JOURNAL OF VIROLOGY, Nov. 1992, p. 6470–6479
0022-538X/92/116470-10$02.00/0

Vol. 66, No. 11

Transcription of Viral Late Genes Is Dependent on Expression of the Viral Intermediate Gene G8R in Cells Infected with an Inducible Conditional-Lethal Mutant Vaccinia Virus

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Received 1 June 1992/Accepted 10 August 1992

There are three temporal classes of vaccinia virus genes: early, intermediate, and late. The object of this study was to determine the effects on virus replication of regulating the expression of G8R, an intermediate gene that encodes a late transcription factor. We inserted the *lac* operator adjacent to the RNA start site of the G8R gene in a recombinant vaccinia virus that constitutively expresses the *Escherichia coli lac* repressor to make expression of the G8R gene dependent on the inducer isopropyl-β-D-thiogalactopyranoside (IPTG). In case repression would not be complete, we also weakened the promoter of the G8R gene by making a single-nucleotide substitution designed to reduce its basal level of transcription. The mutant virus replicated well in the presence of the inducer, although synthesis of the G8R-encoded 30,000-M_r protein was only 10% of that of the wild-type virus. In the absence of IPTG, (i) synthesis of the G8R protein was inhibited by more than 99% relative to that of the wild-type virus, (ii) synthesis of early and intermediate mRNAs appeared to be unaffected, (iii) intermediate proteins accumulated to higher than normal levels, (iv) synthesis of late mRNA and protein was reduced by about 90%, (v) viral DNA was replicated but incompletely resolved concatemeric molecules accumulated, (vi) not even the earliest stages of virion assembly were detectable by transmission electron microscopy, and (vii) virus yield under one-step growth conditions and plaque formation were 10^{-3} and 10^{-4} times the wild-type values, respectively. The defect in late gene expression could be overcome by transfection of a G8R gene that was not under *lac* operator control, as well as by addition of IPTG, further demonstrating the specificity of the repression. The correlation between decreased expression of the G8R intermediate gene and inhibition of late mRNA synthesis is consistent with the notion that the G8R product serves as an essential late transcription factor and supports a cascade mechanism of vaccinia virus gene regulation. In addition, the inducer-dependent vaccinia virus mutant provided a tool for selective inhibition of late gene expression while allowing synthesis of early and intermediate mRNAs and proteins.

Vaccinia virus, a member of the poxvirus family, encodes most, if not all, of the proteins needed for transcription and replication of its 200,000-bp genome within the cytoplasm of infected cells (reviewed in reference 41). The existence of early, intermediate, and late classes of genes was inferred from kinetic analyses of protein synthesis in the presence and absence of specific metabolic inhibitors (43, 45). Early genes are expressed immediately after infection, since the enzymes and other proteins required for synthesis of functional early mRNA are packaged within the virion (36, 44, 54), whereas intermediate and late genes are successively activated after DNA replication has occurred. There is compelling evidence for a cascade mechanism of regulation in which early genes encode intermediate transcription factors (53), intermediate genes encode late factors (37), and late genes encode early factors (2, 6, 28). Considerable progress has been made in defining the promoter sequences specific for each class of genes (3, 19, 20, 29) and in identifying their cognate transcription factors. In addition to a requirement for a viral multisubunit core RNA polymerase, early transcription is dependent on the vaccinia virus early transcription factor, a virus-encoded heterodimeric protein with promoter-specific binding and DNA-dependent ATPase activities (5–9, 28, 58), and RAP94, a virus-encoded RNA polymerase-associated polypeptide (2). Intermediate transcription requires capping enzyme and a partially purified transcription factor (51, 52). At least three virus-encoded proteins, the products of genes A1L, A2L, and G8R, are necessary for late transcription (37, 56, 57).

Since the transcription factors and other proteins involved in mRNA synthesis were discovered by biochemical fractionation and in vitro assays or by transfection and reporter gene *trans* activation, the roles of these proteins during virus replication are largely inferred. Analyses of conditional-lethal mutants could be useful to confirm and extend such studies. Temperature-sensitive mutants have been mapped to genes that encode some RNA polymerase subunits (25, 30, 31, 49), the small subunit of capping enzyme (10), late transcription factor A1L (11), and RAP94 (34). Further searches through existing mutant collections may provide additional examples of viruses that encode altered transcription factors. However, the limited number of temperature-sensitive mutants available and the unsuitability of some for biochemical studies make it desirable to attempt more directed genetic approaches.

The demonstration that the *Escherichia coli lac* repressor could be used to regulate the expression of genes under vaccinia virus late promoter control (27, 46) led to the development of a new class of conditional-lethal mutants that are inducer dependent. Two such mutants of vaccinia virus, which are blocked in virion assembly under nonpermissive conditions, have been described (60–62). A related approach was used to obtain two mutants that require an

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inducer for efficient release of infectious virions from cells (22, 46). In the above examples, the *lac* repressor was used to regulate the expression of genes that encode late structural proteins. As these components are required in stoichiometric amounts, severe phenotypic effects can occur even when repression is not 100% effective. The efficiency of the *lac* repressor system in regulating vaccinia virus genes that belong to other regulatory classes or encode catalytic proteins has not been reported.

In this study, we extended the use of the *lac* repressor system to vaccinia virus intermediate promoters and to a gene, G8R, that encodes one of the three known late transcription factors (37). Evidence that expression of G8R is required for transcription of late genes in vivo is presented. In addition, the inducer-dependent vaccinia virus mutant selectively inhibited late gene expression while allowing synthesis of intermediate mRNA and proteins to continue. Under these conditions, incompletely resolved concatemeric viral DNA molecules accumulated and even the earliest stages of virion assembly were blocked, indicating that late transcription is essential for both events.

MATERIALS AND METHODS

Cells and viruses. BS-C-1 and CV-1 cells were grown in minimum essential medium supplemented with 10% fetal calf serum. Vaccinia virus strain WR, referred as wild-type (WT) virus, and recombinant virus vlacI, which expresses the *E. coli lac* repressor (27), were propagated and purified as previously described (23).

Recombinant virus construction. Protocols for construction of *lac* operator-regulated vaccinia viruses have already been published (60–62). For construction of a new recombinant virus in which the G8R gene is under *lac* operator control, overlap extension polymerase chain reaction (PCR) (32) was used to assemble a 1,121-bp DNA fragment containing, in sequential order, an *Sph*I restriction endonuclease recognition site, 510 bp of vaccinia virus DNA upstream of the translation initiation codon of the G8R gene with a T-to-G base substitution within the promoter (see Fig. 1), the 22-bp symmetric *E. coli lac* operator segment (47), an *Xho*I restriction endonuclease recognition site, a new methionine translation initiation codon, 570 bp of the G8R open reading frame, and the *Sac*I restriction endonuclease recognition site. The sequences of the four oligonucleotide primers prepared for PCR are (i) GGGGGGCATGCCAATAATT CTGCTAGTTCTTGAGAC, (ii) GGGGGGAGCTCCCTG GAAAATGCATCAGTATTACC, (iii) GCGCTCACAATT CCCTCGAGAATTGTGAGCGCTCACAATTCTATTTT AAATTTTTGTAAATTATTTACAGTTAAATG, and (iv) TCGAGGGAATTGTGAGCGCTCACAATTCTCGAGAT GAGCATCCGTATAAAAATCGATAAACTG. After amplification, the assembled PCR fragment was digested with restriction endonucleases *Sac*I and *Sph*I and ligated to a vector, pUC-gpt (60), that had been cut with the same two restriction endonucleases to form plasmid pgpt-op-G8R. The latter plasmid was transfected into cells that were infected with vlacI, and homologous recombination led to replacement of the natural G8R promoter with the *lac* operator regulated one to form vRO-G8R (see Fig. 1). The procedures for infection, transfection, selection, and screening of *lac* operator-positive recombinant virus were essentially the same as described previously (60–62). Two oligonucleotide primers, GAATTAATAGATATATCTCTTAATACAGATT GC and CCGCCCAAGGCGGCAAAAATATAC, were used to screen *lac* operator-positive recombinant virus by PCR.

One-step growth of vRO-G8R. BS-C-1 cells (5×10^5) were infected with the WT virus or recombinant vaccinia virus vRO-G8R at a multiplicity of infection (MOI) of 10 PFU per cell in the presence or absence of 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Progeny viruses were harvested at various times after infection, and their titers were determined by plaque formation on BS-C-1 cells in the presence of 5 mM IPTG.

Electron microscopic analysis. BS-C-1 cells (5×10^6) were infected with the WT virus or recombinant vaccinia virus vRO-G8R at an MOI of 10 PFU per cell in the presence or absence of 5 mM IPTG. Cells were fixed 8 h after infection as described previously (61). The electron micrographs were prepared by Advanced Biotechnologies, Inc. (Columbia, Md.).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of viral proteins. BS-C-1 cells (10^6) were infected with the WT virus or recombinant vaccinia virus vRO-G8R at an MOI of 10 PFU per cell in the presence or absence of 5 mM IPTG. At various times, the minimum essential medium was removed and the cells were washed with phosphate-buffered saline and then incubated with 1 ml of methionine-free medium containing 30 μCi of [^{35}S]methionine for 30 min. Two different protocols were used to prepare cell lysates. For analysis of infected cell proteins synthesized at different times and for immunoprecipitation of the vaccinia virus 11-kDa late protein encoded by the F18R gene, infected cells were lysed with a buffer containing β-mercaptoethanol and SDS as described previously (61). For immunoprecipitation of the intermediate proteins encoded by the G8R and A1L genes of vaccinia virus, infected cells were vortexed in 150 μl of lysis buffer containing 0.5% Nonidet P-40, 100 mM Tris-HCl (pH 8.0), and 100 mM NaCl and incubated on ice for 10 min before nuclei were pelleted in a microcentrifuge (HERMLE Z-230M; National Labnet) at 12,000 rpm for 10 min at 4°C. The supernatants were used for immunoprecipitation.

The procedure for immunoprecipitation was essentially the same as reported previously (61). The antisera raised to F18R and G8R proteins were described previously (35, 56). The antiserum against the A1L protein was obtained from a rabbit that had been immunized with a bacterially expressed A1L gene product that will be described in detail elsewhere.

Proteins from cell lysates and immunoprecipitations were incubated in 10 mM Tris-HCl (pH 6.8)–1% SDS–1% mercaptoethanol–10% glycerol for 10 min at 100°C and applied to a 12 or 15% polyacrylamide–SDS gel (38).

Viral RNA analysis. RNA was isolated from BS-C-1 cells that were infected with the WT virus or recombinant vaccinia virus vRO-G8R at an MOI of 10 PFU per cell in the presence or absence of 5 mM IPTG. At various times after infection, the cells were lysed and the RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform extraction method (12) using RNAzol (Tel-Test, Inc., Friendwood, Tex.). Approximately 20 μg of total RNA was obtained from BS-C-1 cells.

DNA templates for generation of ^{32}P-labeled RNA probes were prepared either with or without a cloning step. For the first method, a DNA fragment of about 400 to 600 bp including 200 to 300 bp up- and downstream of the RNA start site of a gene of interest was synthesized by PCR and then cloned into plasmid pGEM-4Z (Promega). The plasmid was linearized with a restriction endonuclease at a site 400 to 600 bp downstream of the bacteriophage T7 promoter, and the linearized template was transcribed by T7 RNA polymerase in the presence of ATP, GTP, CTP, and ^{32}P-labeled UTP.

For the second method of template preparation, one of the primers contained the 18-nucleotide T7 promoter, and the PCR product was used without cloning as a template for T7 RNA polymerase.

RNA (5 to 10 μg) isolated from infected cells was incubated with the ^{32}P-labeled RNA probe (200,000 cpm) in 15 μl of hybridization buffer containing 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4), 400 mM NaCl, 1 mM EDTA (pH 8.0), and 80% deionized formamide for 18 h at 46°C. Unhybridized RNA was digested in a volume of 300 μl with 500 U of nuclease S1 (Boehringer Mannheim) for 1 h at 42°C or with 1.2 μg of RNase A plus 3 U of RNase T1 for 1 h at room temperature. In the latter case, the digestion was terminated by addition of 50 μg of proteinase K and 10 μl of 20% SDS and incubation at 37°C for 15 min. With both procedures, the RNA was extracted with phenol-chloroform and precipitated with 95% ethanol and applied to a 6% polyacrylamide gel containing 8 M urea. After electrophoresis, the gel was dried and autoradiographed.

Pulsed-field gel electrophoresis of DNA from infected cells. BS-C-1 cells (10^6) were infected with 10 PFU of WT vaccinia virus or vRO-G8R per cell in the presence or absence of 5 mM IPTG or with 5 PFU of a temperature-sensitive mutant, *ts*21 (16) per cell. The infected cells were harvested after 24 h at 37°C and embedded in agar plugs as previously described (40). Total infected-cell DNA in the agar plug was electrophoresed for 40 h at 220 V with a switching time of 1 min at 13°C by using a transverse alternating-field electrophoresis apparatus (Beckman). Transfer of the DNA to GeneScreen Plus (Dupont NEN) membranes and hybridization with ^{32}P-labeled DNA probes were carried out as previously described (40).

Transient expression. BS-C-1 cells (5×10^5) were infected with WT vaccinia virus or recombinant vRO-G8R at an MOI of 10 PFU per cell in the presence or absence of 5 mM IPTG. $CaPO_4$-DNA precipitates were made by sequentially mixing 5 μg of plasmid DNA; 225 μl of sterilized, deionized H_2O; 25 μl of 2.5 M $CaCl_2$; and 250 μl of 0.28 M NaCl–10 mM KCl–2 mM Na_2HPO_4–0.2% glucose–40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (adjusted to pH 7.05 with 0.5 M NaOH) and sterilized. The precipitates were allowed to form at room temperature for 20 min and then added dropwise to 2 ml of medium covering infected cells in individual wells of a 12-well plate. The medium was changed at 4 h after addition of the DNA, and the infection was continued for a total of 16 h. The following plasmids were used for transfection: p11Xβ (37), pMJ480, and pMJ496 (20), which have late promoters that regulate the *lacZ* gene, and p26Xβ and p30Xβ, which contain the 30-bp promoters of the A2L and G8R genes of vaccinia virus strain WR, respectively, fused to the *lacZ* gene (3).

Approximately 1.5×10^6 OST7-1 cells (24) were infected with vRO-G8R as described above for BS-C-1 cells. $CaPO_4$-DNA precipitates were formed with 1.3 μg of plasmid p11xβ and, where indicated, with 5 μg of plasmid pT7-G8R or pTM-1 (42). Medium changes were carried out as described above, except that the infected cells were incubated for a total of 24 h. Plasmid pT7-G8R was constructed by cloning the PCR-derived G8R open reading frame into plasmid pTM-1.

β-Galactosidase activity and protein assays. Infected cells (0.5×10^6 to 1.5×10^6) were suspended in 500 μl of phosphate-buffered saline, subjected to three freeze-thaw cycles, and then sonicated. The samples were centrifuged to remove the cell debris, and the supernatants were collected. For β-galactosidase assays, 50 μl of 4% *o*-nitrophenyl-β-D-galactopyranoside solution was added to a well of a 96-well microtiter plate containing 50 μl of cell extract and 150 μl of Z buffer (60 mM Na_2PO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4, 50 mM β-mercaptoethanol, pH 7.0). A_{415} and A_{590} were determined simultaneously with a Vmax Kinetic Microplate Reader (Molecular Devices Corp., Menlo Park, Calif.). Optical density at the latter wavelength was subtracted as a control for light scattering caused by residual debris in the lysate. Protein assays were carried out by using the protein assay reagent (Bio-Rad, Richmond, Calif.) in accordance with the protocol supplied by the manufacturer by using the microplate reader set at 590 nm. The specific activity of β-galactosidase was calculated by using the formula β-galactosidase units = $(1.3 \times 10^3 \times A_{415}/5.3)$ (time in minutes × milligrams of protein).

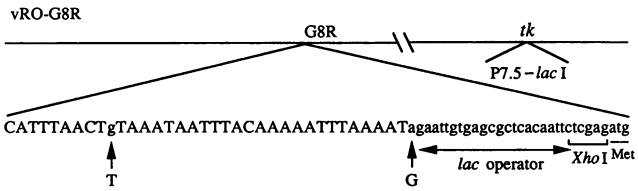

FIG. 1. Genomic structure of the vRO-G8R recombinant virus. The vaccinia virus genome is represented at the top. The positions of the G8R and thymidine kinase (*tk*) genes are indicated. The sequence of the G8R promoter is shown with original nucleotides in uppercase letters and point mutations and insertions of the *lac* operator, the *Xho*I restriction endonuclease site, and the shifted methionine translation initiation codon in the recombinant virus are shown in lowercase letters. The *E. coli lacI* repressor gene regulated by the vaccinia virus P7.5 promoter is inserted into the *tk* gene.

RESULTS

Construction of a conditional-lethal mutant of vaccinia virus with the G8R gene regulated by the *lac* repressor. The strategy for construction of an inducible conditional-lethal mutant vaccinia virus that has the G8R gene regulated by the *E. coli lac* repressor was adapted from procedures developed in our laboratory (60–62). We started with vlacI, a recombinant vaccinia virus that contains the *E. coli lacI* repressor gene under control of the vaccinia virus P7.5 early-late promoter inserted into the *tk* gene locus (27). Our aim was to place the 22-bp symmetrical *lac* operator DNA sequence (47) just 2 bp downstream of the RNA start of the G8R gene. Although this operator location worked well with vaccinia virus late promoters, it had not been tested with an intermediate promoter. The nature of the G8R gene product raised additional concerns. As the G8R protein is a transcription factor, it is probably required in lower amounts than most structural proteins and might even be made in excess. In that case, stringent repression would be needed to produce a significant biological effect. To alleviate this potential problem, we decided to lower the basal level of G8R expression by mutagenesis of the G8R promoter. We knew, from a recently completed study (3), the effect of almost every single-base substitution on the activity of the G8R promoter in regulating expression of a reporter gene. Thus, the specific T-to-G substitution shown in Fig. 1 should decrease activity by approximately 80%. We speculated that this level of G8R expression would be adequate for replication but that a further reduction mediated by *lac* repressor binding would be conditionally lethal.

A segment of vaccinia virus DNA with the G8R promoter

mutation described above, as well as the *lac* operator, was constructed by overlap extension PCR (32). These modifications did not alter the sequence of the G8R open reading frame. The amplified DNA was cloned, and the plasmid was used to generate recombinant vaccinia virus vRO-G8R (Fig. 1) by using transient dominant selection procedures (26) as previously described in detail (60–62). Individual plaques were analyzed by PCR for the presence of *lac* operator DNA. Two recombinant viruses, from a total of 100 plaques screened, were positive. One of these was plaque purified three additional times and amplified to make a high-titer stock of vRO-G8R virus.

Inducer-dependent virus growth. Initial evidence that vRO-G8R is an inducer-dependent virus was obtained by plaque assay. The titer of the virus stock was nearly 10,000-fold higher in the presence of 5 mM IPTG than in its absence (Fig. 2A). The vRO-G8R plaques that formed in the presence of IPTG, however, were smaller than those of the WT virus. This difference was not attributed to toxicity, since 5 mM IPTG had no effect on the number or size of WT virus plaques (data not shown). In the absence of IPTG, vRO-G8R plaques did not form, even after 15 days of incubation. Nevertheless, addition of IPTG at any time during this period resulted in visible plaques in 24 h.

The IPTG dependence of vRO-G8R was further demonstrated by one-step growth analysis (Fig. 2B). An MOI of 10 PFU was used to ensure synchronous infection of all cells. In the presence of the inducer, the yield of vRO-G8R approached that of the WT virus, whereas there was a 1,000-fold difference in its absence.

Repressor-mediated effects on synthesis of viral intermediate and late proteins. In vaccinia virus-infected BS-C-1 cells, viral early protein synthesis begins within 1 h, viral DNA replication starts between 2 and 3 h, and by 6 h host protein synthesis is largely inhibited and intermediate and late proteins can be selectively labeled with [^{35}S]methionine. The labeled proteins resolved by SDS-PAGE from cells infected with the WT virus and cells infected with vRO-G8R in the presence of IPTG were very similar at all times (Fig. 3). Between 6 and 10 h after infection with vRO-G8R in the absence of the inducer, however, the intensities of many labeled bands were markedly decreased (Fig. 3, dashes) while those of other bands were increased or at least unaffected (Fig. 3, asterisks). These results are consistent with the existence of two distinct classes of postreplicative proteins; the bands that increase and decrease in intensity are candidate intermediate and late class proteins, respectively.

The [^{35}S]methionine-labeled proteins from cells infected with WT virus or vRO-G8R in the presence or absence of IPTG were immunoprecipitated with antisera to the G8R protein and to other intermediate and late viral proteins. Because of the promoter mutation, synthesis of the 30-kDa G8R protein in vRO-G8R-infected cells was greatly reduced compared with that in WT virus-infected cells, even in the presence of IPTG (Fig. 4). In the absence of IPTG, synthesis of the 30-kDa protein was virtually undetectable by autoradiography. Quantitation with a PhosphorImager (Molecular Dynamics Inc.) revealed that the amounts of G8R protein made in vRO-G8R-infected cells in the presence and in the absence of IPTG were 10% and less than 1% of WT levels, respectively. The effect of IPTG omission was attributed to *lac* repressor binding to the *lac* operator placed just downstream of the G8R gene RNA start site and not to a general effect on intermediate gene expression. The latter interpretation was confirmed by determining the synthesis of the

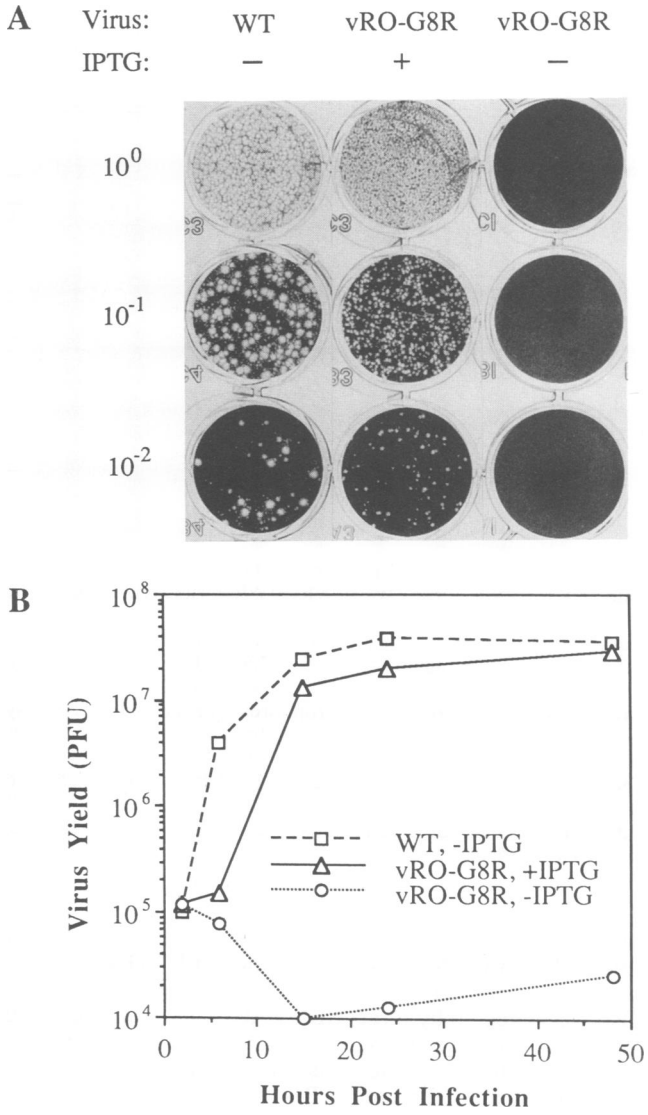

FIG. 2. vRO-G8R has an inducible conditional-lethal phenotype. (A) Plaque titrations. Dilutions of the WT and vRO-G8R viruses were prepared and inoculated onto BS-C-1 monolayers in the presence or absence of IPTG. After 48 h, the monolayers were stained with crystal violet. (B) One-step growth analyses. BS-C-1 cells (5×10^5) were infected with 10 PFU of the WT or vRO-G8R virus per cell in the presence or absence of 5 mM IPTG and harvested at the indicated times after infection. Virus yields were determined by plaque assay in the presence of 5 mM IPTG.

17-kDa product of the A1L intermediate gene, which encodes another late transcription factor (37). Synthesis of the 17-kDa protein was highest in cells infected with vRO-G8R in the absence of IPTG and lowest in cells infected with the WT virus (Fig. 4). A 17-kDa protein that exhibited a similar pattern of synthesis with respect to IPTG addition was detected directly by SDS-PAGE of infected cell lysates (Fig. 3) and found to comigrate with the immunoprecipitated protein (data not shown).

As a representative late protein, we selected the 11-kDa product of the F18R open reading frame (35, 55, 61). The pattern of synthesis was precisely the reverse of that obtained for the A1L intermediate protein: the amount of

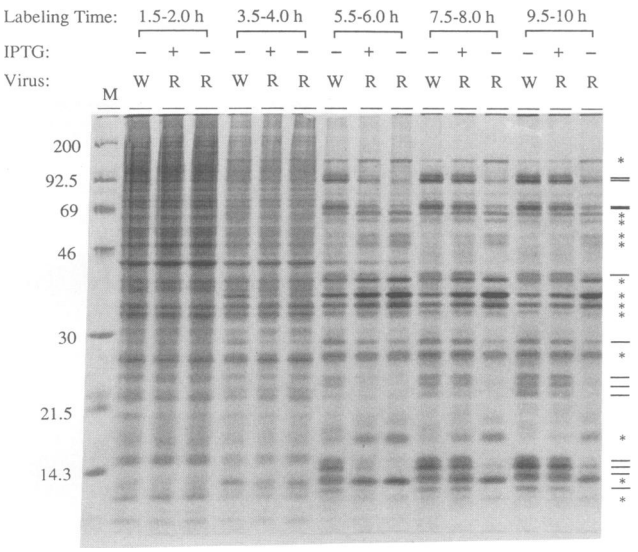

FIG. 3. SDS-PAGE of proteins metabolically labeled in cells infected with WT virus (W) or vRO-G8R virus (R). BS-C-1 cells (10^6) were infected with 10 PFU of virus in the presence (+) or absence (−) of IPTG and pulse-labeled with [^{35}S]methionine for 30-min periods starting at 1.5, 3.5, 5.5, 7.5, and 9.5 h after infection. Immediately after labeling, the cells were washed and lysed and the proteins were analyzed by electrophoresis on an SDS–12% polyacrylamide gel. Marker proteins (^{14}C labeled) were analyzed on the same gel (lane M). The numbers on the left of the autoradiograph are the molecular masses of the markers in kilodaltons. The positions of migration of proteins that are over- and underexpressed in cells infected with vRO-G8R in the absence of IPTG are indicated by asterisks and dashes, respectively.

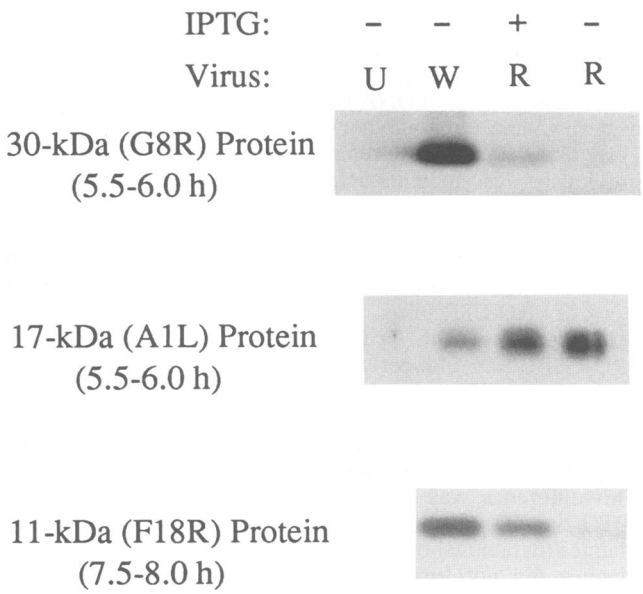

FIG. 4. SDS-PAGE of immunopurified viral intermediate and late proteins. Lysates from mock (U)-, WT (W)-, and vRO-G8R (R)-infected BS-C-1 cells that had been incubated with (+) or without (−) IPTG and pulse-labeled with [^{35}S]methionine at the times indicated were incubated with specific antisera raised to the 30-kDa product of the intermediate G8R gene, the 17-kDa product of the intermediate A1L gene, or the 11-kDa product of the late F18R gene. The antigen-antibody complexes were bound to protein A-Sepharose beads, which were then washed and treated with buffer containing SDS at 100°C. The eluted proteins were resolved by electrophoresis on a 15% polyacrylamide gel. Relevant portions of the autoradiographs are shown.

labeled 11-kDa late protein immunoprecipitated from cells infected with vRO-G8R in the absence of IPTG was 12% of that obtained with the WT virus (Fig. 4). The 11-kDa protein also was detected by direct SDS-PAGE analysis (Fig. 3) but was not completely resolved from an adjacent band under the electrophoretic conditions used. The amounts of two other late structural proteins, P4a and P4b, were quantitated directly from Tricine-buffered SDS-polyacrylamide gels as previously described (61). In cells infected with vRO-G8R in the absence of IPTG, the amounts of P4a and P4b were about 7% of those that occurred during WT virus infection (data not shown).

These results supported the idea that repression of G8R expression results in decreased late and increased intermediate protein syntheses.

Analysis of early, intermediate, and late mRNAs. The RNA made in vRO-G8R-infected cells was analyzed to determine whether the effect of IPTG omission on viral late protein synthesis has a transcriptional basis. For this analysis, complementary RNA probes were made by transcribing segments of individual early, intermediate, and late vaccinia virus genes with bacteriophage T7 RNA polymerase in the presence of ^{32}P-labeled UTP. The labeled probes were hybridized to RNA from infected cells (or to tRNA as a control), and the remaining single-stranded materials were digested with RNase or nuclease S1. The sizes and amounts of the resistant RNAs were determined by polyacrylamide gel electrophoresis and autoradiography. Preliminary experiments were carried out to ensure that each of the probes was in excess and that optimal conditions were used for RNase and nuclease S1 digestions.

The early mRNAs for the vaccinia virus growth factor (59) and DNA polymerase (33, 50) were analyzed previously, and their RNA start sites were determined. Vaccinia virus early mRNAs can still be detected after the onset of DNA replication in BS-C-1 cells, and therefore 3- and 8-h time points were chosen. The protected probe lengths were of the predicted sizes, and the mRNAs were made in the presence of cytosine arabinoside, an inhibitor of DNA replication, as expected for early transcripts (Fig. 5, upper panel). Significantly, IPTG had little effect on the amounts of early mRNAs isolated at 3 or 8 h after infection with the WT virus or vRO-G8R recombinant virus.

The mRNAs that correspond to intermediate genes A1L and A2L (37) and late gene F18R (4) were present at 8 h after infection with the WT virus or vRO-G8R recombinant virus as determined by the predicted size bands formed with the labeled probes (Fig. 5, lower panel). IPTG had little effect on the amounts of the intermediate mRNAs present in WT or mutant virus-infected cells. In contrast, the amount of viral late mRNA was specifically reduced in cells infected with vRO-G8R in the absence of IPTG (Fig. 5). Quantitation with a PhosphorImager indicated that the amount of F18R gene mRNA in cells infected with vRO-G8R in the absence of IPTG was approximately 10% of that present in cells infected with the WT virus. Thus, the effects on late protein synthesis could be fully attributed to decreased late gene transcription.

Regulation of expression of transfected reporter genes. Genes regulated by intermediate and late promoters can be expressed by transfecting plasmids that contain them into

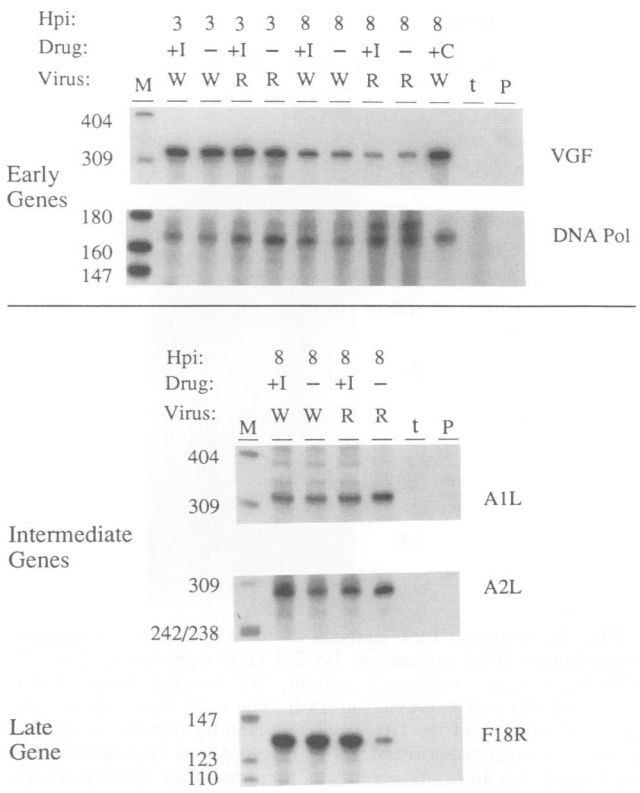

FIG. 5. Analysis of viral mRNAs. Total BS-C-1 cell RNA was isolated at 3 or 8 h postinfection (Hpi) with the WT (W) or vRO-G8R (R) virus in the presence of IPTG (+I) or cytosine arabinoside (+C) or in the absence of both (−). Infected-cell RNA or tRNA (t) was hybridized with individual ^{32}P-labeled RNA probes produced by in vitro transcription of the vaccinia virus growth factor (VGF) or DNA polymerase (DNA Pol) early gene, the A1L or A2L intermediate gene that encodes a late transcription factor, or the F18R late gene that encodes an 11-kDa structural protein. Hybridized samples were digested with either nuclease S1 or a combination of RNase A and RNase T_1. The resistant RNAs were resolved by electrophoresis on a 6% polyacrylamide gel containing 8 M urea. Only the relevant portions of the autoradiographs are shown. Lanes: M, radioactive DNA size markers (sizes are given in bases on the left); P, probe alone. The full-length probes were all greater than 450 nucleotides long, and the expected lengths of the protected segments (in nucleotides) were as follows: VGF, 316; DNA Pol, 160; A1L, 300; A2L, 300; F18R, 126.

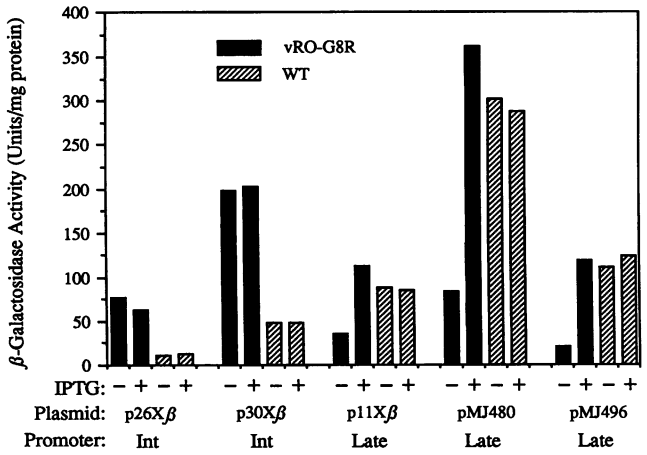

FIG. 6. Transient expression of intermediate (Int) and late promoter-*lacZ* plasmids in vRO-G8R- or WT-infected cells. BS-C-1 cells infected with the WT or vRO-G8R virus at an MOI of 10 PFU per cell in the presence or absence of 5 mM IPTG were transfected with plasmids containing the *lacZ* gene controlled by either intermediate (p26Xβ or p30Xβ) or late (p11Xβ, pMJ480, and pMJ496) promoters. The cells were harvested at 16 h after infection, and the lysates were assayed for β-galactosidase activity and protein concentration. The β-galactosidase specific activities shown are averages of duplicate transfections.

cells infected with vaccinia virus (3, 13, 37, 53). We carried out transfection experiments to further investigate the effects of repression of the G8R gene on the expression of genes controlled by intermediate and late promoters. An advantage of this approach is that different promoters can be used to regulate the same reporter gene. Plasmids p11Xβ, pMJ480, and pMJ496 contain the *lacZ* gene regulated by the vaccinia virus late P11 and strong synthetic and cowpox virus ATI promoters, respectively (20, 37). When these plasmids were transfected into cells that had been infected with vRO-G8R, IPTG significantly increased β-galactosidase activity (Fig. 6). As expected, IPTG had no effect when the plasmids were transfected into WT virus-infected cells. As additional controls, we transfected plasmids p26Xβ and p30Xβ, which contain the *lacZ* gene regulated by promoters derived from intermediate genes A2L and G8R, respectively (3). Under these conditions, β-galactosidase activities were unaffected by IPTG in cells infected with either the WT virus or vRO-G8R (Fig. 6). We cannot explain the higher expression of reporter genes regulated by intermediate promoters in cells infected with vRO-G8R compared with those infected with the WT virus except to note that a similar result was obtained when expression of the genomic A1L gene was measured (Fig. 3). The point we wish to make in this section is that IPTG only enhances the expression of reporter genes regulated by late promoters in plasmids transfected into cells infected with vRO-G8R.

A *lac* operator-negative G8R gene *trans* activates expression of a late promoter-regulated reporter plasmid in the absence of an inducer. The experiments carried out thus far suggested that the effects of the *lac* repressor on late gene expression were due to repression of the *lac* operator-modified G8R gene in vRO-G8R. The possibility existed, however, that expression of neighboring genes was also affected by the placement of the operator. This concern could be eliminated if the defect in late gene expression were corrected by transfection of a plasmid containing only an unrepressible G8R gene into cells that were infected with vRO-G8R in the absence of an inducer. An experiment designed to answer this question, by transfecting plasmids containing genes regulated by the bacteriophage T7 promoter into a cell line that constitutively expresses T7 RNA polymerase (24), is outlined in Fig. 7A. This cell line was infected with either vRO-G8R or the WT virus and then transfected with p11Xβ, a late promoter reporter plasmid that expresses β-galactosidase, alone or with either plasmid pT7-G8R, which contains the G8R gene under control of a bacteriophage T7 promoter, or pTM-1, a control vector plasmid used to make pT7-G8R. When p11Xβ alone was transfected, synthesis of β-galactosidase was IPTG dependent (Fig. 7B). IPTG was not required for expression of p11Xβ, however, if pT7-G8R (but not pTM-1) was cotransfected. Transfection with pT7-G8R in the absence of IPTG also produced a small (less than 10-fold) but reproducible increase in the infectious titer of vRO-G8R

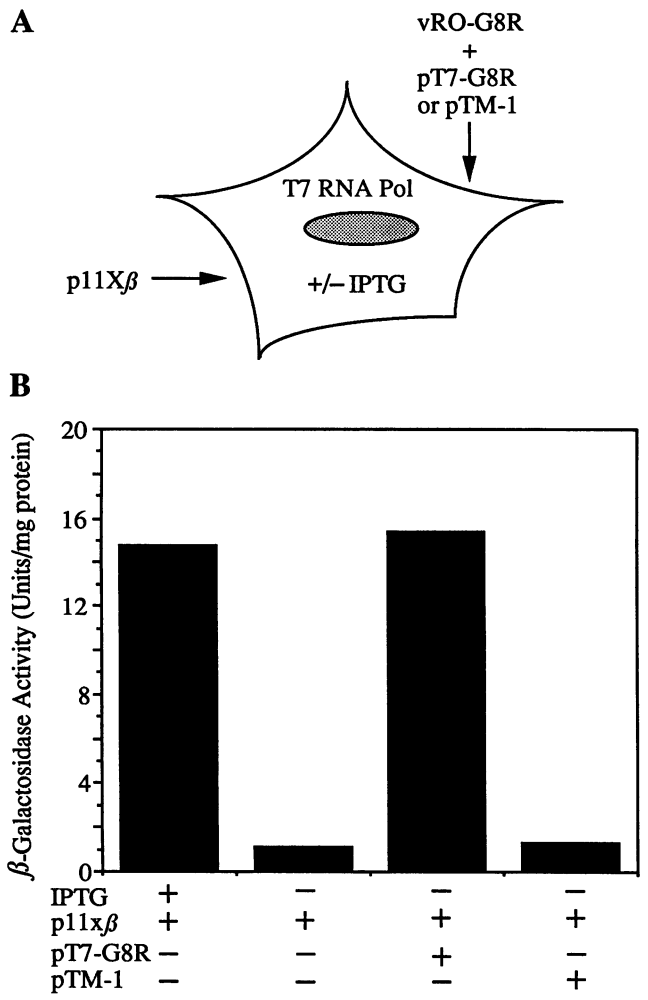

FIG. 7. *trans* activation of late gene expression by a bacteriophage T7 promoter-regulated G8R plasmid. (A) Experimental outline. OST7-1 cells, which constitutively express bacteriophage T7 RNA polymerase, were infected with vRO-G8R at an MOI of 10 PFU per cell in the presence or absence of 5 mM IPTG. The infected cells were cotransfected with plasmid p11xβ, pT7-G8R, or pTM-1 as indicated. Cells were harvested at 24 h after infection, and β-galactosidase specific activities were determined. (B) Transfections were carried out in duplicate, and the β-galactosidase activities were averaged.

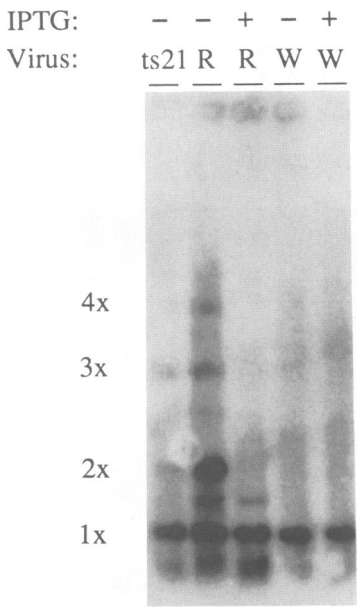

FIG. 8. Southern blot analysis of viral genomic and concatemeric length DNA molecules. BS-C-1 cells were infected at 37°C with the vaccinia virus *ts*21 mutant, WT vaccinia virus (W), or vRO-G8R (R) and harvested at 24 h. The cells were lysed within agarose blocks, and the DNA was resolved by transverse alternating-field gel electrophoresis and transferred to a GeneScreen Plus membrane. An autoradiograph was prepared after hybridization to ^{32}P-labeled vaccinia virus DNA. Monomeric and concatemeric molecules of lambda DNA were run as size markers, and the interpolated positions of 1×, 2×, 3×, and 4× vaccinia virus genome lengths are indicated.

(data not shown). The ability to fully restore expression of a cotransfected reporter plasmid but to only partially enhance infectivity is not surprising, owing to the relative inefficiency of transfection procedures.

Deficient resolution of concatemeric forms of viral DNA in vRO-G8R-infected cells. vRO-G8R can provide a useful tool for investigation of viral intermediate and late gene functions. For example, previous studies with temperature-sensitive mutants and metabolic inhibitors demonstrated that expression of early genes was not sufficient for complete resolution of concatemeric forms of vaccinia virus DNA (21, 39, 40). As seen in a Southern blot (Fig. 8), a ladder of viral DNA bands was resolved by pulse-field gel electrophoresis of DNA from cells infected at the nonpermissive temperature with *ts*21 (16), which has a lesion that maps to gene D7R (48), which encodes an RNA polymerase subunit (1). A similar ladder of bands was also detected upon examination of viral DNA from cells infected with vRO-G8R in the absence of IPTG but not in its presence (Fig. 8). Bacteriophage lambda DNA multimers analyzed on the same gel (data not shown) indicated that the increment between two adjacent vaccinia virus DNA bands was approximately 200,000 bp, equivalent to the unit genome length. A band corresponding to about 1.5 times the monomer genome length was found in DNA from vRO-G8R-infected cells in the presence or absence of IPTG and was not investigated further. Restriction enzyme analysis demonstrated the accumulation of concatemer junctions in cells infected with vRO-G8R in the absence of IPTG (data not shown). Thus, early gene expression and intermediate gene expression are not sufficient for complete concatemer resolution.

Absence of viral morphogenesis in cells infected with vRO-G8R. The requirements for intermediate and late gene expression in virion morphogenesis were examined in cells infected with vRO-G8R. Electron micrographs of sections of cells infected with vRO-G8R for 8 h in the presence of the inducer displayed normal immature viral particles which appeared as circular membrane-bound structures (Fig. 9A). In contrast, when the inducer was omitted, immature viral forms were not observed. Careful examination of the sections, however, revealed cytoplasmic regions composed of relatively dense, amorphous material from which cellular organelles were largely excluded (Fig. 9B). Similar regions of viroplasm have been noted previously during the first 2 to 3 h after infection with WT vaccinia virus (18) and as the only viral structures discerned after infection with certain temperature-sensitive mutants (17).

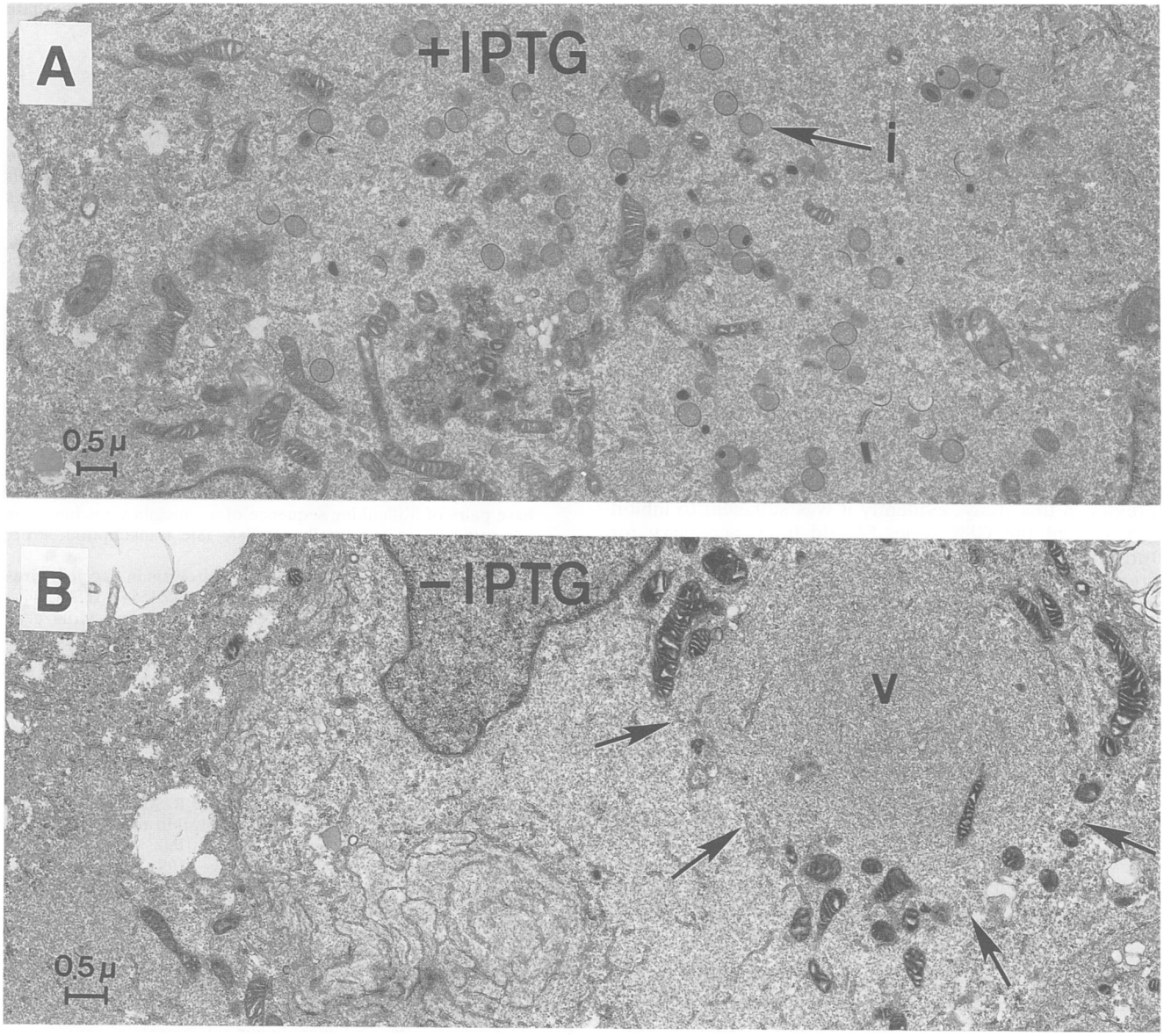

FIG. 9. Electron micrographs of sections of BS-C-1 cells infected with vRO-G8R under permissive and nonpermissive conditions. Infections were carried out at an MOI of 10 PFU per cell in the presence (A) or absence (B) of 5 mM IPTG for 8 h. i, immature viral particles; v, viroplasm, the boundary of which is marked by arrows. Magnification, ×12,000.

DISCUSSION

We have described the construction and characterization of a new inducible conditional-lethal mutant of vaccinia virus, vRO-G8R. The mutant virus expresses the *E. coli lac* repressor, which can bind to the *lac* operator DNA sequence which was inserted adjacent to the RNA initiation site of the G8R gene. This vaccinia virus gene is naturally regulated by an intermediate promoter and encodes a 30,000-Da protein that is synthesized after DNA replication. Evidence that the product of the vaccinia virus G8R gene is a late transcription factor was initially obtained by transfection studies (37) and supported by in vitro experiments (56). We have demonstrated in this study that repression of the G8R gene selectively inhibits late gene expression in vivo. Moreover, late gene expression could be restored either by addition of the inducer IPTG, which inactivates the *lac* repressor, or by transfection of a *lac* operator-negative G8R gene into cells infected with the vRO-G8R mutant.

To ensure that the level of G8R expression was reduced sufficiently, we mutated the G8R promoter in addition to inserting the *lac* operator. For this reason, the amount of G8R protein made in the presence of the inducer was only 10% of that made in cells infected with the WT virus. Nevertheless, this level of expression was adequate for nearly normal levels of virus replication. In the absence of the inducer, G8R protein synthesis was inhibited by more than 99%, causing 3- and 4-log-unit reductions in virus yield and plaque titer, respectively. Whether such stringent regulation could have been achieved without lowering the basal level of G8R transcription was not determined.

Under nonpermissive conditions, there was a 90% reduction in the steady-state amounts of late mRNA but there was little or no effect on the amounts of early or intermediate mRNAs; this is consistent with the notion that G8R serves as a specific late transcription factor. A corresponding decrease in late protein synthesis also was noted. Intermediate proteins generally increased in amount under nonpermissive conditions, perhaps because of reduced competition for the translation apparatus by late mRNAs. The additional possibility that some late gene products serve as regulators of intermediate gene expression needs to be examined.

The *E. coli lac* repressor gene in the recombinant virus was regulated by the vaccinia virus P7.5 promoter, which contains early and late transcriptional elements of about equal activities (14). Previous calculations had indicated that in cells infected with a related recombinant virus, the repressor was made in 1,000-fold excess over the DNA copy number (27). Because of the role of the G8R-encoded protein in late transcription, we considered that inhibition of G8R expression might have caused a 50% reduction in repressor synthesis. Although we did not measure the amount of the repressor in this study, evidently it was sufficient to inhibit expression of the G8R gene. Should it be necessary for future experiments, the amount of the *lac* repressor could be increased either by using a stronger early promoter or by employing an intermediate promoter.

Recently, Carpenter and DeLange (11) mapped the lesion in *ts*C63 (15, 16) to the A1L gene, which encodes another one of the three late transcription factors (37). Under nonpermissive conditions, the phenotypes of *ts*C63 and vRO-G8R are similar, as one would expect for two proteins that act at the same step in the regulation of gene expression. Mutant *ts*C63 makes small plaques and is partially defective in late protein synthesis, even at the permissive temperature, suggesting that the altered protein is not fully active under any conditions. No mutants have been reported for the A2L gene, which encodes a third late transcription factor.

The ability to selectively inhibit late gene expression while allowing continued synthesis of early and intermediate proteins, as well as viral DNA, provides a useful adjunct to inhibitors of DNA replication which prevent the expression of both classes of postreplicative genes. Thus, resolution of concatemeric forms of vaccinia virus DNA is defective under nonpermissive conditions with both *ts*C63 (11) and vRO-G8R, indicating that late transcription or gene expression is required. In a similar manner, we demonstrated that early and intermediate gene expression is insufficient for even the first stages of virion morphogenesis. In cells infected with vRO-G8R, only dense, relatively amorphous regions of viroplasm were observed in the absence of the inducer whereas morphogenesis appeared normal in the presence of the inducer. In contrast, when the *lac* repressor was used to regulate genes that encode specific late structural proteins, morphogenesis was interrupted at more advanced stages (61, 62).

Both the vRO-G8R and *ts*C63 mutants appear to be useful for determining which of the postreplicative vaccinia virus genes belong to the intermediate and late classes. We detected at least 13 possible intermediate proteins and an equal number of late ones on the basis of whether synthesis was increased or decreased, respectively, upon repression of the G8R gene. Since one-dimensional SDS-PAGE of [^{35}S] methionine-labeled proteins typically resolves only the most dominant species, the potential number of intermediate proteins may be quite large. We are now trying to identify the genes that encode some of the candidate intermediate proteins.

ACKNOWLEDGMENTS

We thank the following members of our laboratory: C. J. Baldick, for sharing his unpublished G8R promoter mutagenesis data with us and for advice and materials for RNase protection analysis and transient expression, and M. Merchlinsky, for advice and materials for viral DNA analysis. We also thank C. Wright (Armed Forces Institute of Pathology) for antiserum against the G8R protein.

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