JOURNAL OF VIROLOGY, Nov. 1996, p. 7669–7677 Vol. 70, No. 11
0022-538X/96/$04.00+0

De Novo Synthesis of the Early Transcription Factor 70-Kilodalton Subunit Is Required for Morphogenesis of Vaccinia Virions

XIAOLEI HU, LAWRENCE J. CARROLL,† ELIZABETH J. WOLFFE, AND BERNARD MOSS*

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892-0445

Received 12 April 1996/Accepted 23 July 1996

Vaccinia virus early transcription factor (VETF) is a heterodimeric protein that is packaged in virus particles for expression of early genes during the next round of infection. To investigate additional roles of VETF, we constructed a conditionally lethal recombinant vaccinia virus in which the D6R gene, encoding the 70-kDa subunit of VETF, is under stringent *Escherichia coli lac* operator control. When cells were infected with the recombinant virus in the absence of an inducer, synthesis of the 70-kDa protein was undetectable and the yield of infectious virus was severely reduced. Under these nonpermissive conditions, DNA replication and synthesis of viral proteins other than the one encoded by D6R occurred, suggesting that de novo synthesis of VETF is not required for expression of early or late genes during the virus growth cycle. Electron microscopy, however, revealed that immature virus particles and masses of electron-dense material accumulated in the absence of an inducer. We concluded that VETF has a direct role in virion morphogenesis or is required for expression of a novel subset of genes that have such a role.

Vaccinia virus, a member of the poxvirus family, is a large, double-stranded DNA virus that replicates within the cytoplasm of infected cells (30). The nearly 200 viral genes are expressed in a temporally regulated fashion. Vaccinia virus particles contain RNA polymerase and other factors for transcription of early genes, which occurs immediately after infection (21, 33). Viral early genes encode proteins required for intermediate transcription, which follows viral DNA replication (36, 37, 41, 42); viral early and intermediate genes encode factors for late transcription (22–24, 45, 46), and late genes encode early transcription factors that are packaged into assembling virions (8, 10, 19).

The proteins needed for transcription of early genes include the viral multisubunit RNA polymerase (6, 31, 40), the RNA polymerase-associated protein (RAP94) (1, 2, 11), the vaccinia virus early transcription factor (VETF) (8, 10, 19), and the capping enzyme-termination factor (28, 39), as well as several other enzymes whose roles remain to be determined. Additional virus-encoded enzymes, such as the mRNA (nucleoside 2′-*o*)-methyltransferase (5, 38) and poly(A) polymerase (18, 32), are required for mRNA modification.

It seems unlikely that all of the enzymes and factors needed for the synthesis of early mRNA are randomly incorporated into virions. A clue to the possible packaging mechanism was obtained by analysis of a conditionally lethal mutant of vaccinia virus with an inducible RAP94 gene. Zhang et al. (48) demonstrated that when RAP94 expression was repressed, normal-appearing virus particles lacking detectable amounts of the viral RNA polymerase were formed. Also absent from these particles were a multitude of enzymes known or suspected to be involved in the synthesis and processing of early mRNA. VETF was present in these defective particles, and Zhang et al. (48) suggested that the protein is targeted to early promoter sequences in the viral genome and functions as a receptor for RAP94 and the associated RNA polymerase. They further speculated that additional enzymes form a multicomponent complex with RNA polymerase, ensuring their packaging. This model is attractive for several reasons. First, the assembly of a multiprotein transcription complex obviates the need for each protein to have an independent virion targeting signal. Second, the model is consistent with the DNA binding property of VETF (10, 10a, 47), the ability of VETF to recruit RNA polymerase for transcription (4, 25), and the proposed role of RAP94 in mediating the VETF-RNA polymerase interaction (2). Studies by Li et al. (27) with a temperature-sensitive VETF mutant are consistent with this model. Virions which formed at the nonpermissive temperature had approximately half the expected amount of VETF and corresponding amounts of RNA polymerase, capping enzyme, and nucleotide phosphohydrolase I.

VETF contains 82- and 70-kDa subunits encoded by the A8L and D6R genes, respectively (8, 19). DNA-protein cross-linking studies indicated that the large and small subunits interact with nucleotides in the core region of the promoter and downstream of the RNA start site, respectively (10a). The DNA-dependent ATPase of VETF (7, 9) is associated with the 70-kDa subunit (26), whereas no activity has been reported for the 82-kDa subunit. Here, we describe the construction and properties of a conditionally lethal vaccinia virus mutant in which expression of the gene encoding the 70-kDa subunit is stringently regulated. The results indicated that VETF has an earlier role in virion maturation than anticipated.

MATERIALS AND METHODS

Cells and viruses. BS-C-1 (ATCC CCL26) and CV-1 (ATCC CCL70) cells were grown in Eagle minimum essential medium (EMEM; Quality Biologicals) containing 10% fetal bovine serum (FBS). Suspension and monolayer cultures of HeLa S3 cells (ATCC CCL2.2) were grown in EMEM modified for spinner culture (Quality Biologicals) containing 5% horse serum and Dulbecco minimum essential medium (Quality Biologicals) containing 10% FBS, respectively. Vaccinia virus strain WR (ATCC Vr119) stocks were prepared in HeLa S3 monolayers containing 2% FBS at 37°C as previously described (14). Cells were harvested 72 h after infection, centrifuged, resuspended in EMEM with 2% FBS, frozen and thawed three times, sonicated for 1 min, and stored at −80°C. Stocks

* Corresponding author. Mailing address: Laboratory of Viral Diseases, Building 4, Room 229, National Institutes of Health, 4 Center Dr., MSC 0445, Bethesda, MD 20892-0445. Phone: (301) 496-9869. Fax: (301) 480-1147. Electronic mail address: bmoss@nih.gov.

† Present address: U.S. Patent Office, Arlington, VA 22241.

of conditionally lethal mutant virus were made in a similar fashion, except that the medium was supplemented with 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG).

Construction of a plasmid transfer vector containing an inducible D6R gene. A copy of the D6R open reading frame was made by PCR with primers that added a *Hin*dIII site followed by nucleotides T and C at the 5′ end of the gene (which generated a *Bsp*HI site) and a *Pst*I site at the 3′ end and cloned between the *Hin*dIII and *Pst*I sites of pUC18. The resulting plasmid, pLJC157, was digested with *Bsp*HI to generate a 5′ *Bsp*HI-*Bsp*HI fragment of the D6R gene, which was cloned into the *Nco*I site of pMITEO*lac*.20/3. The latter plasmid is a *lacZ*-containing precursor of pVOTE1 containing the vaccinia virus hemagglutinin (HA) gene interrupted by the *Escherichia coli gpt* gene, which is regulated by the vaccinia virus P7.5 promoter, and the *lacZ* gene, which is preceded by the T7 promoter, *lacO*, and the encephalomyocarditis virus leader (43). A plasmid having the D6R open reading frame in the correct orientation was obtained and then cut with *Afl*II and *Bam*HI, which removed part of the D6R sequence and all of the *lacZ* sequence; an *Afl*II and *Bam*HI fragment from pLJC157 was then inserted to generate the complete open reading frame of D6R, followed by a polylinker. The resulting transfer vector, pLJC157/MITEOlac.20/3, contained, from left to right, the left flank of the vaccinia virus HA gene, the *gpt* gene-P7.5 promoter, the T7 promoter-*lacO*-encephalomyocarditis virus leader-D6R open reading frame, and the right flank of the HA gene.

Generation of recombinant vaccinia virus containing an inducible D6R gene. The parental virus vT7lacOI (3) contains the T7 RNA polymerase gene under the control of the vaccinia virus late P11 promoter and *E. coli lacO*, as well as the *E. coli lac* repressor (*lacI*) gene regulated by the vaccinia virus early-late P7.5 promoter, inserted into the thymidine kinase gene of vaccinia virus strain WR. Homologous recombination was carried out in a T-25 flask of CV-1 cells essentially as previously described (14). The cells were incubated with mycophenolic acid (MPA)-containing medium for 2 h and then infected with approximately 0.05 PFU of VT7lacOI per cell in serum-free EMEM containing MPA. After 1 h, the inoculum was removed and replaced with 1 ml of EMEM plus MPA containing 5 μg of pLJC157/MITEO*lac*.20/3 DNA and 10 μl of transfectam (Promega). After 4 h, the transfection mixture was removed and replaced with EMEM plus MPA. After 72 h, the cells were harvested and the recombinant viruses were plaqued under agar in the presence of MPA. After three successive rounds of plaque purification, small virus stocks were prepared and their genotype was verified by Southern blotting. This recombinant virus, containing both an inducible copy and an endogenous copy of the D6R gene, was designated vD6ind$^+$end$^+$ (code name vLJC2).

Construction of a plasmid transfer vector containing a deleted copy of the D6R gene. The neomycin resistance gene (*neo*) was prepared from pVVNEO (15) by *Sal*I digestion, followed by Klenow treatment and subsequent *Bam*HI digestion. The 5′ oligonucleotides used to PCR amplify the 5′ flank of the D6R gene were 5′-GGGGGGAAGCTTCCTGTTTTTGATAGGATAGATAACG-3′ and 5′-GGGGGGGTCGACTTCTATAAATATATGAGCATATATT-3′. The oligonucleotides used to amplify the 3′ flank of the D6R gene were 5′-GGGGGGAGCTCTTCGTGTTTATACTTACCGCTATTC-3′ and 5′-GCGCGGATCCGGTATCTTCTCCATAAAACTGATG-3′. The three DNA segments were ligated into a pUC18 derivative to form pD6KO. This plasmid retained no D6 5′ coding sequence and only 13 bp of the D6 3′ coding sequence.

Deletion of the endogenous copy of the D6 gene. CV-1 cells were infected with vD6ind$^+$end$^+$ and transfected with pD6KO by using lipofectamine (Promega) as described above, except that 50 μM IPTG was present in the medium used to replace the transfection mixture. After 3 days, the cells were harvested and 0.1 volume of the lysate was used to infect a T-25 flask of BS-C-1 cells that had been incubated for the previous 24 h in EMEM supplemented with 10% FBS, 50 μM IPTG, and 2 μg of G418 (Life Technologies) per ml. After 2 days, the infected cells were harvested and passaged two more times in the presence of G418 and IPTG. After the third round of enrichment, the virus was plaqued in BS-C-1 cells that had been preincubated with G418 and IPTG by using 1% agar containing both drugs. After 2 days, the materials from isolated plaques were placed in 0.5 ml of EMEM supplemented with 10% FBS and 50 μM IPTG but without G418 and sonicated. Each suspension was then used to overlay a single well of a 12-well plate of BS-C-1 cells. The cells were harvested after extensive cytopathic effects were evident and resuspended in 0.2 ml of phosphate-buffered saline. Half of each suspension was used to purify DNA for Southern blot analysis.

The DNA was digested with *Sal*I, separated on a 0.8% agarose gel, and blotted onto a nylon membrane by using standard procedures. The blot was probed in QuikHyb solution (Stratagene) with a ^{32}P-labeled, randomly primed fragment corresponding to the C-terminal 1,700 bp of the D6R open reading frame. Potential knockout viruses were identified and assayed for plaque formation in the absence of IPTG. Viruses that were unable to form normal-size plaques in the absence of IPTG were then subjected to two additional rounds of plaque purification in the presence of G418 and IPTG. Isolated plaques were selected and amplified.

Plaque assay. BS-C-1 cells, grown as monolayers in six-well plates, were infected with 10-fold serial dilutions of viruses for 1 h and incubated at 37°C for 2 days in EMEM supplemented with 2% FBS and 50 μM IPTG when appropriate. The cells were then stained with crystal violet, and the plaques were counted.

One-step virus growth. Monolayers of BS-C-1 cells were inoculated with 10 PFU of virus per cell for 1 h at 37°C. The incubation was continued with or without IPTG, and the cells were harvested at various times, frozen and thawed three times, sonicated, and stored at −80°C. Virus titers were determined by plaque assay.

Immunoprecipitation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). BS-C-1 cells in six-well plates were inoculated with 10 PFU of vaccinia virus per cell at 37°C for 1 h and then incubated at the same temperature for various periods. The infected cells were lysed in RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) at 4°C for 10 min, and cell debris was sedimented by full-speed centrifugation in a microcentrifuge at 4°C for 5 min. The supernatant was incubated with antibody at 4°C overnight and then with excess protein A agarose beads for 2 h. The beads were sedimented and washed twice with RIPA buffer and once with 25 mM Tris buffer (pH 8.8). SDS-PAGE sample buffer was added to the beads and heated at 100°C for 3 min, the beads were removed, and the supernatant was applied to the gel.

Western blot (immunoblot) analysis. Virus-infected BS-C-1 cell monolayers were lysed in RIPA buffer at 18 h after infection. Cell debris was removed by centrifugation in a microcentrifuge, and SDS-PAGE sample buffer was added to the supernatant. After SDS-PAGE, the proteins were transferred to a nylon filter membrane, which was subsequently incubated with antibodies and stained in accordance with the manufacturer's (Bio-Rad) protocol.

DNA slot blot analysis. Virus-infected BS-C-1 cells were incubated for 6 h at 37°C in lysis buffer (20 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.75% SDS, 0.6 mg of proteinase K per ml) and the DNA was extracted with phenol and phenol-chloroform, precipitated in ethanol, and resuspended in TE (10 mM Tris-HCl [pH 8.0]), 1 mM EDTA). Probe DNA was prepared from the vaccinia virus genome by labeling with [α-^{32}P]dCTP by using the random-priming procedure. DNA was denatured in 0.25 N NaOH for 10 min, diluted in 0.125 N NaOH–0.125× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and applied to a nylon membrane in a vacuum manifold. Hybridization was carried out by using the QuikHyb protocol (Stratagene). Briefly, the membrane was incubated in QuikHyb solution at 68°C for 30 min and this was followed by hybridization with the probe at 68°C for 1 h. The membrane was washed twice with 2× SSC–0.1% SDS at 60°C for 15 min each time and then with 0.1× SSC–0.1% SDS at 60°C for 30 min. The membrane was air dried for autoradiography.

Pulse-labeling and chase. BS-C-1 monolayer cells in 60-mm-diameter dishes were infected with 10 PFU of virus per cell and labeled with 50 μCi of [^{35}S]methionine for 30 min and then chased in nonradioactive medium containing normal amounts of methionine. Cells were lysed in SDS-PAGE sample buffer, and the lysates were applied to a 10% gel.

Gel mobility shift assay. A 77-bp DNA segment containing the vaccinia virus growth factor promoter, excised from plasmid Vep40 (4), was labeled with [α-^{32}P]dCTP by using the random-priming procedure. Cell lysates were prepared as for Western blotting. Approximately 10,000 cpm of probe (3 ng) and various amounts of poly(dI)-poly(dC) were mixed with each lysate in binding buffer (50 mM Tris-HCl [pH 7.4], 250 mM KCl, 25% glycerol, 0.25% Nonidet P-40, 1 mM EDTA) and incubated on ice for 30 min. The mixtures were applied to a 4% agarose gel, and electrophoresis was done at 4°C.

Electron microscopy. Monolayers of BS-C-1 cells in 60-mm-diameter dishes were infected with virus in the presence or absence of 50 mM IPTG and subsequently fixed in 2% glutaraldehyde (EM Sciences) in 100 mM phosphate buffer, pH 7.4, for 1 h. Cells were then prepared in Epon resin for transmission electron microscopy by osmication, dehydration, and embedding. Thin sections were cut, collected on Formvar-coated copper mesh grids (Polysciences), and stained with 2% uranyl acetate and Reynolds' lead citrate (34). Samples were viewed with a Philips CM100 electron microscope.

RESULTS

Construction of a recombinant vaccinia virus with the D6R gene regulated by *E. coli lacO*. The method chosen to regulate expression of the D6R gene, encoding the 70-kDa subunit of VETF, differed from the standard one in which *lacO* is placed adjacent to the promoter of the target gene (16, 35, 49). The stringency of regulation was increased by using *lacO* to repress two successive steps: transcription of a vaccinia virus promoter-regulated bacteriophage T7 RNA polymerase gene and a T7 promoter-regulated target gene. This approach was developed for expression of foreign genes (43) but has since been adapted for control of vaccinia virus genes (44).

A two-step procedure was used to construct the desired recombinant vaccinia virus. We started with recombinant vaccinia virus vT7lacOI, which contains the T7 RNA polymerase gene (regulated by the vaccinia virus late P11 promoter and *lacO*) and the *E. coli lacI* gene (regulated by the vaccinia virus

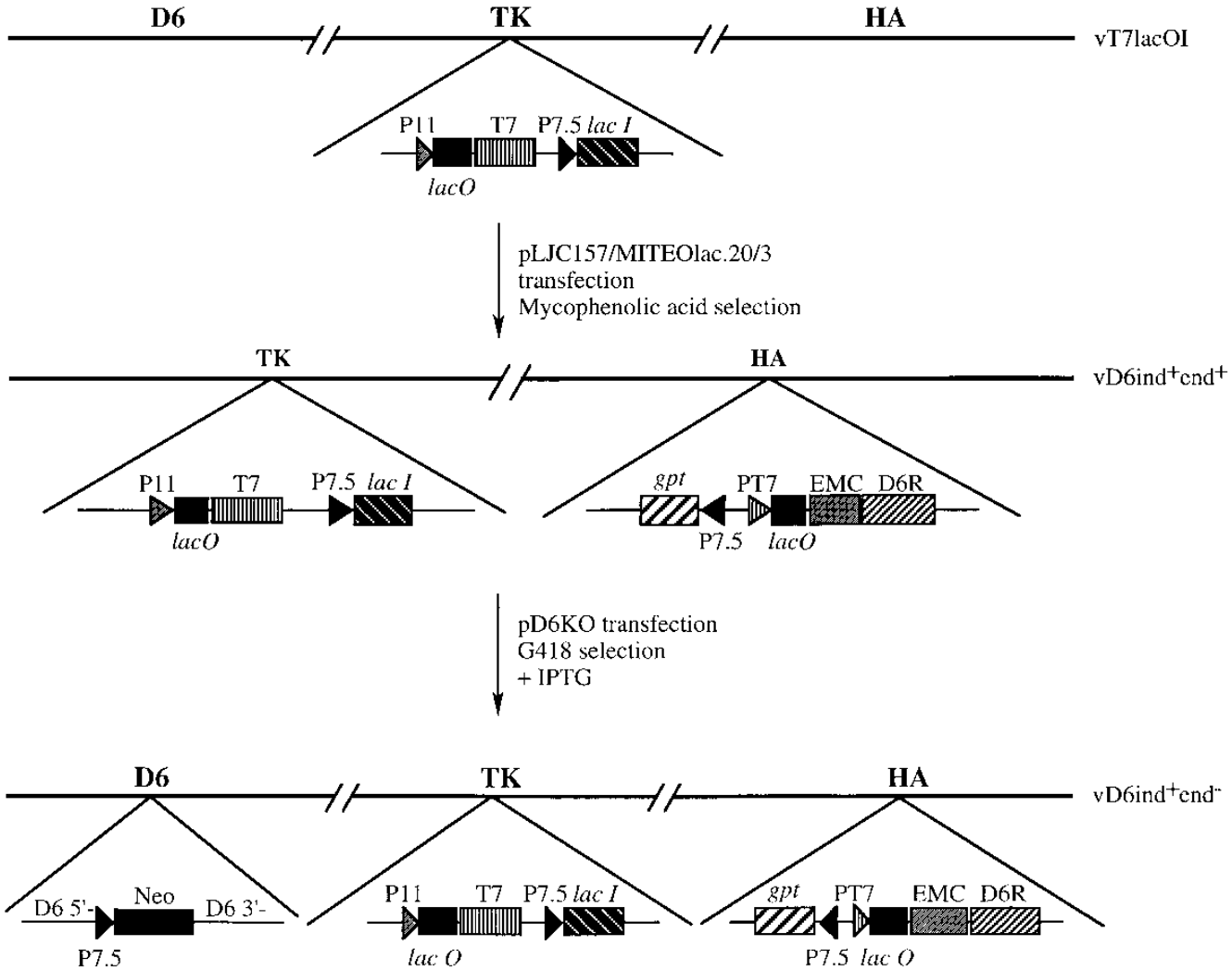

FIG. 1. Steps in the construction of an inducible D6R conditionally lethal vaccinia virus mutant. Plasmid transfer vector pLJC157/MITEOlac.20/3 was used to insert a regulatable copy of the D6R gene and the *gpt* gene as a selection marker into the HA region of a previously made recombinant vaccinia virus, vT7lacOI. Recombinant vaccinia virus vD6ind$^+$end$^+$, containing both inducible and endogenous copies of the D6R gene, was isolated. A second plasmid transfer vector, pD6KO, was then used to delete the endogenous D6R gene of vD6ind$^+$end$^+$ by inserting the *neo* gene as a selection marker. The final virus, vD6ind$^+$end$^-$ (also called vD6ind for short), was isolated. Vaccinia virus promoter elements P11 and P7.5 are active at late times and at early and late times, respectively. PT7 refers to the phage T7 ϕ10 promoter (12). Directions of transcription are indicated by arrowheads. EMC and T7 refer to a copy of the encephalomyocarditis virus untranslated region which confers cap-independent translation and the T7 RNA polymerase gene, respectively. TK, thymidine kinase.

early-late P7.5 promoter) in the thymidine kinase locus (Fig. 1). The D6R gene (under T7 promoter and *lacO* control) plus the *E. coli gpt* gene (under vaccinia virus P7.5 promoter control) for antibiotic selection were then inserted into the HA locus. The resulting virus, vD6ind$^+$end$^+$, contains inducible and endogenous copies of the D6R gene. Next, the endogenous D6R gene was replaced with the *neo* gene (under vaccinia virus P7.5 promoter control). The latter step was carried out in the presence of IPTG to induce D6R expression and G418 to select for antibiotic-resistant recombinant virus. The resulting virus, vD6ind$^+$end$^-$ (vD6ind for short) contains only the inducible copy of the D6R gene. The presence of endogenous and inducible copies of the D6R gene in vD6ind$^+$end$^+$ but only the inducible copy in vD6ind was demonstrated by Southern blotting (data not shown).

Effect of IPTG on virus growth. The abilities of wild-type vaccinia virus (WR), vD6ind$^+$end$^+$, and vD6ind to produce plaques in the presence and absence of IPTG were determined (Fig. 2). For WR and vD6ind$^+$end$^+$, the number and size of plaques were unaffected by the presence or absence of IPTG because both contain endogenous, unregulated copies of the D6R gene. For vD6ind, however, plaque formation was IPTG dependent because the endogenous D6R gene was deleted (Fig. 2). With prolonged incubation times, some tiny plaques could be detected in the absence of the inducer (data not shown). We noted that the plaques formed by both vD6ind$^+$end$^+$ and vD6ind under permissive conditions were smaller than those formed by the wild-type virus, perhaps because of the multiple genetic alterations in the recombinant viruses.

Virus yields were determined under one-step growth conditions. IPTG dependence was noted only with vD6ind (Fig. 3). Without IPTG, the 48-h yield of vD6ind was severely reduced whereas that of vD6ind$^+$end$^+$ was unaltered. The yields were similar at IPTG concentrations of 20 to 200 μM (data not shown). Maximal yields of vD6ind were obtained when IPTG

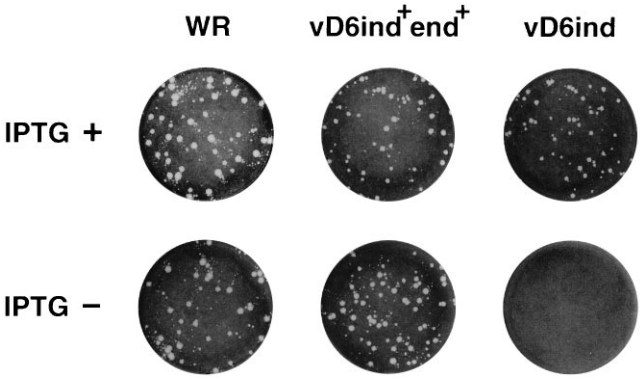

FIG. 2. Effect of IPTG on plaque formation. Monolayers of BS-C-1 cells in six-well plates were infected with the wild-type virus (WR), vD6ind$^+$end$^+$, or vD6ind in the presence (+) or absence (−) of 50 μM IPTG. After 48 h, the cells were stained with crystal violet.

was given at the time of infection, and progressively lower yields were obtained when administration was delayed by more than a few hours (data not shown). Conversely, maintenance of IPTG was required for maximal vD6ind yields. The higher maximal yields of WR than recombinant viruses, were consistent with the plaque data. Since the wild-type and recombinant viruses have multiple genetic differences and replicate to slightly different levels, the most important comparisons in the following sections are between the same virus in the presence and absence of IPTG.

Inducer-dependent synthesis of the 70-kDa subunit of VETF. Several experiments were carried out to ensure that synthesis of the 70-kDa subunit of VETF was stringently regulated and that functional VETF was made in the presence of the inducer. At 4 h, the labeled 70-kDa protein was immunoprecipitated from lysates of cells infected with wild-type vaccinia virus but not from cells infected with vD6ind (Fig. 4A). At 6 h, synthesis of the 70-kDa protein was detected in cells infected with vD6ind in the presence of IPTG but not in its absence. There was still no detectable 70-kDa protein made by vD6ind in the absence of IPTG at 12 or 18 h (Fig. 4A and B), whereas in the presence of IPTG the amount synthesized was about six times greater than that made by the wild-type virus. Further studies demonstrated that maximal synthesis of the 70-kDa protein occurred with IPTG concentrations between 20 and 100 μM (Fig. 4B). Corresponding data demonstrating IPTG dependence were obtained by Western blotting in experiments carried out for 72 h after infection (data not shown).

Detectable amounts of the 82-kDa VETF subunit were not coprecipitated from lysates of wild-type-virus-infected or vD6ind-infected cells by a polyclonal antibody to the 70-kDa subunit (Fig. 4A and B), and additional studies are needed to determine whether this is due to the ratios of the two VETF polypeptides, the specificity of the antibody, or the conditions of immunoprecipitation and washing. However, the 70-kDa subunit was precipitated by an antibody to the 82-kDa subunit when cells were infected with vD6ind in the presence of IPTG but not in its absence (Fig. 5). This experiment also showed that synthesis of the 82-kDa subunit was unaffected by omission of IPTG, although less protein was immunoprecipitated after the chase period under these conditions. The 82-kDa protein was resolved as a doublet, a feature not previously noted.

The formation of native VETF was examined by a gel mobility shift assay with a ^{32}P-labeled early promoter probe. A protein extract prepared from cells infected with vD6ind in the presence of 50 μM IPTG shifted the probe, to the same position as did purified VETF, in a poly(dI)-poly(dC)-resistant manner (Fig. 6). By contrast, the complex formed by extracts from cells infected in the absence of IPTG was faint and had a slightly faster mobility than authentic VETF; this band corresponded to a complex formed by extracts of uninfected cells and was efficiently competed against by poly(dI)-poly(dC) (Fig. 6).

Taken together, the above-described experiments established that expression of the 70-kDa subunit of VETF was stringently regulated by IPTG. We next sought to determine the steps in virus replication that were dependent on this protein.

Effect of IPTG on viral DNA replication. Vaccinia virus DNA replication occurs in the cytoplasm of infected cells and can be detected by hybridization to a ^{32}P-labeled probe. In cells infected with the wild-type virus or vD6ind, viral DNA increased between 2 and 6 h after infection (Fig. 7). Importantly, IPTG had no significant effect on the accumulation of viral DNA in cells infected with vD6ind, although more was detected in cells infected with the wild-type virus. We concluded that de novo synthesis of the 70-kDa subunit of VETF is not required for viral DNA replication.

Effect of IPTG on viral protein synthesis. Since host protein synthesis is inhibited during vaccinia virus infection, the synthesis of viral proteins can be readily discerned by amino acid pulse-labeling. Generally, the late pattern of viral protein synthesis and inhibition of host protein synthesis is established at about 6 h after infection, although the timing is sensitive to the multiplicity of infection and titer of the virus stock. In the experiment whose results are depicted in Fig. 8, we infected replicate BS-C-1 cell monolayers with vD6ind$^+$end$^+$ or vD6ind in the presence or absence of IPTG. At the indicated times,

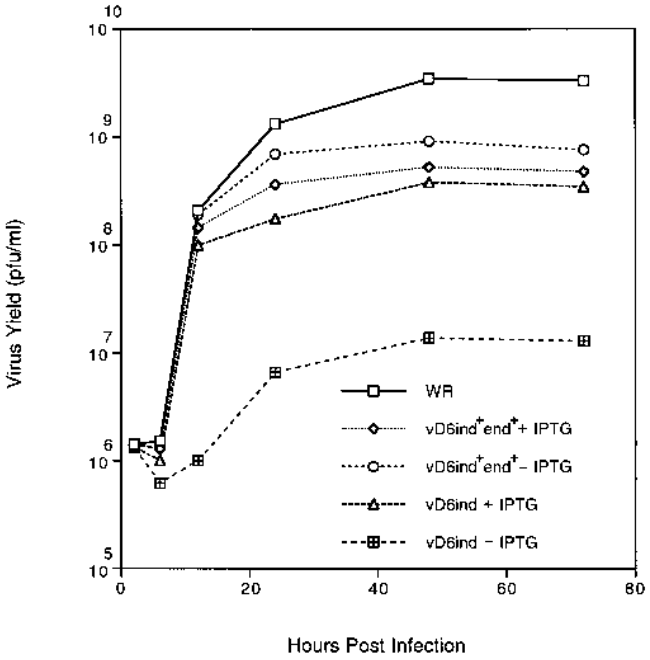

FIG. 3. Effect of IPTG on virus yields. BS-C-1 cells were infected with 10 PFU of wild-type vaccinia virus (WR), or vD6ind$^+$end$^+$, or vD6ind per cell in the presence (+) or absence (−) of 50 μM IPTG. The cells were harvested at the indicated times after infection, and the virus titers were determined by plaque assay in the presence of 50 μM IPTG. The results of two separate experiments were averaged.

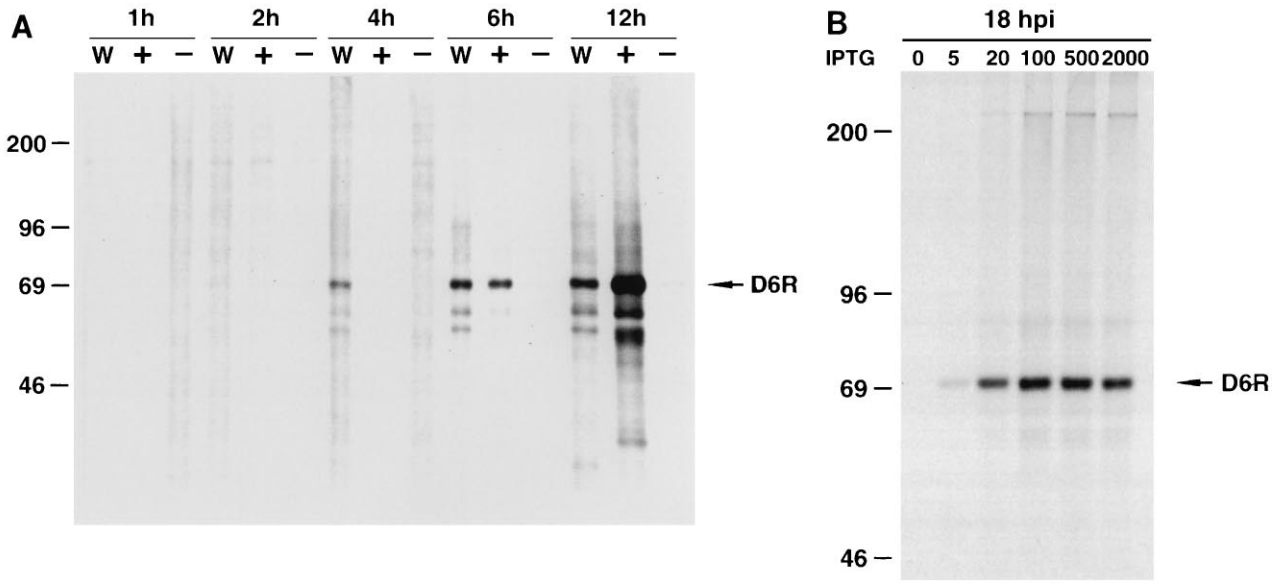

FIG. 4. Inducible synthesis of the D6R subunit of VETF. (A) BS-C-1 cells were infected with the wild-type virus (W) or vD6ind in the presence (+) or absence (−) of 50 μM IPTG and labeled with [^{35}S]methionine at the indicated times after infection. Cell lysates were incubated successively with a polyclonal antibody to the D6R protein and protein A agarose beads, and the bound materials were analyzed by SDS-PAGE. The position of the D6R protein is indicated. Protein marker sizes are indicated in kilodaltons on the left. (B) Same as panel A, except that the concentration of IPTG was varied from 0 to 2,000 μM and labeling was done at 18 h after infection.

individual plates were incubated with [^{35}S]methionine for 30 min. The cells were then harvested, lysed, and analyzed by SDS-PAGE and autoradiography. The pattern of viral late protein synthesis was established between 6 and 12 h with the shutoff of host protein synthesis being somewhat delayed for vD6ind. Except for induction of the D6R and T7 polymerase polypeptides, IPTG had little or no effect on the number or

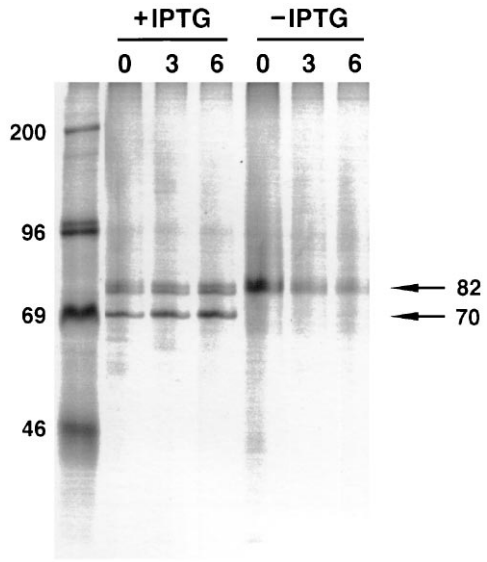

FIG. 5. Coprecipitation of the induced D6R subunit of VETF with the A8L subunit. Replicate wells containing BS-C-1 cells were infected with vD6ind in the presence or absence of 50 μM IPTG for 12 h and incubated with [^{35}S]methionine for 30 min. Individual wells were chased for 0, 3, or 6 h, and lysates were prepared. Proteins immunoprecipitated with an antibody to the A8L protein were analyzed by SDS-PAGE and autoradiography. Marker sizes (in kilodaltons) are shown at the left, and the positions of the A8L 82-kDa and D6R 70-kDa VETF subunits are on the right.

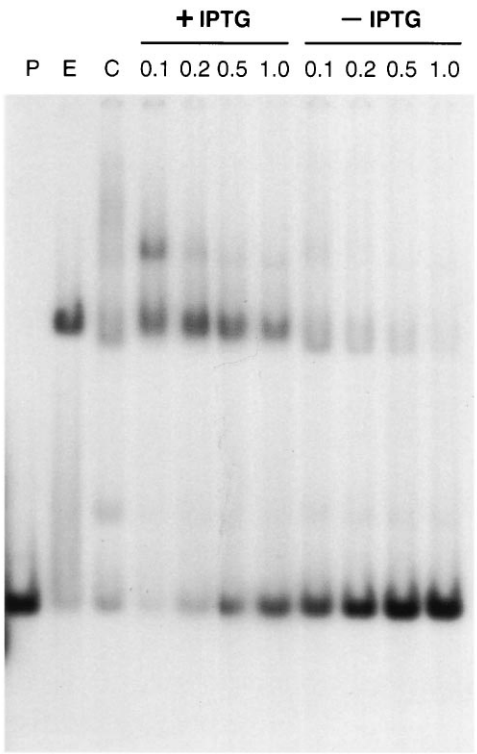

FIG. 6. Induction of early promoter DNA-binding activity. BS-C-1 cells were infected with 10 PFU of vD6ind per cell in the presence or absence of 50 μM IPTG. Extracts, prepared from the cells at 18 h after infection, were incubated with 3 ng of a ^{32}P-labeled DNA segment containing a vaccinia virus early promoter, 0.1 to 1.0 μg of poly(dI)-poly(dC), and subjected to agarose gel electrophoresis. As controls, the probe alone (P), the probe incubated with purified VETF (E), and the probe incubated with a lysate of mock-infected cells (C) were analyzed.

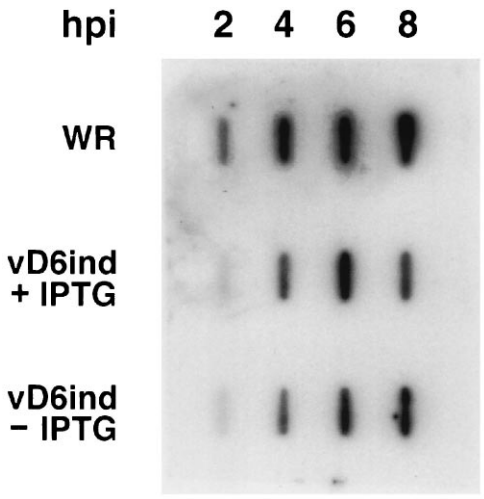

FIG. 7. Synthesis of viral DNA. BS-C-1 cells were infected at 10 PFU of wild type (WR) or vD6ind per cell in the presence (+) or absence (−) of 50 μM IPTG. Cells were harvested at the indicated number of hours postinfection (hpi). DNA from the infected cells was applied to a nylon filter membrane in a slot blot apparatus and hybridized with [α-^{32}P]dCTP-labeled vaccinia virus genomic DNA.

intensity of polypeptides for either vD6ind^{+}end^{+} or vD6ind, even though IPTG was required for replication of the latter. A minor band of approximately 60 kDa, that was increased in the presence of IPTG, corresponded to a polypeptide of that size immunoprecipitated with a specific antibody (Fig. 4A), suggesting that it may be an altered form of the D6R protein. In other experiments (data not shown), the patterns of proteins made in cells infected with WR and vD6ind were compared; except for the additional recombinant polypeptides, the patterns were identical, although the kinetics of infection were more rapid with WR. The absence of any detectable difference in the spectrum of viral polypeptides made by vD6ind, with or without IPTG, led us to seek other explanations for the dependence on IPTG for infectious virus formation.

Morphogenesis of viral particles under permissive and nonpermissive conditions. Attempts to purify virions by sucrose gradient centrifugation from lysates of cells infected with vD6ind under nonpermissive conditions were unsuccessful, suggesting a defect in their formation (data not shown). To examine this possibility, we fixed cells at various times from 12 to 48 h after infection and examined sections by transmission electron microscopy. In the absence of IPTG, clusters of typical immature virions (including some with dense spots called nucleoids) were seen at all of the times examined (Fig. 9A). However, the immature virions were frequently adjacent to large granular masses that were uncommon in productive vaccinia virus infection. Some of the immature virions were filled with a dense material that lacked an internal structure typical of mature virions. In addition, these dense immature particles consistently appeared round, suggesting that they are spherical rather than brick shaped in three dimensions. Mature-appearing virions were rare in the absence of IPTG, even after 48 h in most cells. By contrast, the images of cells infected with vD6ind in the presence of IPTG (Fig. 9B) were similar to those of wild-type-virus-infected cells: fields containing both immature and typical mature virions were found at all times examined between 12 and 48 h.

DISCUSSION

The D6R gene of vaccinia virus encodes the 70-kDa subunit of the early transcription factor VETF. This report describes

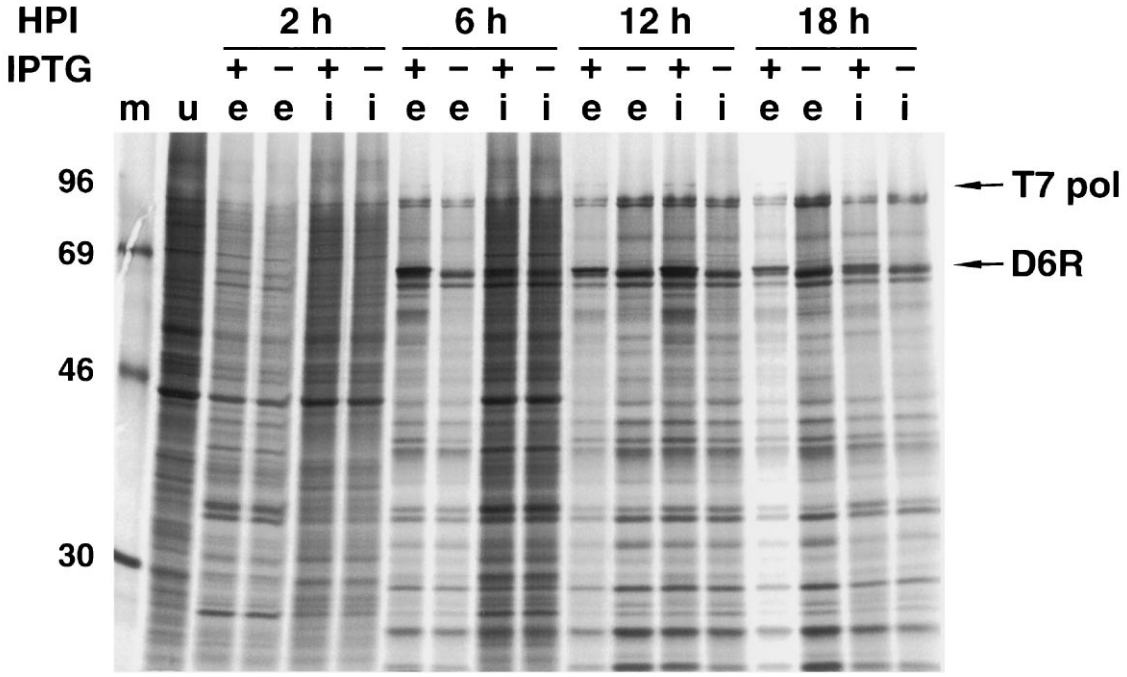

FIG. 8. Synthesis of viral proteins. BS-C-1 cells were infected with 20 PFU of vD6ind^{+}end^{+} (lanes e) or vD6ind^{+}end^{-} (lanes i) per cell in the presence (+) or absence (−) of 50 μM IPTG. Cells were pulse-labeled for 30 min with [^{35}S]methionine at the indicated number of hours postinfection (HPI), lysed in SDS sample buffer, and analyzed by SDS-PAGE. An uninfected-cell lysate (lane u) was used as a control. Protein marker (lane m) sizes are indicated in kilodaltons on the left. The arrows on the right point to the faint band corresponding to T7 RNA polymerase (T7 pol) and to the D6R subunit of VETF, which appears as the upper band of a doublet.

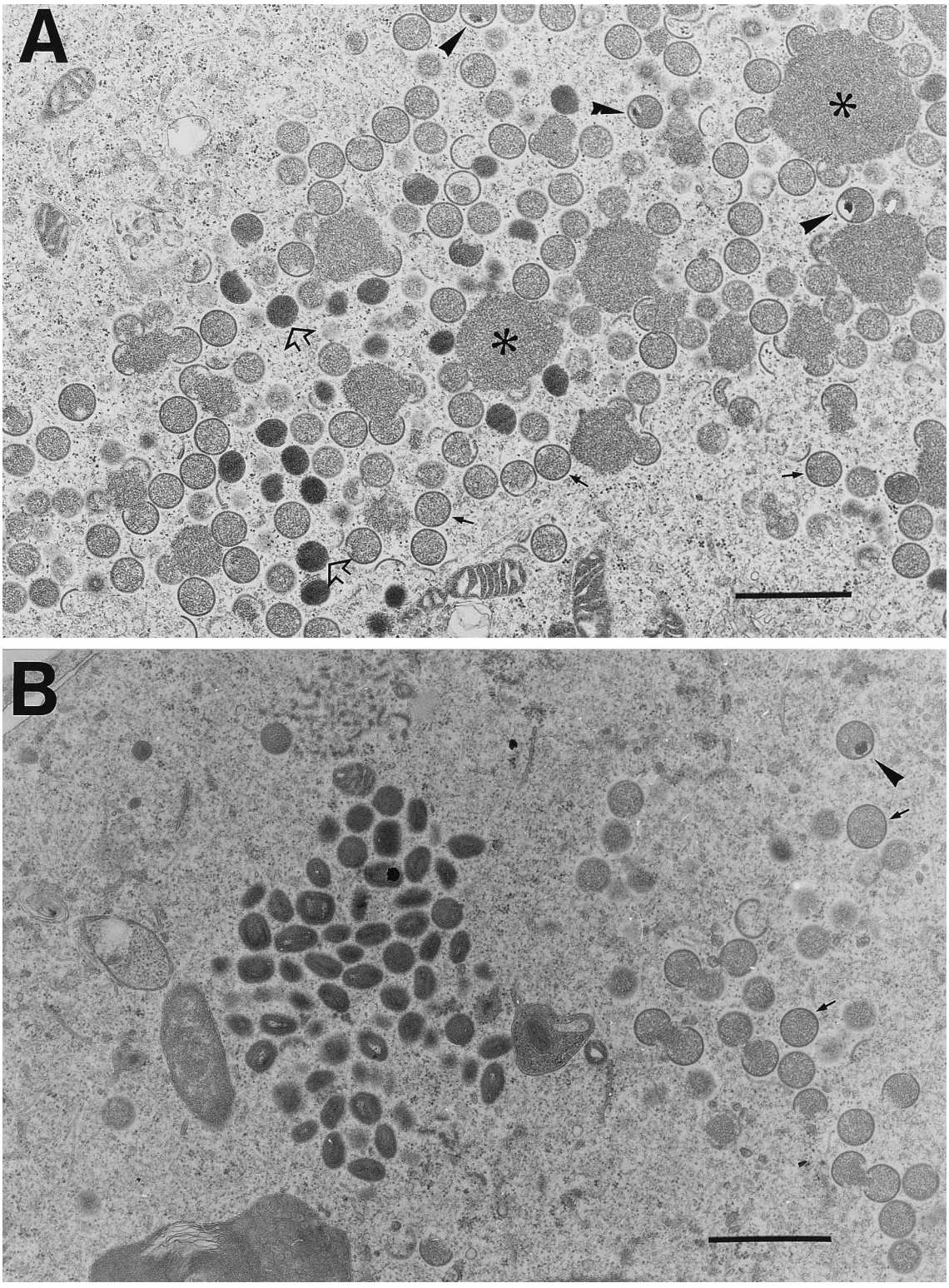

FIG. 9. Electron microscopy of ultrathin sections of infected BS-C-1 cells infected with vD6ind in the absence (A) and presence (B) of 50 μM IPTG. Examples of typical immature particles (→), immature particles with nucleoids (➤), dense immature particles (⇒), and large granular masses (*) are indicated. Bars, μm.

the construction and biological properties of two genetically modified vaccinia viruses: vD6ind$^+$end$^+$, which contains endogenous and inducible copies of the D6R gene, and vD6ind, which contains only the inducible copy. The yield of vD6ind was severely reduced in the absence of IPTG, whereas that of vD6ind$^+$end$^+$ was unaffected. Although the yields of the two recombinant viruses in the presence of IPTG were similar to each other, neither replicated to as high a level as the wild-type

virus, presumably because of the extensive genetic alterations. For this reason, the most important comparisons were between cells infected with the same virus in the presence and absence of IPTG.

Stringent transcriptional regulation of the D6R was achieved by modification of a recently described inducible vaccinia virus-bacteriophage T7 hybrid expression system (43). Tight repression and high inducibility resulted from placement of the vaccinia virus D6R gene under the control of the T7 RNA polymerase and regulation of both the polymerase gene and the D6R gene with copies of *E. coli lacO*. Synthesis of the 70-kDa protein by recombinant virus vD6ind ranged from undetectable in the absence of IPTG to about six times normal at >50 μM IPTG. Since the T7 RNA polymerase gene is under the control of a vaccinia virus late promoter, the onset of synthesis of the D6R gene product was slightly delayed compared with that of the wild-type virus. The high level of expression of the 70-kDa protein in the presence of IPTG suggests that vD6ind (or its precursor vD6ind$^+$end$^+$) might be used to overproduce this VETF subunit for biochemical studies. In the absence of IPTG, vD6ind might be useful for preparation of small quantities of the free 82-kDa subunit of VETF.

Both subunits of VETF are ordinarily synthesized late in infection and packaged as a heterodimer in progeny virus particles. Since vD6ind is propagated in the presence of IPTG, the virions formed contain functional VETF. Accordingly, when this virus was used to infect cells in the absence of IPTG, early genes were transcribed by using the packaged VETF but de novo synthesis of the 70-kDa subunit was prevented. Therefore, our studies examined the consequences of prevention of synthesis of the D6R gene product on late events of the virus replication cycle. We anticipated that noninfectious virions lacking the transcription factor and, possibly, other enzymes would be formed. Indeed, omission of IPTG did not affect vD6ind DNA replication, inhibition of host protein synthesis, or synthesis of viral late proteins other than the 70-kDa subunit of VETF. When the infection with vD6ind was compared to that with either the wild-type virus or vD6ind$^+$end$^+$, however, the early shutoff of host protein synthesis was delayed somewhat. Structural and functional studies of purified vD6ind virions, made in the presence of IPTG, might provide some explanation for this.

Despite the relatively normal progress of the infection, attempts to purify noninfectious virus particles by sucrose gradient centrifugation were unsuccessful. In addition, pulse-chase experiments indicated reduced proteolytic processing of certain structural proteins under nonpermissive conditions, consistent with an assembly block (data not shown). Upon examination of infected-cell sections by transmission electron microscopy, we noted that morphogenesis of vD6ind in the presence of IPTG was indistinguishable from that of the wild-type virus. In the absence of the inducer, however, the cytoplasm was filled with immature, electron-lucent, enveloped viral forms which were frequently adjacent to much larger granular masses. Some electron-dense viral forms were also noted, but the majority of these were round instead of brick shaped and lacked evidence of an organized core structure.

The interruption of virion morphogenesis caused by the absence of the de novo-synthesized 70-kDa subunit of VETF was unanticipated because Zhang et al. (48) had shown that morphologically mature-appearing virions formed when RAP94 expression was repressed. The latter virions, however, were defective since they lacked RNA polymerase and several other enzymes. The packaging of VETF under those conditions led to the hypothesis that this protein binds to early promoter sites on the genome and serves as a receptor for a complex including RAP94-containing RNA polymerase (48). The present results, while not invalidating the latter model, indicate that VETF has an earlier and more profound role on morphogenesis than RAP94. One attractive possibility is that binding of VETF to the genome is a prerequisite for entry of the nucleoprotein into immature particles. However, we did notice immature virions with dense spots thought to represent nucleoprotein (29) and failed to see the large DNA crystalloids that accumulate in the cytoplasm in the presence of the drug rifampin, which blocks an early stage of morphogenesis (20).

Although a direct role of VETF in morphogenesis is the simplest interpretation of the data, we have considered alternative models. The most interesting is that VETF has a new and unprecedented role in the transcription of a subset of putative very late genes. The proteins encoded by these very late genes would include some required for morphogenesis. The transcriptional regulation of this gene class would be unique, as VETF, but not RAP94, would be required, since repression of the latter protein did not perturb morphogenesis (48). The reported transcriptional reactivation of certain early promoters late in infection (17) could be a related phenomenon.

We are trying to develop the ability to colocalize genomic DNA and viral proteins, including VETF, at high resolution to study the packaging of DNA into immature particles. In addition, construction of a mutant vaccinia virus with an inducible 82-kDa subunit of VETF is in progress.

ACKNOWLEDGMENTS

We thank Michael Merchlinsky for many helpful discussions, Norman Cooper for providing cell cultures and virus stocks, and Andrea Weisberg for electron microscopy.

REFERENCES

1. **Ahn, B.-Y., P. D. Gershon, and B. Moss.** 1994. RNA-polymerase associated protein RAP94 confers promoter specificity for initiating transcription of vaccinia virus early stage genes. J. Biol. Chem. **269:**7552–7557.
2. **Ahn, B.-Y., and B. Moss.** 1992. RNA polymerase-associated transcription specificity factor encoded by vaccinia virus. Proc. Natl. Acad. Sci. USA **89:**3536–3540.
3. **Alexander, W. A., B. Moss, and T. R. Fuerst.** 1992. Regulated expression of foreign genes in vaccinia virus under the control of bacteriophage T7 RNA polymerase and the *Escherichia coli lac* repressor. J. Virol. **66:**2934–2942.
4. **Baldick, C. J., M. C. Cassetti, N. Harris, and B. Moss.** 1994. Ordered assembly of a functional preinitiation transcription complex, containing vaccinia virus early transcription factor and RNA polymerase, on an immobilized template. J. Virol. **68:**6052–6056.
5. **Barbosa, E., and B. Moss.** 1978. mRNA (nucleoside-2′-)-methyltransferase from vaccinia virus. Purification and physical properties. J. Biol. Chem. **253:**7692–7697.
6. **Baroudy, B. M., and B. Moss.** 1980. Purification and characterization of a DNA-dependent RNA polymerase from vaccinia virions. J. Biol. Chem. **255:**4372–4380.
7. **Broyles, S. S.** 1991. A role for ATP hydrolysis in vaccinia virus early gene transcription. J. Biol. Chem. **266:**15545–15548.
8. **Broyles, S. S., and B. S. Fesler.** 1990. Vaccinia virus gene encoding a component of the viral early transcription factor. J. Virol. **64:**1523–1529.
9. **Broyles, S. S., and B. Moss.** 1988. DNA-dependent ATPase activity associated with vaccinia virus early transcription factor. J. Biol. Chem. **263:**10761–10765.
10. **Broyles, S. S., L. Yuen, S. Shuman, and B. Moss.** 1988. Purification of a factor required for transcription of vaccinia virus early genes. J. Biol. Chem. **263:**10754–10760.

10a. **Cassetti, M. C., and B. Moss.** Interaction of the 82-kDa subunit of the vaccinia virus early transcription factor heterodimer with the promoter core sequence directs downstream DNA binding of the 70-kDa subunit. Proc. Natl. Acad. Sci. USA, in press.

11. **Deng, S., and S. Shuman.** 1994. A role for the H4 subunit of vaccinia RNA polymerase in transcription initiation at a viral early promoter. J. Biol. Chem. **269:**14323–14329.
12. **Dunn, J. J., and F. W. Studier.** 1983. Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. J. Mol. Biol. **166:**477–535.

13. **Earl, P. L., N. Cooper, and B. Moss.** 1991. Preparation of cell cultures and vaccinia virus stocks, p. 16.16.1–16.16.7. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. Greene Publishing Associates & Wiley Interscience, New York.
14. **Earl, P. L., and B. Moss.** 1991. Generation of recombinant vaccinia viruses, p. 16.17.1–16.17.16. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. Greene Publishing Associates & Wiley Interscience, New York.
15. **Franke, C. A., C. M. Rice, J. H. Strauss, and D. E. Hruby.** 1985. Neomycin resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants. Mol. Cell. Biol. **5:**1918–1924.
16. **Fuerst, T. R., M. P. Fernandez, and B. Moss.** 1989. Transfer of the inducible *lac* repressor/operator system from *Escherichia coli* to a vaccinia virus expression vector. Proc. Natl. Acad. Sci. USA **86:**2549–2553.
17. **Garcés, J., K. Maternak, B. Kunz, and R. Wittek.** 1993. Reactivation of transcription from a vaccinia virus early promoter late in infection. J. Virol. **67:**5394–5401.
18. **Gershon, P. D., B. Y. Ahn, M. Garfield, and B. Moss.** 1991. Poly(A) polymerase and a dissociable polyadenylation stimulatory factor encoded by vaccinia virus. Cell **66:**1269–1278.
19. **Gershon, P. D., and B. Moss.** 1990. Early transcription factor subunits are encoded by vaccinia virus late genes. Proc. Natl. Acad. Sci. USA **87:**4401–4405.
20. **Grimley, P. M., E. N. Rosenblum, S. J. Mims, and B. Moss.** 1970. Interruption by rifampin of an early stage in vaccinia virus morphogenesis: accumulation of membranes which are precursors of virus envelopes. J. Virol. **6:**519–533.
21. **Kates, J. R., and B. R. McAuslan.** 1967. Poxvirus DNA-dependent RNA polymerase. Proc. Natl. Acad. Sci. USA **58:**134–141.
22. **Keck, J. G., C. J. Baldick, and B. Moss.** 1990. Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late transactivator genes. Cell **61:**801–809.
23. **Keck, J. G., G. R. Kovacs, and B. Moss.** 1993. Overexpression, purification, and late transcription factor activity of the 17-kilodalton protein encoded by the vaccinia virus A1L gene. J. Virol. **67:**5740–5748.
24. **Kovacs, G. R., R. Rosales, J. G. Keck, and B. Moss.** 1994. Modulation of the cascade model for regulation of vaccinia virus gene expression: purification of a prereplicative, late-stage-specific transcription factor. J. Virol. **68:**3443–3447.
25. **Li, J., and S. S. Broyles.** 1993. Recruitment of vaccinia virus RNA polymerase to an early gene promoter by the viral early transcription factor. J. Biol. Chem. **268:**2773–2780.
26. **Li, J., and S. S. Broyles.** 1993. The DNA-dependent ATPase activity of vaccinia virus early gene transcription factor is essential for its transcription activation function. J. Biol. Chem. **268:**20016–20021.
27. **Li, J., M. J. Pennington, and S. S. Broyles.** 1994. Temperature-sensitive mutations in the gene encoding the small subunit of the vaccinia virus early transcription factor impair promoter binding, transcription activation, and packaging of multiple virion components. J. Virol. **68:**2605–2614.
28. **Martin, S. A., E. Paoletti, and B. Moss.** 1975. Purification of mRNA guanylyltransferase and mRNA (guanine 7-)methyltransferase from vaccinia virus. J. Biol. Chem. **250:**9322–9329.
29. **Morgan, C.** 1976. The insertion of DNA into vaccinia virus. Science **193:**591–592.
30. **Moss, B.** 1996. *Poxviridae*: the viruses and their replication, p. 2637–2671. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, third ed., vol. 2. Lippincott-Raven Publishers, Philadelphia.
31. **Moss, B., B.-Y. Ahn, B. Y. Amegadzie, P. D. Gershon, and J. G. Keck.** 1991. Cytoplasmic transcription system encoded by vaccinia virus. J. Biol. Chem. **266:**1355–1358.
32. **Moss, B., E. N. Rosenblum, and E. Paoletti.** 1973. Polyadenylate polymerase from vaccinia virions. Nature (London) New Biol. **245:**59–63.
33. **Munyon, W. E., E. Paoletti, and J. T. Grace, Jr.** 1967. RNA polymerase activity in purified infectious vaccinia virus. Proc. Natl. Acad. Sci. USA **58:**2280–2288.
34. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17:**208.
35. **Rodriguez, J. F., and G. L. Smith.** 1990. IPTG-dependent vaccinia virus: identification of a virus protein enabling virion envelopment by Golgi membrane and egress. Nucleic Acids Res. **18:**5347–5351.
36. **Rosales, R., N. Harris, B.-Y. Ahn, and B. Moss.** 1994. Purification and identification of a vaccinia virus-encoded intermediate stage promoter-specific transcription factor that has homology to eukaryotic transcription factor SII (TFIIS) and an additional role as a viral RNA polymerase subunit. J. Biol. Chem. **269:**14260–14267.
37. **Rosales, R., G. Sutter, and B. Moss.** 1994. A cellular factor is required for transcription of vaccinia viral intermediate stage genes. Proc. Natl. Acad. Sci. USA **91:**3794–3798.
38. **Schnierle, B. S., P. D. Gershon, and B. Moss.** 1992. Cap-specific mRNA (nucleoside-$O^{2'}$-)-methyltransferase and poly(A) polymerase stimulatory activities of vaccinia virus are mediated by a single protein. Proc. Natl. Acad. Sci. USA **89:**2897–2901.
39. **Shuman, S., S. S. Broyles, and B. Moss.** 1987. Purification and characterization of a transcription termination factor from vaccinia virions. J. Biol. Chem. **262:**12372–12380.
40. **Spencer, E., S. Shuman, and J. Hurwitz.** 1980. Purification and properties of vaccinia virus DNA-dependent RNA polymerase. J. Biol. Chem. **255:**5388–5395.
41. **Vos, J. C., M. Sasker, and H. G. Stunnenberg.** 1991. Promoter melting by a stage-specific vaccinia virus transcription factor is independent of the presence of RNA polymerase. Cell **65:**105–114.
42. **Vos, J. C., M. Sasker, and H. G. Stunnenberg.** 1991. Vaccinia virus capping enzyme is a transcription initiation factor. EMBO J. **10:**2553–2558.
43. **Ward, G. A., C. K. Stover, B. Moss, and T. R. Fuerst.** 1995. Stringent chemical and thermal regulation of recombinant gene expression by vaccinia virus vectors in mammalian cells. Proc. Natl. Acad. Sci. USA **92:**6773–6777.
44. **Wolffe, E. J., D. M. Moore, P. J. Peters, and B. Moss.** 1996. Vaccinia virus A17L open reading frame encodes an essential component of nascent viral membranes that is required to initiate morphogenesis. J. Virol. **70:**2797–2808.
45. **Wright, C. F., and A. M. Coroneos.** 1993. Purification of the late transcription system of vaccinia virus: identification of a novel transcription factor. J. Virol. **67:**7264–7270.
46. **Wright, C. F., J. G. Keck, and B. Moss.** 1991. A transcription factor for expression of vaccinia virus late genes is encoded by an intermediate gene. J. Virol. **65:**3715–3720.
47. **Yuen, L., A. J. Davison, and B. Moss.** 1987. Early promoter-binding factor from vaccinia virions. Proc. Natl. Acad. Sci. USA **84:**6069–6073.
48. **Zhang, Y., B.-Y. Ahn, and B. Moss.** 1994. Targeting of a multicomponent transcription apparatus into assembling vaccinia virus particles requires RAP94, an RNA polymerase-associated protein. J. Virol. **68:**1360–1370.
49. **Zhang, Y., and B. Moss.** 1991. Inducer-dependent conditional-lethal mutant animal viruses. Proc. Natl. Acad. Sci. USA **88:**1511–1515.