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Vaccinia Virus A17L Open Reading Frame Encodes an Essential Component of Nascent Viral Membranes That Is Required To Initiate Morphogenesis

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We generated an antiserum to the predicted C-terminal peptide of the A17L open reading frame (ORF), which encodes a 23-kDa polypeptide with hydrophobic regions characteristic of membrane proteins. Immunoelectron microscopy of infected cells indicated that the A17L protein is intimately associated with the earliest characteristic viral membranes, even those formed in the presence of the drug rifampin. To study the role of the A17L protein in morphogenesis, we constructed recombinant vaccinia viruses in which the endogenous A17L ORF was deleted and a copy of the ORF under the control of the bacteriophage T7 RNA polymerase and the *Escherichia coli lac* repressor was inserted into an alternative site in the vaccinia virus genome. Growth of these recombinant viruses was entirely dependent on the induction of A17L expression by isopropyl-β-D-thiogalactopyranoside. Electron microscopic examination of cells infected in the absence of inducer revealed the accumulation of large, well-demarcated electron-dense aggregates but no characteristic membrane-associated viral structures. Viral late protein synthesis occurred under these conditions, although the maturational proteolytic processing of structural proteins was inhibited. We conclude that the product of the A17L gene is an essential component of the immature viral membrane and has an early function in viral morphogenesis.

Poxviruses are large enveloped DNA viruses that replicate within the cytoplasm of vertebrate and invertebrate cells. Although many of the steps in the infectious cycle have been illuminated by molecular genetic studies, little is known regarding the mechanism of virus entry, assembly, and egress. Two related but antigenically distinct infectious, enveloped viral forms are generally recognized; intracellular mature virions (IMV) constitute the majority of the virus obtained by lysing the cell, whereas extracellular enveloped virions (EEV) are spontaneously released into the culture medium (21, 26, 32). During morphogenesis, a population of IMV become enwrapped by a double membrane derived from the trans-Golgi network or elements of the endocytic pathway to form the intracellular enveloped virions (IEV), which are then transported to the periphery of the cell (20, 43, 53). The outer IEV membrane fuses with the plasma membrane, resulting in the deposition of large numbers of cell-associated enveloped virions (CEV) on the exterior of the cell (5). Some of the CEV are detached to form the EEV. It is thought that the CEV and EEV are responsible for cell-to-cell and long-range spread, respectively.

Different proteins comprise the outer membranes of the IMV and EEV. There are at least five EEV-specific integral membrane proteins encoded by the open reading frames (ORFs) A56R (45), B5R (14, 22), A34R (11), A36R (30), and A33R (41a). Many, if not all, of these proteins are glycosylated. The nonglycosylated, palmitylated 37-kDa product of the F13L ORF is also an EEV component (20, 31). Mutagenesis studies have shown that the products of at least three genes, F13L, B5R, and A27L, are required for IMV wrapping to form IEV (4, 15, 30, 41, 44, 58); of these, the A27L protein is associated with the IMV and may form a bridge between the IMV and EEV membranes (41).

In contrast to the detailed picture of EEV formation that is now emerging, very little is known about the structure or formation of the IMV membranes. Conventional electron microscopy studies suggested that the initial steps of virion morphogenesis consisted of the de novo formation of a viral membrane (50, 51). A recent immunoelectron microscopy study, however, provided evidence for the derivation of a double viral membrane from cisternae of the intermediate compartment between the endoplasmic reticulum and the Golgi complex (48). At least four viral proteins are thought to be associated with the IMV membrane: p65 (D13L), which is responsible for sensitivity to the drug rifampin (2, 49, 52); p32 (D8L), an integral membrane protein with 36% homology to carbonic anhydrase (24, 28, 47); and two proteins that elicit strong neutralizing antibodies, the fusion protein p14 (A27L) (9, 38) and the myristylated protein M25 (L1R) (33, 60). Of these, p32 and p14 are associated with the earliest viral membranes, but neither appears to have any role in IMV morphogenesis, although p14 is essential for the formation of EEV (41). While p65 and M25 are involved in IMV morphogenesis, their absence affects a stage after the formation of a recognizable viral membrane (34, 63). Thus, it is of extreme interest to identify the viral proteins responsible either for de novo formation or recruitment of the IMV membrane precursor.

We first became interested in the product of the A17L gene because of the presence of possible transmembrane sequences (18). Subsequently, Rodriguez et al. (37) reported that a 21-kDa N-terminally cleaved form of the protein encoded by the A17L gene coprecipitated with the 14-kDa protein when an

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antiserum against the latter was used. This N-terminal cleavage occurs at amino acid residue 16 within an AGA sequence that is a consensus motif for proteolytic processing of some structural proteins of vaccinia virus (55). From results of assays using extraction with Nonidet P-40 and dithiothreitol, Rodriguez et al. (37) suggested that the A17L protein is an IMV membrane protein and is responsible for anchoring the 14-kDa fusion protein. To investigate the synthesis, localization, and putative function of the A17L protein and to determine its role in vaccinia virus morphogenesis, we made an antipeptide antiserum and recombinant viruses in which the endogenous A17L ORF was replaced by a stringently regulated isopropyl-β-D-galactopyranoside (IPTG)-inducible copy of the gene. Here, we present immunoelectron microscopic studies showing that the A17L protein is associated with the earliest viral membranes. Furthermore, in the absence of A17L expression, no characteristic viral membranes are detectable and only aberrant viral structures are formed.

Our results were presented at the 14th Annual Meeting of the American Society for Virology (25), and a description of an A17L mutant virus with a similar phenotype was reported by Rodriguez et al. (36).

MATERIALS AND METHODS

Cells and viruses. CV-1 cells (ATCC CCL 70) and BS-C-1 cells (ATCC CCL26) were maintained in Earle's modified Eagle's medium (Quality Biological Inc.) containing 10% fetal bovine serum in a 5% CO_2 atmosphere. Vaccinia virus strain WR (ATCC Vr119) and recombinant vaccinia virus vT7lacOI (1) were propagated in HeLa cells as previously described (13). Recombinant virus vT7lacOI-A17L, code named vDM-321, which contains both the endogenous and an IPTG-inducible copy of the A17L ORF, was isolated and propagated in cells grown in medium containing mycophenolic acid, hypoxanthine, and xanthine. For vT7lacOI-A17LΔ5 (vA17LΔ5) and vT7lacOI-A17LΔ6 (vA17LΔ6), code named vEW5 and vEW6, respectively, which both lack the endogenous copy of the A17L ORF but contain an IPTG-inducible copy of the gene, the cell culture medium included 1.5 to 2 mg of Geneticin (Gibco Life Technologies) per ml, depending on the effective concentration of the drug in any particular lot. Cells were typically infected with crude, trypsin-treated vaccinia virus stocks at a multiplicity of 10 PFU in medium containing 2.5% fetal bovine serum in the presence or absence of 100 μM IPTG as required.

Antibodies. An antipeptide antiserum, made to the C-terminal 12 amino acids of the predicted A17L protein sequence, has been described elsewhere (60). An antiserum against a C-terminal peptide of the protein encoded by the A18R ORF was kindly provided by R. Condit, University of Florida, Gainesville (46). Monoclonal antibody C3, against the vaccinia virus 14-kDa fusion protein encoded by A27L, was generously provided by M. Esteban (Centro Nacional de Biotechnología, Madrid, Spain) (39).

Plasmid construction. A copy of the A17L gene, containing an *Nco*I site as the initiation codon and a TAA termination signal after codon number 203 flanked by *Bam*HI sites, was generated by PCR using vaccinia virus genomic DNA and oligonucleotide primers EW46 (GGGCCATGGCTTATTTAAGATATTACAA TATGCTT) and EW47 (GGGGGATCCTTAATAATCGTCAGTATTTAAA CT) (restriction endonuclease sites are underlined, and initiation and termination codons are in italics). To insert the *Nco*I site and maintain the ATG codon at position 1, AGT (Ser) at position 2 was altered to GCT (Ala) during the PCR. The PCR product was cut with *Nco*I and *Bam*HI and inserted into plasmid pMITEOlac.20/3 (a *lacZ*-containing precursor of pVOTE 1 [57]), from which the *lacZ* gene had been excised with *Nco*I and *Bam*HI to produce plasmid pDMA17L2.

A 640-bp DNA fragment corresponding to the downstream-flanking region of the A17L gene (right flank) was produced by PCR from viral DNA, using primers DMM510 (CCCGGATCCGCTACTATTAAAGATG) and DMM511 (CCCTCTAGATGATTCATCCAAGATG), which contain *Mae*III and *Xba*I sites (underlined) at their respective 5′ ends. A synthetic 81-bp DNA fragment was produced by annealing two overlapping oligonucleotides and filling in the recessed portions with deoxynucleoside triphosphates and Klenow enzyme. Oligonucleotide DMM506a (CCCGGATCCAAAAATTGAAAATAAATACAAA GGTTCTTG*AGGGTTGTGTT*) was the left-end oligonucleotide containing a *Bam*HI site, and oligonucleotide DMM507 (CCCTCTAGAGTGACATTTAT GATTATTTCTCGCTTTCAATTT*AACACAACCCT*) was the right-end oligonucleotide containing an *Mae*III site (sites are underlined, and overlapping sequences are in italics). This synthetic DNA fragment contained the H5 early/late promoter sequences (originally designated H6) (42) from positions −107 through −39, with the late transcriptional initiation consensus sequence TAA AT(G) contiguous with the A18R gene. The G at position −102 of the early promoter was changed to T to remove a potential ATG initiation site and to optimize early promoter activity (10). The synthetic H5 promoter fragment was cut with *Mae*III and *Bam*HI, the 640-bp PCR DNA fragment was cut with *Mae*III and *Xba*I, and the two fragments were cloned into the *Bam*HI and *Xba*I sites of plasmid pBluescript II SK (Stratagene), producing plasmid pH5RF.5c. This plasmid contained the right flank of the A17L gene and the H5 early/late promoter designed to drive transcription of the A18R gene.

A 686-bp DNA fragment corresponding to the downstream-flanking region of the A17L gene (alternate right flank) was produced by PCR from viral DNA, using primers DMM514 (CCCGGATCCAAGCATATTGTAATATCTT) and DMM511 (CCCTCTAGATGATTCATCCAAGATG), which contain *Bam*HI and *Xba*I sites, respectively, at their 5′ ends. The end of the alternate right flank was 45 bp upstream of the A18R ATG and included the late promoter sequences of the A18R gene (29). This fragment was cloned into the *Bam*HI and *Xba*I sites of plasmid pBluescript II SK (Stratagene), producing plasmid pALFRF.8c.

A 638-bp DNA fragment corresponding to the upstream-flanking region of the A17L gene (left flank) was produced by PCR from viral DNA, using primers DMM513 (CCCGGTACCTTTTGCACGTAACC) and DMM512 (CCCCGTCGACATATACCAACATTTAACAGTTTAAATA), which contain *Kpn*I and *Sal*I sites (underlined) at their respective 5′ ends. The oligonucleotide DMM512 primed DNA synthesis in the A17L gene, starting 43 bp upstream of the A16L ATG. The left-flank PCR DNA was cut with *Kpn*I and *Sal*I and inserted into like sites in plasmids PH5RF.5c and pALFRF.8c to produce plasmids pLFH5RF.5c3c and pLFALFRF.8c8c, which contain a synthetic H5 early/late promoter and the native A18R late promoter sequences regulating the A18R gene, respectively.

Finally, a *Sal*I-to-*Bam*HI fragment cut from plasmid pVV:neo (16) containing the vaccinia virus P7.5 promoter and the bacterial neomycin resistance gene was cloned into the *Sal*I and *Bam*HI sites between the A17L left flank and right flank in plasmids pLFH5RF.5c5c and pLFALFRF.8c8c to produce the A17L knockout vectors pEWDM5 (synthetic H5 early/late promoter regulating the A18R gene) and pEWDM6 (native late A18R promoter regulating the A18R gene), respectively.

Generation of recombinant viruses. The transfer vectors pDMA17L2, pEWDM5, and pEWDM6 were used as follows to generate recombinant viruses. pDMA17L2, which contains a copy of the A17L gene under the control of a T7 promoter and regulated by the *lac* operator and a copy of the *Escherichia coli gpt* gene under the control of the vaccinia P7.5 promoter flanked by vaccinia virus hemagglutinin (HA) DNA sequence was used to insert a second copy of the A17L gene into the vaccinia virus *Hin*dIII-A sequence interrupting the vaccinia virus HA gene (ORF A56R). CV-1 cells were infected with vT7lacOI and transfected with pDMA17L2. After 48 h, cells were harvested and diluted lysates were used to infect BS-C-1 cell monolayers in the presence of mycophenolic acid. The infected cells were covered with agar, and mycophenolic acid-resistant plaques were picked and used to infect fresh monolayers of BS-C-1 cells. In this way, the recombinant virus vT7lacOI-A17L was purified by three successive rounds of plaque isolation under mycophenolic acid selection. This virus was then used to generate vA17LΔ5 and vA17LΔ6. CV-1 cells were infected with vT7lacOI-A17L and transfected with pEWDM5 or pEWDM6. Cells were harvested after 48 h, and diluted lysates were used to infect BS-C-1 cell monolayers in the presence of Geneticin and 50 μM IPTG. After two rounds of enrichment under Geneticin selection, cell lysates were used to infect BS-C-1 cells. The infected cells were covered with agar containing 50 μM IPTG, and plaques were picked and used to infect fresh monolayers of BS-C-1 cells. Plaque-purified viruses vA17LΔ5 and vA17LΔ6 were checked for neomycin resistance, and stocks were amplified in the presence of Geneticin and IPTG.

Southern blot analysis of viral DNA. Southern blot analysis of DNA prepared from uninfected BS-C-1 cells and cells infected with vaccinia virus WR or recombinant viruses was carried out as previously described (12). DNA was digested with restriction enzyme *Bam*HI (New England Biolabs), and the products were separated by electrophoresis on 0.8% agarose, transferred to nylon membranes (GeneScreen Plus; Dupont), and cross-linked to the membrane by UV cross-linking (Stratalinker). By using ethidium bromide staining as a guide, similar amounts of DNA were loaded onto the gel, and staining of the gel after blotting revealed that the DNA transfer had been efficient. The membrane was hybridized to a PCR fragment generated by using primers EW46 and EW47, which contained the sequence of the entire A17L gene. This fragment was fluorescein labeled by random priming and visualized by luminescence detection of alkaline phosphatase-conjugated antifluorescein antibody as described by the manufacturer (Fluorescein Gene Images; Amersham).

Analysis of [^{35}S]methionine-labeled polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins synthesized during early and late stages of vaccinia virus infection of BS-C-1 cells were analyzed as described previously (61) except that a 12.5% polyacrylamide gel was used. For pulse-labeling, infected BS-C-1 cells were first incubated in methionine-free medium for 15 min and then labeled for 30 min in methionine-free medium containing 5% dialyzed fetal bovine serum and 100 mCi of [^{35}S]methionine (Amersham) per ml. For pulse-chase experiments, BS-C-1 cells were infected for 12 h, methionine starved for 15 min, labeled for 30 min with 100 mCi of [^{35}S]methionine per ml, and then incubated in medium containing unlabeled methionine for 12 h.

Electron microscopy. For immunoelectron microscopy, infected and uninfected HeLa cells were grown in the presence or absence of 100 μg of rifampin per ml, fixed with increasing amounts of paraformaldehyde (2 to 8%), and

prepared for cryosectioning as previously described (6). Ultrathin frozen sections were cut with a Leica/Reichert Ultracryomicrotome FCS. Thawed sections were incubated with A17L antiserum at a dilution of 1:150 and then with 10-nm colloidal gold-conjugated protein A (Department of Cell Biology, Utrecht University School of Medicine, Utrecht, The Netherlands) diluted 1:45 in 1% bovine serum albumin–0.1 M phosphate buffer (pH 7.4). Finally, sections were stained with 0.3% uranyl acetate in 1.8% methylcellulose. For conventional electron microscopy, infected BS-C-1 cells were grown in the presence or absence of 100 mM IPTG for 18 h. Cells were fixed in 2% gluteraldehyde (EM Sciences) in 100 mM phosphate buffer (pH 7.4) for 1 h. Cells were prepared for transmission electron microscopy by osmication, dehydration, and embedding in Epon resin. Thin sections were cut, collected on Formvar-coated copper mesh grids (Polysciences), and stained with 2% uranyl acetate and Reynold's lead citrate (35). Samples were viewed with a Philips CM100 electron microscope.

RESULTS

The A17L protein is associated with the vaccinia virus membrane at early stages of morphogenesis. To study the synthesis, localization, and putative membrane association of the A17L protein, we generated a polyclonal antiserum to the predicted C-terminal 12 amino acids of the A17L ORF (18). This antiserum recognized both the precursor and mature forms of the protein in Western blot (immunoblot) analysis and by radioimmunoprecipitation (60). Using this antibody for immunoelectron microscopy of ultrathin sections of infected cells, we demonstrated immunogold staining of the crescent-shaped membranes and the membranes enclosing immature virions (Fig. 1A). The labeling was predominantly on the inner face of these membranes. There was significantly less staining of mature viral forms and no obvious staining of the nuclear membrane, Golgi apparatus, or other intracellular membrane organelles. However, we did observe staining of coated pits and vesicles, which was apparently due to cross-reactivity of the antiserum with a cellular component of these structures, as a similar pattern of staining was evident in uninfected cells (data not shown). Preimmunization serum from the same rabbit did not show any significant labeling of either infected (Fig. 1B) or uninfected (data not shown) cells.

We also examined the distribution of the A17L protein in infected cells that had been grown in the presence of rifampin, a drug which prevents formation of the crescent-shaped membranes and subsequent steps in virus morphogenesis without significant effect on viral DNA or protein synthesis (27). In these cells, there was labeling of the irregular membranes surrounding the so-called rifampin bodies. However, the gold particles were not clearly associated with one or the other side of the membrane (Fig. 1C). The effects of rifampin on morphogenesis are readily reversed by washing the drug out of cells (27). Within minutes of rifampin removal, crescent-shaped membranes form at the periphery of the rifampin bodies. When such cells were stained with the A17L antiserum, we observed that the gold label was now associated predominantly with the newly forming viral crescents, consistent with their origin from rifampin bodies (Fig. 1D).

Generation of recombinant vaccinia viruses with an A17L-inducible gene. Having established that the A17L protein is associated with the very early membrane forms of vaccinia virus, we investigated its possible function in morphogenesis. Our strategy was to construct recombinant vaccinia viruses in which the native copy of the A17L gene was deleted and a stringently regulated inducible copy of the A17L gene was introduced into an alternative site in the viral genome. As depicted in Fig. 2, the recombinant vaccinia virus encoded bacteriophage T7 RNA polymerase transcribes the A17L ORF in the presence of IPTG. In the absence of IPTG, the *E. coli lac* repressor binds to operators strategically placed adjacent to both the T7 RNA polymerase gene and the A17L gene, preventing the transcription of both, which accounts for the very high stringency. The A17L inducible virus was constructed by using a recently described mammalian expression system which consists of a recombinant vaccinia virus and a plasmid transfer vector (57). One of the viruses used in the system, vT7lacOI (1), contains (i) the *E. coli lac* repressor gene (*lacI*) attached to a vaccinia virus early/late promoter and (ii) the bacteriophage T7 RNA polymerase gene regulated by a modified *E. coli lac* operator (*lacO*) placed several nucleotides downstream of the vaccinia virus P11 late promoter. The plasmid transfer vector has (i) an expression cassette consisting of a T7 promoter, *lacO*, encephalomyocarditis virus translation enhancer, sites for the insertion of a desired ORF, and transcriptional terminators plus (ii) the *gpt* gene for antibiotic selection and (iii) vaccinia virus sequences for insertion into the HA (ORF A56R) site of the vaccinia virus genome.

First, we made a PCR copy of the A17L ORF, digested it with restriction endonucleases *Nco*I and *Bam*HI, and ligated the product into the *Nco*I- and *Bam*HI-cleaved vector pMiteolac 20/3 (Fig. 3A). This placed the A17L ORF under control of the T7 promoter and *lacO*. The resulting plasmid, pDMA17L2, was transfected into cells infected with vT7lacOI, and when homologous recombination into the HA site occurred, selection for *gpt* expression was used to isolate a recombinant virus, vT7lacOI-A17L, that expressed both the endogenous copy of the A17L gene and a second copy which was regulated by T7 RNA polymerase and the *lac* repressor. The genomic DNA of the three-times plaque-purified recombinant virus was analyzed by PCR using primers located within the HA locus to check that the virus was a product of a double-recombination event (data not shown). In addition, we demonstrated by Western blotting that the product of the A17L gene was overexpressed upon addition of IPTG (data not shown).

The second step, replacement of the endogenous copy of the A17L gene by insertion of the neomycin resistance gene, was complicated by the proximity of the neighboring vaccinia virus genes. Analysis of the DNA sequence revealed that the coding regions of the genes flanking the A17L ORF, A18R and A16L, lie within a few base pairs of A17L and that their promoter elements overlap the A17L gene. The function of the protein encoded by the A16L gene is undetermined, but the A18R gene product was shown to be essential for virus replication (3, 46). Analysis of temperature-sensitive mutants showed that the loss of the A18R gene results in a virus with a late abortive phenotype (29). In these studies, Pacha et al. mapped the transcription start sites of the A16L, A17L, and A18R genes of vaccinia virus by using S1 nuclease and primer extension analyses (29). The A16L gene has a late transcription start site that overlaps the sequence encoding the C terminus of the A17L protein, and the A18R gene is expressed at both early and late times during infection. A late transcription start site was mapped to a region within the 5′ coding region of the A17L gene, and an early start site was mapped to sequences within the 3′ third of the A17L gene. Thus, deletion of the major portion of the coding sequence of the A17L gene would result in deletion of this early transcription start site. Although the significance of the early A18R transcript was unclear, we decided to make two recombinant viruses to cover all eventualities. For the first virus, vA17LΔ6, we deleted 540 bp of the A17L ORF including the early promoter for A18R but retained A17L-encoding sequence containing the late promoter and also the late promoter of the A16L ORF, which overlapped the C-terminal coding sequence of A17L. For the second virus, vA17LΔ5, we deleted 570 bp of the A17L ORF, retaining the late promoter region for A16L but deleting all natural promoter sequences for A18R. The native promoter sequences were replaced by the H5 promoter

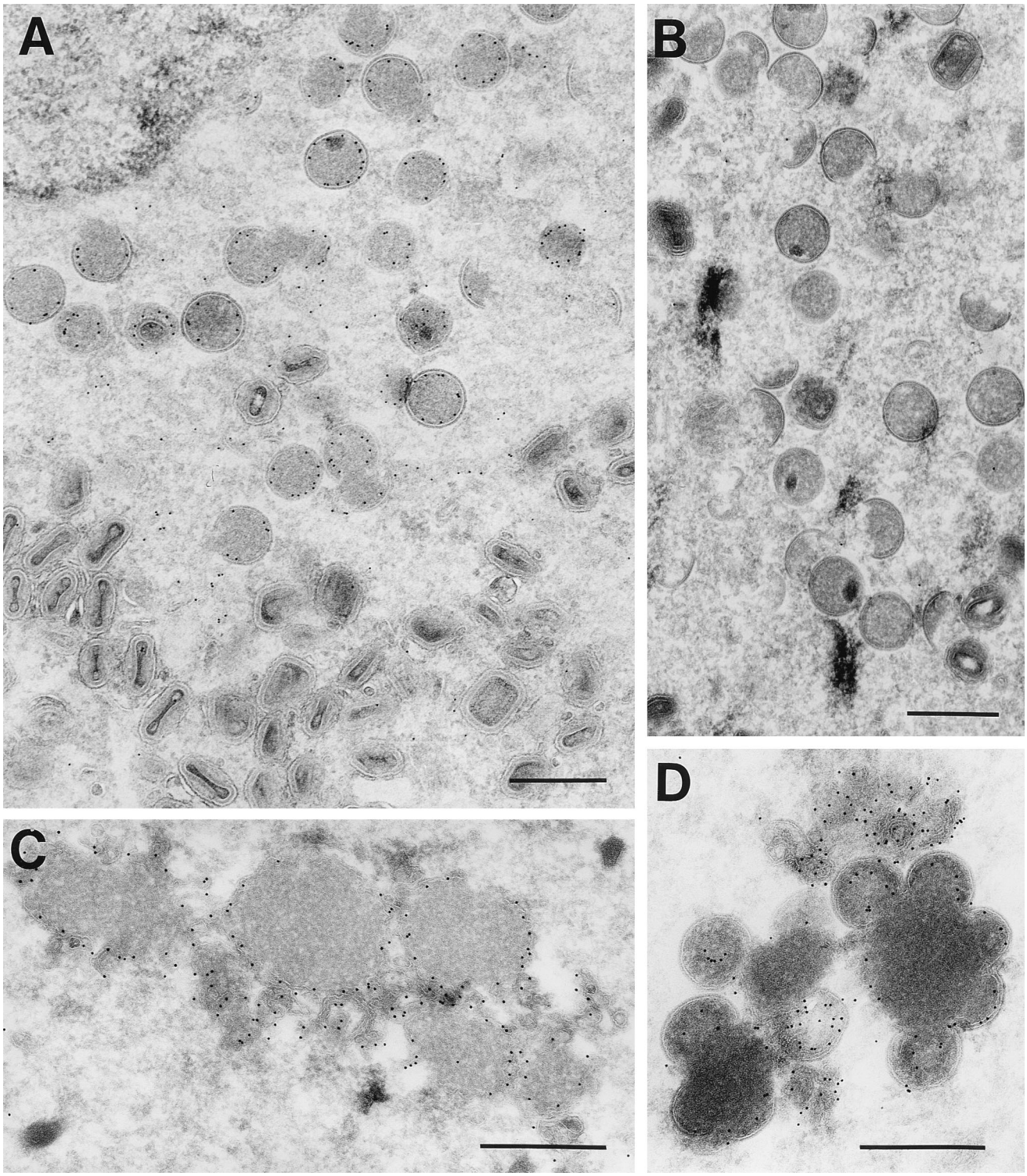

FIG. 1. Immunoelectron microscopy of thawed ultrathin sections of BS-C-1 cells infected with vaccinia virus WR in the absence (A and B) or presence (C and D) of 100 mg of rifampin per ml and stained with either a rabbit antiserum against A17L (A, C, and D) or preimmune serum (B) followed by protein A conjugated to colloidal gold (10 nm). In sections of cells shown in panel D, rifampin was washed out of the cells for 10 min prior to fixation. Scale bars represent 500 nm.

(previously called H6), since it has both early and late regulatory elements (42). Steps in the construction of the transfer vectors pEWDM6 and pEWDM5 are shown in Fig. 3B. The neomycin resistance gene was flanked by sequences at the 3′ end of the A17L gene which included the A16L promoter and either the 5′ portion of the A17L gene encompassing the native A18R late promoter (pEWDM6) or the A18R sequence reconstituted with the H5 promoter element (pEWDM5). CV-1

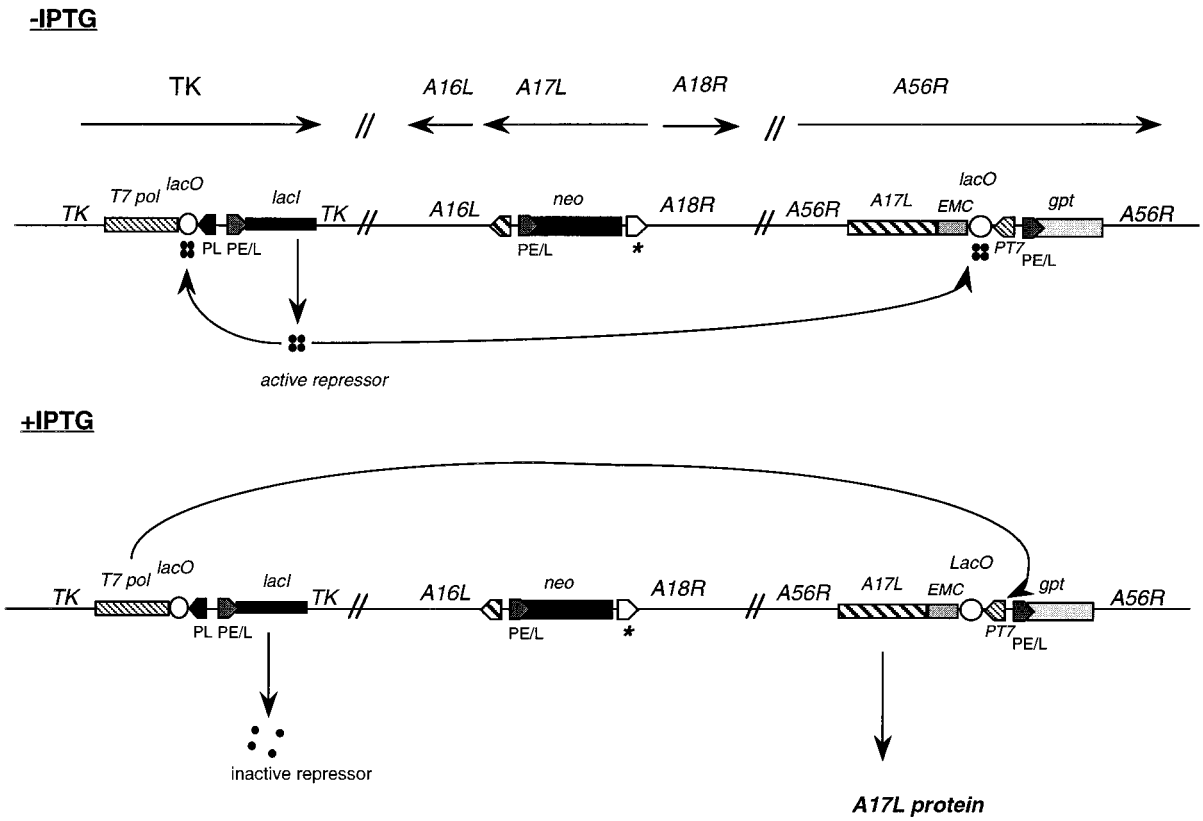

FIG. 2. Schematic diagram illustrating the regulation of expression of the A17L ORF by IPTG. Portions of the genomes of the recombinant viruses are represented with arrows above the sequences indicating the directions and locations of ORFs in the parental virus. In the upper panel, *lac* repressor prevents transcription of the T7 polymerase and new copy of the A17L ORF. In the lower panel, the *lac* repressor is inactivated by IPTG and the ORFs are transcribed. TK, thymidine kinase; EMC, encephalomyocarditis virus; PL, late promoter; PE/L, early/late promoter.

cells were infected with vT7lacOI-A17L and transfected with either of these plasmids. After 48 h, cell lysates were harvested and used to infect BS-C-1 cell monolayers in the presence of Geneticin and IPTG to enrich for neomycin-resistant virus. This enriched stock was subjected to three rounds of plaque purification, and stocks were grown in the presence of Geneticin and IPTG. DNA samples from uninfected BS-C-1 cells or cells infected with WR, vT7lacOI-A17L, vA17LΔ5, or vA17LΔ6 were restriction digested with *Bam*HI and analyzed by Southern blotting. A PCR product encompassing the entire coding sequence of the A17L gene was used to probe the digested DNA. We observed that the A17L probe hybridized with a single fragment of the expected molecular mass (14 kbp) which contains the native A17L gene in WR DNA (Fig. 4). We did not observe a fragment of this size in digests of recombinant viruses vA17LΔ5 and vA17LΔ6. However, a single product with a predicted molecular mass of 7.1 kbp which represents the A17L gene inserted into the HA gene was detected in the correct size range. As expected, we observed both digestion products in the intermediate virus vT7lacOI-A17L as well as two additional bands of 11.3 and 10 kbp which could have arisen by recombination events between the two copies of the A17L gene. We did not detect any hybridizing material in *Bam*HI-digested DNA from uninfected cells.

vA17LΔ5 and vA17LΔ6 are inducer-dependent conditional-lethal mutants. To ensure permissive conditions for the isolation of a potentially conditional-lethal mutant, all steps in the generation of these viruses had been carried out in the presence of 50 μM IPTG. We subsequently determined the IPTG requirements for replication of these viruses. The plaque phenotypes of vT7lacOI-A17L, vA17LΔ5, and vA17LΔ6 were observed on BS-C-1 cells in the absence or presence of 100 μM IPTG. After 48 h, the monolayers were stained with crystal violet (Fig. 5). We noted that vT7lacOI-A17L, which contains both the inducible and endogenous copies of the A17L gene, formed similar numbers of plaques under both growth conditions (Fig. 5A) but that the two inducible viruses vA17LΔ6 and vA17LΔ5 (Fig. 5B and C) formed plaques in the presence but not in the absence of inducer. Furthermore, the plaques of the inducible recombinants were noticeably smaller than those of the parental strain vT7lacOI-A17L, with those of vA17LΔ5 being the smallest. The plaque size of vT7lacOI-A17L was comparable to that of WR (data not shown).

The IPTG requirement was also determined under one-step virus growth conditions. BS-C-1 cells were infected with vaccinia virus WR and the recombinant viruses vA17LΔ5 and vA17LΔ6 in the presence of a range of concentrations of IPTG. After 48 h, both cells and medium were harvested and the virus titers were measured by plaque assay on fresh BS-C-1 monolayers (Fig. 6A). In contrast to wild-type vaccinia virus WR, replication of vA17LΔ5 and vA17LΔ6 was entirely dependent on IPTG; maximal amounts of vA17LΔ5 and vA17LΔ6 were obtained with 100 to 500 μM IPTG. However, the yields were usually about 1 log unit lower than that of wild-type virus. The effect of 100 μM IPTG on infectious virus yield over time was determined for WR, vA17LΔ5, and vA17LΔ6 (Fig. 6B). In the presence of 100 μM IPTG, maximal yields of recombinant viruses as well as WR virus occurred at about 24 h. In the

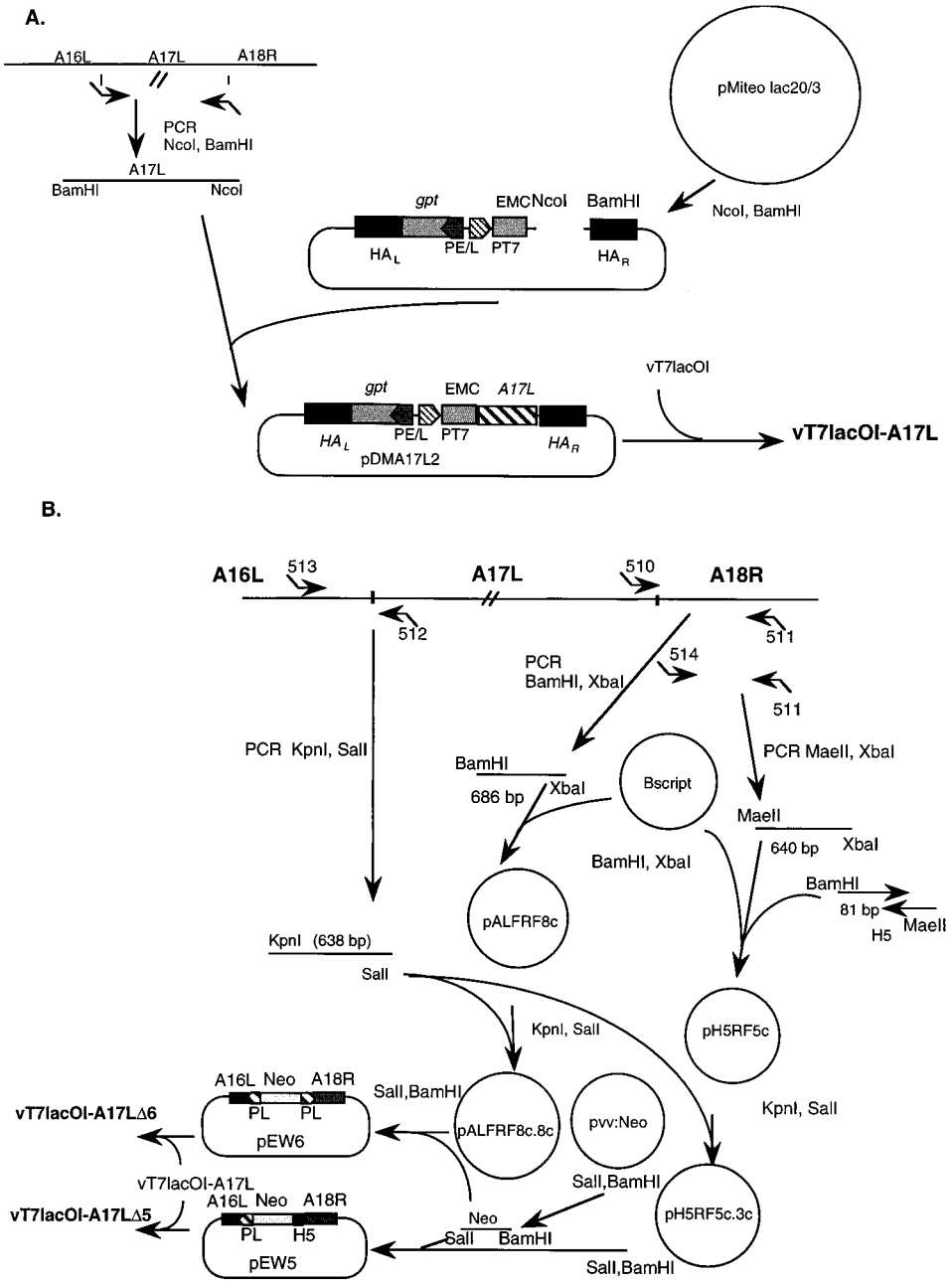

FIG. 3. Schematic showing steps in the construction of transfer vectors pDMA17L2, pEWDM5, and pEWDM6, which were used to make recombinant viruses vT7lacOI-A17L (A), vT7lacOI-A17LΔ5 (B), and vT7lacOI-A17LΔ6 (B), respectively. PT7, T7 promoter. Other abbreviations are as defined in the legend to Fig. 2.

absence of IPTG, no increase in virus yield was observed for either inducible virus even after 72 h (data not shown).

Synthesis of the A17L protein by cells infected with vA17LΔ5 or vA17LΔ6 is dependent on the concentration of IPTG. BS-C-1 cells were infected with wild-type WR or recombinant viruses vA17LΔ5 and vA17LΔ6 and grown for 24 h in the presence of a range of concentrations of IPTG. A protein with a molecular mass of approximately 25 kDa was detected when infected cell extracts were analyzed by SDS-PAGE and Western blotting with the A17L antiserum (Fig. 7). In the absence of IPTG, no A17L protein was detectable in cells infected with either of the recombinant viruses but could be detected in cells grown in the lowest tested concentration of inducer, 5 μM. At 5 to 10 μM IPTG, the amount of A17L protein made by the recombinant virus was similar to that made by the wild-type virus. Maximal amounts of A17L protein were made in the presence of 100 to 500 μM IPTG. For unknown reasons, higher amounts of A17L protein were made in cells infected with vA17LΔ6 than in cells infected with vA17LΔ5. The lower portion of the membrane was probed with monoclonal antibody C3, which recognizes another late IMV protein, the 14-kDa product of the A27L ORF, and then with ^{125}I-labeled sheep anti-mouse immunoglobulin G. We observed that the intensities of the 14-kDa band signal were similar in all lanes, suggesting that equivalent infections had been obtained for WR, vA17LΔ5, and vA17LΔ6.

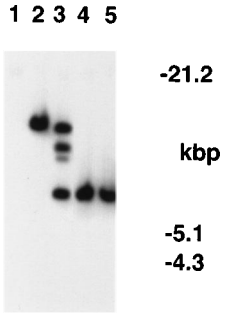

FIG. 4. Southern blot analysis of viral DNA extracted from uninfected BS-C-1 cells (lane 1) and cells infected with vaccinia virus WR (lane 2) and recombinant viruses vT7lacOI-A17L, vA17LΔ5, and vA17LΔ6 (lanes 3, 4, and 5, respectively). The *Bam*HI-digested DNA was transferred to a nylon membrane and hybridized with a probe containing the coding sequence of the A17L gene. Hybridized fluorescein-labeled probe was visualized by luminescence detection of alkaline phosphatase-conjugated antifluorescein antibody. This figure was generated by using Adobe Photoshop 3.0.

Synthesis of the A18R protein. Because of our genetic manipulations, we were interested in comparing the levels of expression of the A18R gene in cells infected with the recombinant viruses. Unlabeled extracts of BS-C-1 cells infected with WR, vA17LΔ5, or vA17LΔ6 in the presence or absence of inducer were made at 2, 4, 6, 8, and 12 h after infection. Samples were analyzed by Western blotting with an antiserum raised against a C-terminal peptide of the protein encoded by A18R and kindly provided by R. Condit (Fig. 8). As previously reported by Simpson and Condit (46), the antiserum reacted with two polypeptides of approximately 57 and 32 kDa. The larger polypeptide corresponds in size to the full-length A18R ORF. The smaller polypeptide was not defined. In our gel system, the band corresponding to the full-length A18R product had a molecular mass of approximately 50 kDa and was detectable in lysates of cells at 2 and 4 h after infection with WR but increased significantly from 6 h, which is consistent with weak early and strong late promoters. Except for higher expression, a similar pattern was obtained with A17LΔ5, which has the A18R gene regulated by the H5 early/late promoter. A18R expression by A17LΔ6, which contains only the A18R late promoter, may have been slightly less than that by WR.

Synthesis and processing of viral late proteins. We were interested in determining whether the lack of synthesis of A17L has any effect on the timing, levels of expression, or processing of other viral proteins. To examine this, we infected BS-C-1 cells with WR, vA17LΔ5, or vA17LΔ6 under permissive or nonpermissive conditions and labeled them at 2, 4, 6, 8, 12, and 23 h after infection with [^{35}S]methionine for 30 min. The labeled, infected cells were lysed directly in Laemmli sample buffer and analyzed by SDS-PAGE. Under each of the conditions used, the general labeling patterns were similar and early and late proteins were synthesized. The 12-h time point, which is representative of the late protein synthesis profile for each virus, is shown in Fig. 9a. In cells infected with vA17LΔ5 and vA17LΔ6 under nonpermissive conditions, a labeled band corresponding to the A17L protein was not observed. However, a band of approximately 25 kDa which likely represents the A17L unprocessed precursor was detected in the presence of inducer, albeit in low amounts in cells infected with vA17LΔ5. In cells infected with vA17LΔ5, shutoff of host protein synthesis was not complete even at 24 h (data not shown). This may be due to the necessity of using a larger inoculum of this relatively low titer virus stock. A labeled band corresponding to a protein with a molecular mass of approximately 50 kDa was visible from 6 h after infection in cells infected with vA17LΔ5 and grown in the presence or absence of IPTG but not in cells infected with vA17LΔ6 or WR. We believe that this protein is the product of the A18R gene, which is under control of the synthetic H5 promoter in vA17LΔ5. Finally, there was a band of approximately 20 kDa in cells infected with vA17LΔ5 and vA17LΔ6 in the presence and absence of inducer, which may represent the product of the *gpt* gene. Several of the major structural proteins of vaccinia virus are derived by proteolytic cleavage of precursor molecules. Cleavage is dependent on virus assembly, as it is inhibited when morphogenesis is blocked with the drug rifampin (19, 23). Figure 9b shows the processing of certain viral proteins that were synthesized during a 30-min pulse with [^{35}S]methionine at 12 h after infection

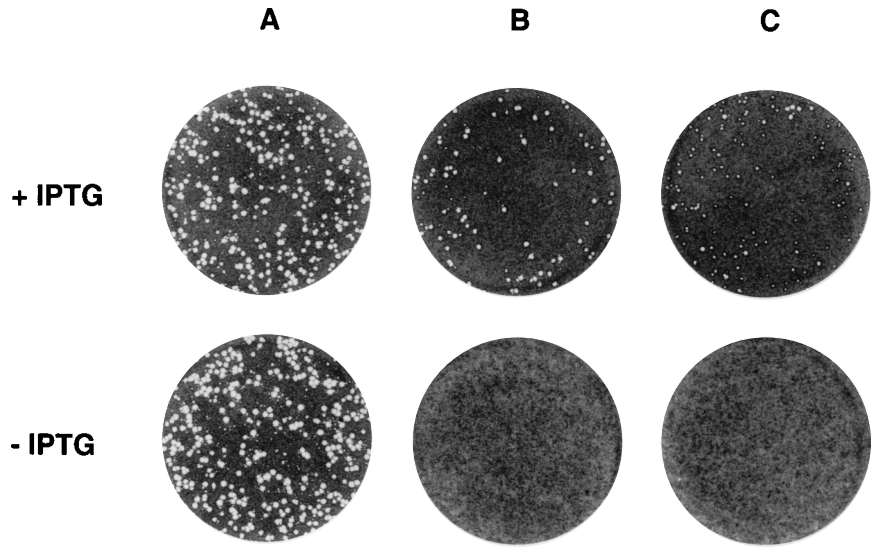

FIG. 5. Comparison of plaques produced by recombinant viruses. Monolayers of BS-C-1 cells were infected with recombinant viruses vT7lacOI-A17L (A), vA17LΔ6 (B), and vA17LΔ5 (C) in the presence or absence of 50 μM IPTG, as indicated. At 48 h after infection, plaques were visualized by staining cells with crystal violet (0.1% in 20% ethanol).

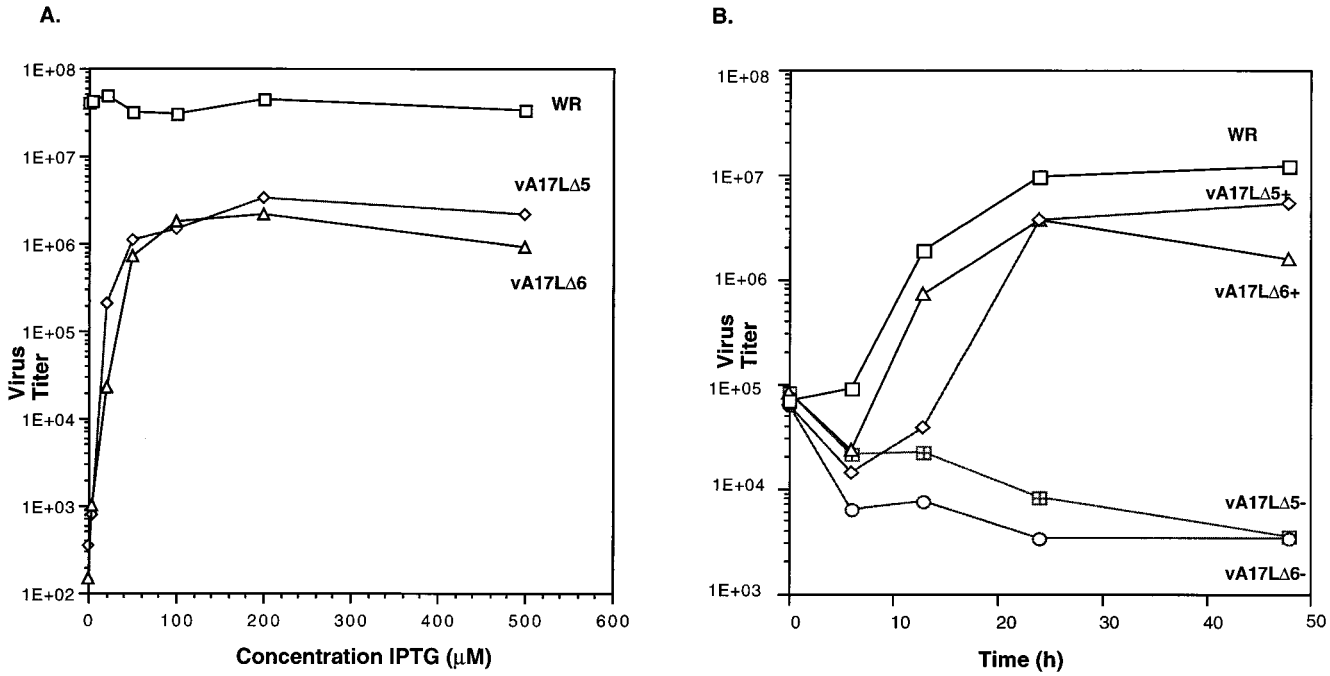

FIG. 6. Yields of infectious recombinant viruses at different concentrations of IPTG and different times after infection. BS-C-1 cells were infected with vaccinia virus WR or recombinant virus vA17LΔ5 or vA17LΔ6. (A) Cells were infected in the presence of the indicated concentrations of IPTG. After 24 h, the cells were lysed and the total virus titers were determined by plaque assay in the presence of 100 μM IPTG. (B) Cells were infected in the presence or absence of 100 μM IPTG and were harvested at 6, 12, 24, and 48 h after infection. Total virus titer for each sample was determined by plaque assay as described above.

and then chased for 12 h in the presence of excess unlabeled methionine. Infected cells were lysed directly in Laemmli sample buffer and analyzed by SDS-PAGE. Under permissive conditions, several proteins, notably 4a and 4b, increased in intensity after the chase. In contrast to the pulse-labeled samples, we did not observe a band migrating at the expected molecular mass of the A17L protein; however, a band of a lower molecular mass was observed in the WR infection in the presence and absence of rifampin and in infections with the recombinant viruses under permissive conditions. This may reflect the N-terminal processing of the A17L protein precursor to a 21-kDa product, which is reported to occur in the presence of rifampin (37). Interestingly, repression of A17L expression mimicked the effects of rifampin, suggesting that there is a block in morphogenesis.

In the absence of inducer, vA17LΔ5 and vA17LΔ6 are blocked at an early stage of morphogenesis. Electron microscopy was used to determine the stage at which the virus replication was blocked in the absence of A17L expression. BS-C-1 cells were infected for 18 h with WR, vA17LΔ5, or vA17LΔ6 in the presence or absence of inducer, fixed, prepared for transmission electron microscopy, and sectioned. Representative sections of BS-C-1 cells infected with vA17LΔ5 and grown in the presence or absence of IPTG are shown in Fig. 10. Cells infected with WR, vA17LΔ5, or vA17LΔ6 in the presence of inducer contained the expected range of viral structures, namely, crescent membranes representative of newly forming virions, immature and mature forms of IMV, and also IEV (Fig. 10C). Although cells infected with the recombinant viruses in the presence of 100 μM IPTG synthesized more A17L protein than those infected with wild-type virus, we did not observe aberrant viral structures. In cells infected with recombinant viruses vA17LΔ5 and vA17LΔ6 in the absence of IPTG, however, we did not detect any characteristic virion membranes or immature virions (Fig. 10A and B). We did see large circular masses of electron-dense material which bore some similarity to the characteristic bodies observed in rifampin-treated cells except for the notable absence of limiting membranes. In some instances, elements of rough endoplasmic reticulum or structures which appeared to be small vesicles

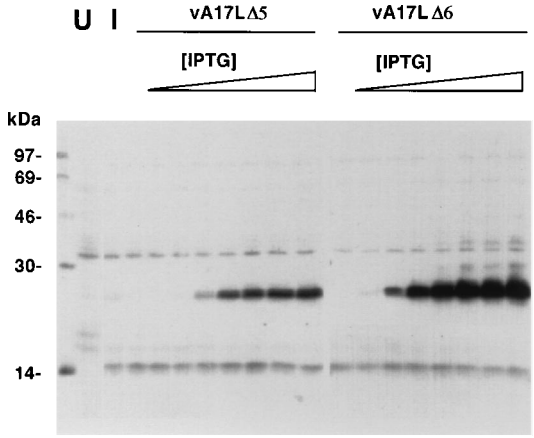

FIG. 7. Western blot analysis showing the synthesis of A17L protein at different concentrations of IPTG. BS-C-1 cells were mock infected (U) or infected (I) with vaccinia virus WR or recombinant virus vA17LΔ5 or vA17LΔ6 in the presence of 0, 5, 10, 20, 50, 100, 200, or 500 μM IPTG. At 24 h after infection, cells were harvested directly into Laemmli sample buffer and separated by SDS-PAGE (12.5% gel). The proteins were transferred to a nitrocellulose membrane, and the membrane was incubated with an anti-A17L antiserum and then with [^{125}I]protein A. Following this procedure, the lower half of the nitrocellulose was incubated with monoclonal antibody C3, against the 14 kDa-fusion protein, and ^{125}I-labeled sheep anti-mouse immunoglobulin G. This figure was generated by using Adobe Photoshop 3.0.

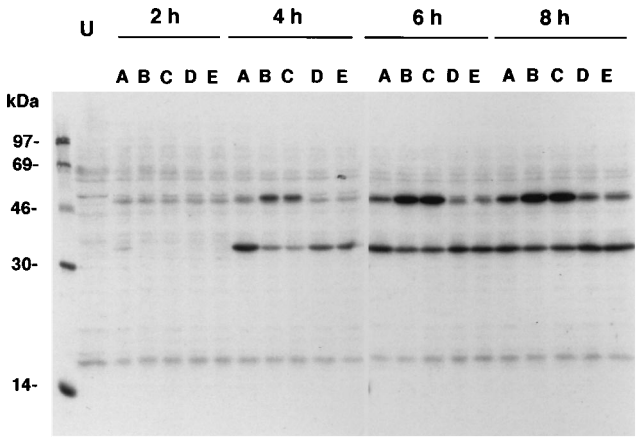

FIG. 8. Western blot analysis to compare the levels of synthesis of the A18R protein in cells infected with vaccinia virus WR and recombinant viruses. BS-C-1 cells were mock infected (U) or infected with vaccinia virus WR (lanes A) or recombinant virus vA17LΔ5 (lanes B and C) or vA17LΔ6 (lanes D and E) in the absence (lanes B and D) or presence (lanes C and E) of 100 μM IPTG. At the times indicated, cells were harvested directly into Laemmli sample buffer, and the proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with an antiserum against a peptide of the A18R protein and then with [^{125}I]protein A. Labeled bands were visualized by autoradiography. This figure was generated by using Adobe Photoshop 3.0.

were seen in close proximity but not obviously associated with the electron-dense material (Fig. 10B). We also observed regions of the cytoplasm which were devoid of cellular organelles and which appeared to have a density lighter than that of the rest of the cellular matrix. These regions were often, but not exclusively, around the dense masses. In some cells, we could see what appeared to be phagocytic vesicles containing cellular debris and the occasional virion, but these were evident in all samples and may represent material from virus stocks that has been phagocytosed by cells during the course of infection.

DISCUSSION

The origin of the vaccinia virus membrane is a central question in poxvirus morphogenesis. Early electron microscopy studies suggested de novo formation (7, 8), whereas more recent studies indicated the recruitment of cellular membranes from the intermediate compartment between the endoplasmic reticulum and the Golgi apparatus (48). Resolution of this conflict will undoubtedly require the identification and characterization of the essential protein components of the earliest observable viral membrane structures. We considered that the product of the A17L gene was a candidate IMV protein because its primary sequence was similar to that of type 1 membrane proteins. Furthermore, Rodriguez et al. reported that the A17L protein coprecipitated with the 14-kDa fusion protein when an antiserum to the latter was used. Here, we provide the first direct evidence that the A17L protein is associated with the membranes of immature viral particles. To obtain this evidence, we first prepared an antiserum to the C-terminal peptides predicted from the A17L ORF.

By immunoelectron microscopy of infected cells, we demonstrated that the A17L protein was associated with virion membranes. Heavy immunogold labeling occurred on the earliest membrane structures identified, the crescent membranes, as well as on the membranes enclosing the spherical immature forms of the virus. We observed less labeling of the brick-shaped mature IMV forms, which may be due to the masking or removal of the epitope. The labeling of the immature virions was noticeably asymmetric, being stronger on the inner concave side of the membrane than on the outer face. As the antibody is directed to the C terminus of this protein, it is evidently oriented to the inside of the immature virion.

Rifampin is a powerful tool for studying vaccinia virus assembly since it blocks an early stage in virion morphogenesis. Instead of the typical viral membrane crescents, irregular membranes lacking spicules form in the presence of rifampin. These membranes delimit electron-dense aggregates, and the entire structures have been called rifampin bodies or membrane-limited domains. We found that in the presence of rifampin, the A17L antiserum labeled the membranes surrounding the rifampin bodies. Thus, the A17L protein is a component of the earliest recognizable viral membrane structures. These data are also supported by observations that A17L protein produced in an in vitro transcription-translation system associates with canine pancreatic microsomal membranes (59). When rifampin was washed out of cells, virus morphogenesis resumed almost immediately and the A17L protein was found associated with the crescent membranes which are derived from the rifampin bodies. Two other viral proteins have been shown to be associated with early viral membranes. One of these proteins, encoded by the D8L ORF, has a molecular mass of 32 kDa, a C-terminal hydrophobic region, and homology with carbonic anhydrase. Recent immunoelectron microscopic studies indicate that this protein, like A17L, is associated with vaccinia virus membranes at all stages of virion assembly, including the membranes of rifampin bodies and viral crescents (47). The other protein, known as the 14 kDa-fusion protein, is encoded by A27L and is also associated with the membranes of rifampin bodies but apparently not those of the crescents (47). However, expression of neither D8L nor A27L is required for IMV formation. Accordingly, these proteins are not essential for formation of the immature IMV membrane. It was therefore of paramount importance to determine whether A17L is required for viral membrane formation.

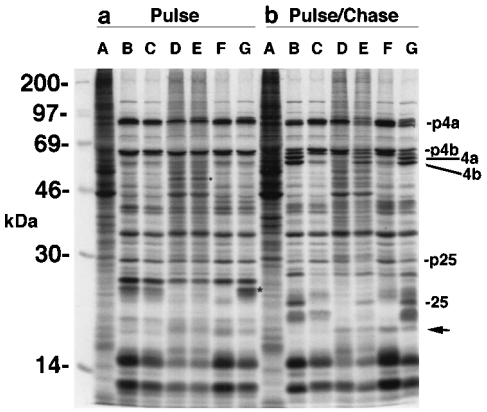

FIG. 9. SDS-PAGE of [^{35}S]methionine-labeled late viral proteins from cells infected with vaccinia virus WR and recombinant viruses. BS-C-1 cells were mock infected (lanes A) or infected with vaccinia virus WR in the absence (lanes B) or presence (lanes C) of 100 μM rifampin or with recombinant virus vA17LΔ5 or vA17LΔ6 in the absence (lanes D and F) or presence (lanes E and G) of 100 μM IPTG. At 12 h after infection, cells were labeled with [^{35}S]methionine for 30 min and either harvested immediately into Laemmli sample buffer (a) or incubated for a further 12 h in medium containing excess unlabeled methionine (b) before harvesting. The positions of migration of the A17L protein (*) and the positions of other bands unique to the recombinant viruses (·, ←) are shown. The positions of bands of the major vaccinia virus precursor proteins (p4a, p4b, and p25) and their mature, processed forms (4a, 4b, and 25) are also indicated. This figure was generated by using Adobe Photoshop 3.0.

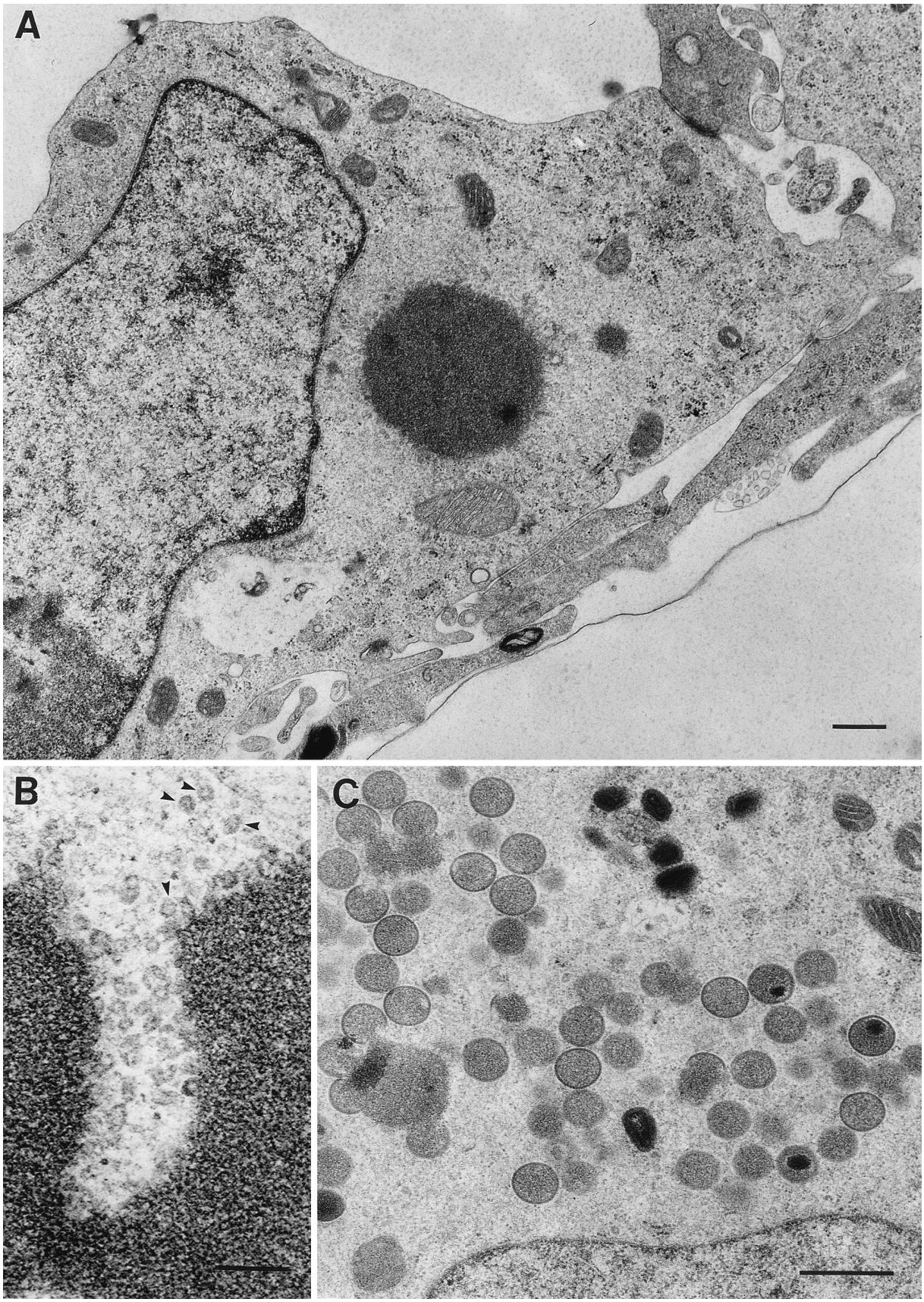

FIG. 10. Electron microscopy of ultrathin sections of BS-C-1 cells infected with vA17LΔ5 for 18 h in the absence (A and B) or presence (C) of 100 μM IPTG. Scale bars represent 500 nm.

To investigate the function of the A17L protein, we made recombinant viruses in which the endogenous copy of the gene was deleted and replaced by an inducible one. Fuerst et al. (17) originally described the use of the *E. coli lac* operator system to regulate the expression of foreign genes recombined into the vaccinia virus genome. Subsequently, the *lac* operator system was used to regulate the expression of endogenous genes encoding both structural proteins and transcription factors (11, 34, 40, 41, 61–65). The strategy involved the incorporation of the *lac* repressor gene under control of a vaccinia virus early/late promoter and the insertion of the *lac* operator adjacent to the RNA start site of the target gene. Although this method provides a powerful way to regulate the expression of intermediate- and late-stage genes, some leakiness was noted. To reduce the leakiness of expression of foreign genes by vaccinia virus, Ward et al. (57) devised a two-tier regulation system. A recombinant vaccinia virus system that contained genes encoding (i) the *E. coli lac* repressor which was synthesized constitutively, (ii) the bacteriophage T7 RNA polymerase controlled by a vaccinia virus late promoter and *lac* operator, and (iii) a target gene controlled by a T7 promoter and *lac* operator was constructed. Thus, in the absence of IPTG, the repressor prevents transcription of the T7 polymerase gene as well as the target gene should any polymerase form. With this system, expression of a reporter gene was undetectable in the absence of IPTG and was induced 10,000- to 20,000-fold in the presence of IPTG. To adapt this system to regulate indigenous vaccinia virus genes, we first inserted a copy of the A17L gene next to the T7 promoter and *lac* operator and then deleted the original A17L. The latter step was problematic, however, because the regulatory elements of the flanking A16L and A18R genes overlapped the A17L gene. The viruses that we constructed were designed to accommodate the expression of these neighboring genes while allowing the deletion of most of the A17L gene. We made two viruses, vA17LΔ5 and vA17LΔ6, both of which preserved the late promoter of the A16L gene, which encodes a protein of unknown function. The difference between the two viruses lay in the sequence preceding the A18R gene, which encodes an essential protein involved in RNA degradation. vA17LΔ6 was constructed to retain only the natural late promoter for the A18R gene and lacked the endogenous early promoter, whereas in vA17LΔ5, the H5 early/late promoter was substituted for the natural promoters. Both of these viruses were IPTG dependent, and their growth could be regulated by IPTG concentrations in the range of 0 to 50 μM. At concentrations above 50 μM, maximal replication was achieved. In the presence of 100 μM IPTG, the virus yields were within 1 log unit of that obtained with wild-type vaccinia virus WR in the same cell line. Most important, no replication was detected in the absence of IPTG. Plaque formation also was absolutely dependent on IPTG. The plaque sizes of the recombinant viruses, however, were smaller than that observed for either WR or the intermediate virus vT7lacOI-A17L, with plaques made by vA17LΔ5 being the smallest. This does not appear to be a consequence of the disruption of the HA gene, with a resulting effect on EEV formation, as the intermediate virus vT7lacOI-A17L, which also has the HA locus disrupted, produces plaques equivalent in size to those formed by WR. At this time, we do not know whether the reduced virus yield and smaller plaque formation in the presence of IPTG are consequences of the levels and timing of A17L expression or those of the neighboring genes. The important point, however, is that virus production is totally blocked in the absence of IPTG.

Electron microscopic analysis, which has given us the most information in the past about vaccinia virus morphogenesis, revealed that all virion forms are present in cells infected with the mutant viruses under permissive conditions. Under nonpermissive conditions, i.e., without IPTG, there were no characteristic viral membrane structures. Instead, we noted regular spherical accumulations of electron-dense material within the cytoplasm of infected cells which were often associated with regions lighter in density than the surrounding cytoplasm and which appeared to displace cellular organelles. A similar observation was recently made by Rodriguez et al. (36) using independently constructed A17L-inducible recombinant vaccinia viruses. Other known mutants which are blocked at a very early stage of morphogenesis include a group of temperature-sensitive mutants, *ts*15, *ts*28, *ts*54, and *ts*61, which when grown at the nonpermissive temperature yield large areas of grainy cytoplasm devoid of cellular organelles and recognizable virion membrane structures. Recent reports show that the defect in these mutants lies in the F10 ORF, which encodes a protein kinase (54, 56). The role of this protein in morphogenesis has not been determined.

We conclude that the A17L protein is an essential component of the immature viral membrane and is required for the formation of vaccinia IMV membranes and all subsequent steps in virion morphogenesis. Further studies on the intracellular trafficking of the A17L protein may resolve the central question regarding the origin of poxvirus membranes.

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