named x41, two induced cs revertants of Cts22 named cs1 and cs4, and one spontaneous, small-plaque, ts^+ revertant of Cts23, named r41. The plaque phenotype of all five mutants relative to parental viruses is shown in Fig. 1. It is noteworthy that in addition to the phenotypic characteristics described above, all five of the new mutants are sensitive to IBT at both 31 and 40°. The mutants were evaluated further by test cross analysis with wt virus, marker rescue of the cs mutants, and by DNA sequence analysis, as described below.

Mutant characterization

Test cross analysis with wt virus. We hypothesize that many or all of the new mutants shown in Fig. 1 should contain mutations in gene G2R. Because mutation of gene G2R can result in an IBT^d phenotype, we reasoned that it might be possible to detect the presence of a G2R mutation by test cross analysis of the mutants with wt virus. Specifically, recombination of double mutant virus with wt virus should yield each of the single mutant viruses among the progeny. Because neither wt virus nor any of the double mutants form plaques in the presence of IBT at either 31 or 40°, an IBT^d mutant should be readily detectable among the progeny of a test cross. The ts progeny virus would be much more difficult to detect because it would be present only as a minor fraction of input wt virus that grows at 31°. The test cross analysis was done in two separate experiments, as described below.

A test cross was first done using x41, which is the Cts23, G2A recombinant, and r41, which is the small-plaque, ts^+ revertant of Cts23. BSC40 cells were infected with wt, x41, or r41, either singly at a m.o.i. of 12 or in pairs at a m.o.i. of 6 for each virus. Infected cells were incubated under permissive conditions, 40° in growth medium lacking drug, for 48 hr. Infected cells were harvested and titered at 31 and 40° + and − IBT. Stocks of G2A and Cts23 were titered in parallel as controls for the plaque assay. The results of this test cross are shown in Table 2. Individual viruses all display an appropriate phenotype when assayed for plaque formation at 31 and 40° + and − IBT. Importantly, none of the parental viruses used in the test cross (wt, x41, and r41) form plaques at either temperature in the presence of IBT. By contrast, progeny from the test crosses (wt + x41 and wt + r41) show a dramatic increase in titer at both 31 and 40° in the presence of IBT, indicating the presence of progeny virus which is altered in its sensitivity to IBT. This result supports the hypothesis that x41 contains the G2A mutation, and it suggests that r41 contains a second mutation conferring either IBT dependence or resistance. Individual progeny from the test cross were analyzed further for the presence of both IBT^d and ts viruses. Twenty-four plaques were picked from the 40° + IBT plaque assays of both the x41 and the r41 test crosses and these isolates were tested for plaque formation at 31 and 40° +

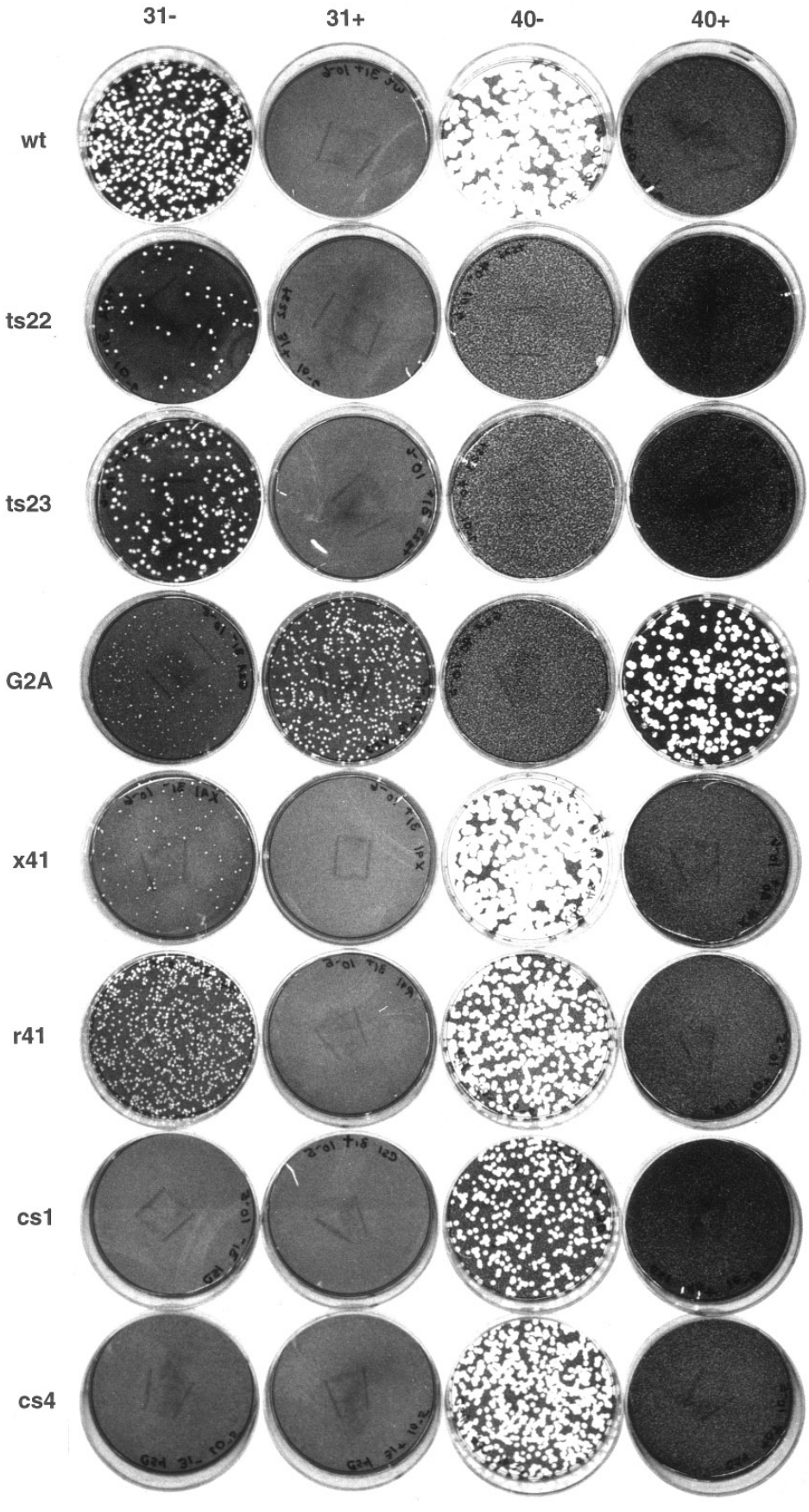

FIG. 1. Plaque phenotypes of A18R and G2R mutant viruses. Confluent monolayers of BSC40 cells were infected in quadruplicate with 50–100 PFU per dish of wt or mutant virus. Infected dishes were incubated at 31 (31) or 40° (40) in the presence (+) or absence (−) of IBT for 1 week under a nutrient agar overlay. Overlays were removed and the cells were stained with crystal violet.

TABLE 2

Test Cross of x41 and r41 with wt Virus

Virus infection[a]	Titer[b]			
	31°		40°	
	−IBT	+IBT	−IBT	+IBT
wt	4.0×10^6	$<1.0 \times 10^2$	4.0×10^6	1.0×10^3
x41	2.0×10^6	$<1.0 \times 10^1$	2.6×10^6	$<1.0 \times 10^1$
r41	1.4×10^6	$<1.0 \times 10^1$	1.2×10^6	$<1.0 \times 10^1$
wt + x41	8.0×10^6	7.0×10^5	7.0×10^6	1.8×10^6
wt + r41	6.0×10^6	5.0×10^5	6.0×10^6	1.0×10^6
G2A	$<1.0 \times 10^3$	1.0×10^7	2.0×10^3	1.5×10^7
Cts23	3.0×10^8	4.0×10^4	3.6×10^5	$<1.0 \times 10^3$

[a] 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.

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

and − IBT, using a spot test (Condit and Motyczka, 1981). In each case, 23 of the 24 isolates formed plaques only in the presence of IBT at both temperatures and thus were IBT^d. Twenty-four plaques were also picked from the 31° − IBT plaque assays of both the x41 and the r41 test crosses and these isolates were also tested for plaque formation at 31 and 40° + and − IBT, using a spot test. Assay of the isolates from the x41 test cross revealed 1 isolate that was ts, 21 that were wt, and 2 that appeared IBT resistant (IBT^r). Assay of the isolates from the r41 test cross revealed 2 isolates that were ts, 19 that were wt, and 1 that appeared IBT^r. These results show that a small fraction of the test cross progeny are ts, as predicted. The presence of a small fraction of IBT^r viruses among these progeny is probably a reflection of the high spontaneous mutation rate to IBT^r. In summary, the results of this test cross support the hypothesis that x41 contains both the G2A and the Cts23 mutations and that r41 contains both the Cts23 mutation and an additional IBT^d mutation.

A second test cross was done with cs1 and cs4, which are the cs revertants of Cts22. The methods used were identical to the methods used for the test cross described above, and the results are shown in Table 3. As was observed in the first x41 and r41 test crosses, mixed infection of cs1 or cs4 with wt virus results in a dramatic increase among the progeny in virus which forms plaques in the presence of IBT. Progeny were analyzed further as for the first test cross. Twenty-four plaques were picked from the 40° + IBT plaque assays for each test cross, and these isolates were tested for plaque formation at 31 and 40° + and − IBT. Of the isolates from the cs1 test cross, 2 contained no virus, and 22 were IBT^d. Of the isolates from the cs4 test cross, 1 contained no virus, and 23 were IBT^d . Twenty-four plaques were picked from the 31° − IBT plaque assays for each test cross, and these isolates were tested for plaque formation at 31 and 40° + and − IBT. For the cs1 test cross, 1 isolate was IBT^r, 1 was IBT^d, 22 were wt, and none were ts. For cs4 backcross, 23 isolates were wt and 1 was ts. The absence of ts progeny in the cs1 backcross most likely reflects the small fraction of ts viruses among progeny that are capable of forming plaques at 31° in the absence of drug. In summary the results of this test cross strongly suggest that both cs1 and cs4 contain IBT^d mutations and that at least cs4 retains the original Cts22 allele as well.

Marker rescue of cs1 and cs4. We mapped the mutations causing cold sensitivity in cs1 and cs4 by marker rescue. BSC40 cells were infected at low m.o.i. with either cs1 or cs4 and transfected with various DNA fragments cloned from wt virus. Infected, transfected cells were incubated under conditions which are nonpermissive for the cs mutants, 31° − IBT, for 6 days, and stained with crystal violet. Successful rescue of the cs marker is indicated by the appearance of plaques on the stained dishes. Rescue was tested with a battery of cosmid clones which span the entire vaccinia genome (not shown), with the 8.9-kb G2R gene-containing *Hin*dIII G fragment (not shown), and with pgG2Rap, a clone which contains only the G2R coding sequence (Fig. 2). The mutant cs1 was rescued well with two overlapping cosmid clones (pWR 45-83 and pWR 67-98), both of which contain the G2R gene (Thompson and Condit, 1986) and did not rescue with any of the other cosmid clones tested, none of which contain the G2R gene. The mutant cs1 was also rescued with *Hin*dIII G, and most importantly, with pgG2Rap (Fig. 2). Rescue of cs4 was not as efficient as that of cs1, and therefore convincing rescue was not observed with pWR 45-83, although positive rescue was observed with pWR 67-98 and with *Hin*dIII G (not shown). Positive marker rescue of cs4 was also observed with

TABLE 3

Test Cross of cs1 and cs4 with wt Virus

Virus infection[a]	Titer[b]			
	31°		40°	
	−IBT	+IBT	−IBT	+IBT
wt	5.0×10^6	7.0×10^2	6.0×10^6	1.0×10^3
cs1	$<1.0 \times 10^1$	$<1.0 \times 10^1$	1.0×10^7	$<1.0 \times 10^1$
cs4	$<1.0 \times 10^1$	$<1.0 \times 10^1$	9.0×10^6	$<1.0 \times 10^1$
wt + cs1	2.0×10^6	1.0×10^5	2.0×10^6	1.0×10^5
wt + cs4	3.0×10^6	1.0×10^5	2.0×10^6	2.0×10^5

[a] Cells were infected at high m.o.i. with the indicated viruses, and lysates were prepared as described under Materials and Methods.

[b] Lysates from high m.o.i. infections were plaque titrated under the indicated conditions.

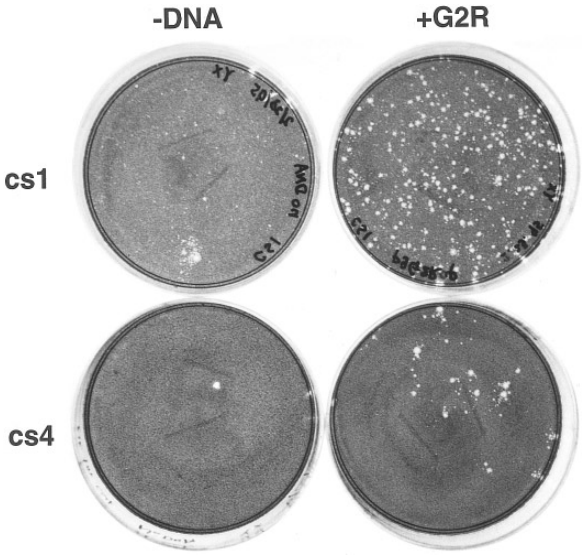

FIG. 2. Marker rescue of cs1 and cs4. Confluent monolayers of BSC40 cells were infected at low m.o.i. with either cs1 or cs4 and transfected with pgG2Rap, which contains the G2R gene (+G2R), or mock transfected (−DNA). Infected, transfected cells were incubated at 31° − IBT for 6 days and then stained with crystal violet. Successful rescue of the cs marker is indicated by the appearance of plaques on the stained dishes.

pgG2Rap (Fig. 2). These results prove that the mutation which causes cold sensitivity in cs1 and cs4 maps to gene G2R.

If cs1 and cs4 are actually double mutants which contain both a G2R mutation and the original Cts22 mutation, then rescue of the G2R mutation should yield a ts virus. This hypothesis was tested using cs1. Marker rescue of cs1 with *Hin*dIII G or pgG2Rap was done as described above except that the infected, transfected dishes were incubated under a nutrient agar overlay and stained with neutral red, so that rescued plaques could be isolated and tested further. A total of 10 plaques were picked from the two marker rescue dishes and each was tested first for plaque formation at both 31 and 40°. In these secondary plaque assays, plaques were observed at both temperatures for each of the 10 isolates. Suspecting that the original plaque isolates from the marker rescue contained more than one virus type, we analyzed plaques from the secondary plaque assays further. For each original rescue isolate, a single plaque was picked from each temperature from the secondary plaque assay, and each of these isolates was tested for plaque formation at both 31 and 40°. We found that 9 of 10 secondary plaques isolated at 31° were ts, and 9 of 10 secondary plaques isolated at 40° were cs. We interpret these results to mean that during the original rescue of cs1 done at 31°, some ts virus was formed which could then complement the growth of unrescued cs1, resulting in formation of plaques which contain both ts and cs virus. When these plaques were picked and tested in the secondary assay, plaque formation was observed at both 31 and 40°, however, the viruses which formed plaques at 31° were virtually all ts and the viruses which formed plaques at 40° were virtually all cs. Significantly, no wt virus was observed at any time in the experiment. These results prove that rescue of the gene G2R cs allele in cs1 produces a ts virus, presumably Cts22.

DNA sequence analysis. To determine the genotype of the mutant viruses, DNA sequence analysis was performed on PCR products from genomic mutant vial DNA corresponding to genes A18R and G2R as described under Materials and Methods. In cases where the sequence of a previously unknown mutation in a gene was being determined, the entire gene in question was sequenced. In cases where the analysis was done in order to confirm the presence of a known mutation, only the relevant region of DNA was sequenced. The results of the sequence analysis are summarized in Table 4. The engineered G2A deletion mutation, which causes a frameshift and premature translation termination, has been described previously (Meis and Condit, 1991). Analysis of the entire A18R gene from Cts22 and Cts23 revealed a single, unique, point missense mutation in each case. Cts22 contains a C to T transition in codon 46 of gene A18R, resulting in substitution of serine for proline. Cts23 contains a C to T transition at codon 432 of gene A18R, resulting in substitution of phenylalanine for serine. DNA sequence analysis of the Cts23, G2A recombinant, x41, confirmed that it contained both the G2A and Cts23 alleles. The ts^+ revertant of Cts23, r41, contained the Cts23 allele of gene A18R and in addition contained a missense mutation in codon 102 of gene G2R, resulting in substitution of aspartic acid for glycine. The cs revertants of Cts22, cs1 and cs4, each contained the Cts22 allele and in addition each contained a missense mutation in codon 102 of the G2R gene. In the case of cs1, the gene G2R codon 102 mutation results in substitution of serine for glycine. Interestingly, in the case of cs4, the gene G2R codon 102 mutation is different than the gene G2R codon 102 mutation found in cs1, and identical to the mutation found in r41. We conclude from the DNA sequence analysis that the recombinant virus x41 contains both parental mutant alleles and that the revertants r41, cs1, and cs4 each contain the parental A18R mutant allele plus a mutation in codon 102 of gene G2R.

DISCUSSION

Our results, summarized in Table 5, demonstrate that mutation of the vaccinia virus gene G2R can compensate for mutations in gene A18R. Major support for this conclusion comes from two separate observations. First, a recombinant, x41, which should contain only an A18R ts mutation and a G2R mutation, is viable at 40°, the nonpermissive temperature for the A18R ts mutant. Second, induced cold-sensitive revertants of an A18 ts mutation

TABLE 4

DNA Sequence Analysis of Mutant Viruses

	Sequence					
	G2R			A18R		
Virus	Codon	DNA	Protein	Codon	DNA	Protein
Cts22	+[a]	+	+	46	CCT → TCT	P → S
Cts23	+	+	+	432	TCC → TTC	S → F
G2A	90	▽ 10 bp	Frameshift	+	+	+
x41	90	▽ 10 bp	Frameshift	432	TCC → TTC	S → F
r41	102	GGC → GAC	G → D	432	TCC → TTC	S → F
cs1	102	GGC → AGC	G → S	46	CCT → TCT	P → S
cs4	102	GGC → GAC	G → D	46	CCT → TCT	P → S

[a] +, wild-type sequence.

can be rescued back to the ts mutant phenotype with a DNA clone containing only the wild-type G2R gene. The conclusion is further supported by the observation that a ts+ phenotypic revertant of an A18R ts mutant contains the same G2R mutation which in a different A18R ts background causes cold sensitivity. We believe that it is specifically *lethal* mutation of G2R that compensates for the A18R ts mutations. The most obvious support for this conclusion is again found in the recombinant x41, in which the Cts23 A18R allele is rescued by a lethal G2R deletion mutation. Implicit in this interpretation is that the gene G2R codon 102 mutations which suppress A18R ts mutations are in fact lethal G2R mutations. This hypothesis is supported by the observation that test crosses of r41, cs1, and cs4 all produce progeny which are IBT^d, a phenotype that has been previously associated with complete inactivation of the G2R gene.

Three subtleties of the genetic analysis deserve comment. First, it is noteworthy that three independently isolated extragenic suppressors of A18R ts mutations all affect the same G2R codon, codon 102. Since it is probably lethal mutation of G2R that causes suppression of A18R ts mutations, this result suggests that codon 102 is extremely critical for G2R function. Second, it is interesting that similar or identical G2R mutations result in two different phenotypes when combined with two different A18R ts alleles. Initially, we expected that recombination of the G2R deletion mutation into Cts23 would result in a cs phenotype, since at 40° both genes would be inactive, resulting in viability, and at 31° only the G2R gene would be inactive, resulting in lethality. Instead, we observe that when G2R mutations are combined with Cts23, the phenotype is ts^+, whereas when similar or identical mutations are combined with Cts22, the phenotype is cs. This observation implies that the two A18R ts alleles are functionally different. Specifically, it seems likely that the Cts23 allele is still somewhat compromised at 31°, accounting for the ts^+ phenotype of the double

TABLE 5

Summary of A18R–G2R Double Mutants

	Genotype[a]		Plaque phenotype[b]				
			31°		40°		
Virus	A18	G2	−IBT	+IBT	−IBT	+IBT	Phenotype summary[c]
wt	+	+	+	−	+	−	ts^+, IBT^s
ts22	P46S	+	+	−	−	−	ts, IBT^s
ts23	S432F	+	+	−	−	−	ts, IBT^s
G2A	+	Δ90–220	−	+	−	+	ts^+, IBT^d
x41	S432F	Δ90–220	+	−	+	−	ts^+, IBT^s
r41	S432F	G102D	+	−	+	−	ts^+, IBT^s
cs1	P46S	G102S	−	−	+	−	cs, IBT^s
cs4	P46S	G102D	−	−	+	−	cs, IBT^s

[a] Summary of the effects of mutations on the protein coding sequence, from Table 4. +, wt.
[b] Summary of the plaque phenotype shown in Fig. 1, +, plaque formation; −, no plaque formation.
[c] ts^+, temperature insensitive; ts, temperature sensitive; cs, cold sensitive; IBT^s, IBT sensitive; $IBRT^d$, IBT dependent.

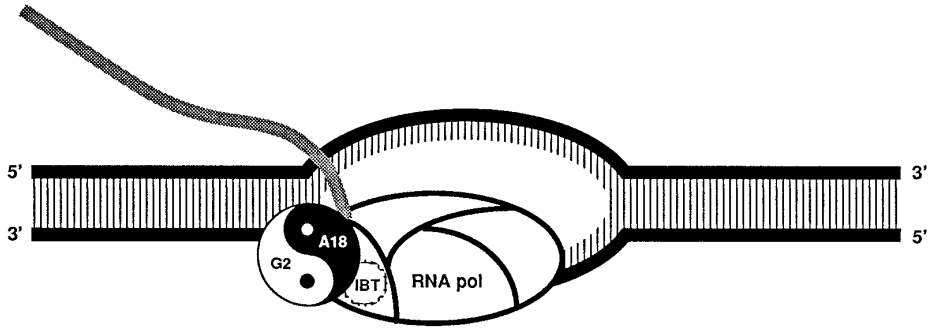

FIG. 3. A model for A18R, G2R, and IBT interaction. RNA polymerase is represented as a segmented ovoid bound to a transcription bubble. A18R and G2R gene products are represented as a complex interacting with the RNA polymerase. Transcribed RNA is represented as a gray single strand emerging from the complex. The proposed site of IBT action on RNA polymerase is indicated.

mutants, while the Cts22 allele is fully functional at 31°, accounting for the cs phenotype. Third, all of the double mutants are IBT^s. Since both IBT treatment and A18R mutations cause promiscuous transcription (Bayliss and Condit, 1993), we originally predicted that any second site mutation which compensated for an A18R mutation would also compensate for the effects of IBT, so that the double mutants would now be insensitive to the effects of IBT, hence IBT^r. The observation that A18R, G2R double mutants are IBT^s implies that the actual target of IBT is a gene other that either A18R or G2R and that promiscuous transcription is only one manifestation of the biochemical activity of IBT. Currently, the most likely candidate for the primary target of IBT is the viral RNA polymerase, since one IBT^r mutant has been mapped to the second largest subunit of this enzyme (Condit *et al.,* 1991). It is not unlikely, however, that continued genetic stratagems employing IBT, A18R, and G2R will uncover additional genes which influence vaccinia transcription in collaboration with A18R and G2R.

Based on the results reported here and on previously published results, we present a model for the biochemical function of A18R and G2R, depicted in Fig. 3. We propose that the A18R and G2R gene products have complementary functions, that they interact either directly or indirectly with each other and with the viral RNA polymerase, and that together A18R and G2R modulate elongation and termination by the viral RNA polymerase during the intermediate and late phases of viral infection. Support for and implications of the model can be summarized as follows. Biochemical analysis of mutants in A18R and G2R strongly suggests that both genes affect the fidelity of transcription (Bayliss and Condit, 1993; Black and Condit, 1995), and the genetic analysis reported here suggests that the genes have complementary activities. The observation that G2R mutants display normal transcription initiation for all three gene classes but produce 3′ truncated intermediate and late gene transcripts focuses attention on the elongation phase of transcription (Black and Condit, 1995). The fact that combination of an A18R mutation with a G2R mutation compensates for the presumed G2R elongation defect implies that A18R also affects transcription elongation or termination. Specifically, since mutation of G2R results in accumulation of intermediate and late transcripts which are too short, we predict that mutation of A18R alone would result in synthesis of intermediate and late transcripts which are too long; if both the A18R and G2R functions are missing, balance would be restored to transcription elongation, and appropriately sized transcripts would be produced. In our model we propose that IBT targets the viral RNA polymerase, because the IBT sensitivity of A18R, G2R double mutants implies a target for IBT other than G2R or A18R, and because IBT resistance maps to an RNA polymerase subunit (Condit *et al.,* 1991). However, we have drawn the site of IBT action on the RNA polymerase so that it overlaps a putative A18R–RNA polymerase interaction domain to indicate that IBT interaction with the RNA polymerase could compromise A18R function, leading to an A18R mutant-like phenotype, promiscuous transcription. Although we have drawn the model as if A18R, G2R, and RNA polymerase undergo direct interactions, evidence to support this concept is currently lacking, and it is not unlikely that additional proteins are involved.

Research in both procaryotic and eucaryotic systems shows that transcription elongation is influenced by *cis* acting sequence elements and *trans* acting factors, and by the RNA polymerase itself (Kane, 1994; Shaaban *et al.,* 1995; Landick *et al.,* 1990). During elongation of transcription, *cis* acting signals in DNA cause RNA polymerase to pause, and at these pause sites, both the RNA polymerase and the *trans* acting factors act to promote either transcription through the pause site or termination near the pause site. A common feature of pause sites is a stretch of T residues in the nontemplate DNA strand, and as few as four Ts may suffice to induce pausing in some systems (Bogenhagen and Brown, 1981). A variety of elongation factors and termination factors have been identified (Kane, 1994), and in addition mutation of the second largest subunit of both procaryotic and eucaryotic RNA polymerase may influence elongation (Shaaban *et al.,* 1995; Landick *et al.,* 1990). Our model for vaccinia

virus intermediate and late gene transcription elongation fits well into this picture. The vaccinia genome is an AT-rich structure, containing an average of one T_4 sequence every 35 nucleotides and one T_5 stretch every 200 nucleotides in each DNA strand. If late during a wild-type virus infection the vaccinia transcription machinery was to pause at short T stretches and terminate with a relatively low frequency at these sites, the extreme heterogeneity of intermediate and late RNA 3′ ends could be explained. The G2R protein could be an elongation factor which normally promotes transcription through pause sites; in the absence of G2R, the frequency of termination at pause sites would increase, resulting in 3′ truncated intermediate and late RNAs, as we have previously observed. The A18R helicase could be a termination factor which normally promotes release of RNA chains near pause sites; in the absence of A18R transcription would frequently read through pause sites, resulting in promiscuous transcription. The fact that IBT resistance maps to the second largest subunit of the vaccinia RNA polymerase implicates this protein in elongation, consistent with mutational analysis of RNA polymerases in other systems. Biochemical experiments to test the predictions of this model are underway.

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