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Vaccinia Virus A21 Virion Membrane Protein Is Required for Cell Entry and Fusion

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We provide the initial characterization of the product of the vaccinia virus A21L (VACWR140) gene and demonstrate that it is required for cell entry and low pH-triggered membrane fusion. The A21L open reading frame, which is conserved in all sequenced members of the poxvirus family, encodes a protein of 117 amino acids with an N-terminal hydrophobic domain and four invariant cysteines. Expression of the A21 protein occurred at late times of infection and was dependent on viral DNA replication. The A21 protein contained two intramolecular disulfide bonds, the formation of which required the vaccinia virus-encoded cytoplasmic redox pathway, and was localized on the surface of the lipoprotein membrane of intracellular mature virions. A conditional lethal mutant, in which A21L gene expression was regulated by isopropyl-β-D-thiogalactopyranoside, was constructed. In the absence of inducer, cell-to-cell spread of virus did not occur, despite the formation of morphologically normal intracellular virions and extracellular virions with actin tails. Purified virions lacking A21 were able to bind to cells, but cores did not penetrate into the cytoplasm and synthesize viral RNA. In addition, virions lacking A21 were unable to mediate low pH-triggered cell-cell fusion. The A21 protein, like the A28 and H2 proteins, is an essential component of the poxvirus entry/fusion apparatus for both intracellular and extracellular virus particles.

The mechanism used by poxviruses to enter cells is poorly understood, partly because of the extreme complexity of the members of this family. Vaccinia virus (VACV), the prototypic member of the *Poxviridae*, contains a large double-stranded DNA genome of approximately 195 kbp that encodes nearly 200 proteins with functions relating to viral ingress, transcription, genome replication, morphogenesis, egress, and host interactions (23). Two major types of infectious VACV particles have been characterized. The most abundant form, called the intracellular mature virion (IMV), can be isolated following cell lysis and consists of a nucleoprotein core surrounded by a lipid envelope that contains more than a dozen viral proteins (19). IMVs are very stable and may mediate spread from host to host. A second membrane, derived from *trans*-Golgi or endosomal cisternae, with at least five unique viral proteins, surrounds the IMV in the extracellular cell-associated enveloped virions (CEVs) and in the dissociated extracellular enveloped virions (EEVs) (43). CEVs and EEVs may mediate spread to adjacent and more distal cells, respectively (6, 7, 26). There is evidence that IMVs penetrate cells by fusing with the plasma membrane at neutral pH (8, 11, 18), although other mechanisms have been suggested (22). In contrast, there is no evidence that the EEV membrane fuses with the cell. Two studies suggest that EEVs enter cells by endocytosis, presumably followed by acid-mediated disruption of the EEV membrane within intracellular vesicles (17, 46).

Many of the IMV membrane proteins have roles in virus assembly, while others have been implicated in VACV attachment and entry. The products of the A27L, D8L, and H3L genes (note that original names for VACV genes was based on location within a HindIII fragment followed by L or R indicating left or right direction of transcription; the latter can be omitted when referring to the protein product of the gene) enhance virus attachment by binding to cell surface glycosaminoglycans (9, 15, 16, 21). An additional role for the A27 protein in fusion was proposed (47), although recent studies with a deletion mutant demonstrate that this protein is not essential for neutral pH virus entry or low pH-triggered cell-cell fusion (37, 49). In contrast, two other IMV membrane proteins, namely A28 and H2, are each required for virus entry and cell-cell fusion (37, 39). Because A28 and H2 are necessary for infection mediated by IMVs and cell-to-cell spread mediated by CEVs and EEVs, there is likely to be a single fusion mechanism involving only the IMV membrane (37). Furthermore, since A28 and H2 are conserved, all poxviruses are likely to penetrate cells in a similar way. Thus far, however, no cellular receptor proteins necessary for fusion have been identified.

We have continued our analysis of poxvirus gene products in order to identify and characterize additional conserved IMV transmembrane proteins, which may have essential roles in virus assembly or entry. Here we provide the initial characterization of the product of the A21L gene (VACWR140; accession number AY243312) of the Western Reserve (WR) strain of VACV. First, we showed that the protein was expressed late in infection and incorporated into the membrane of IMV. Next, we constructed a recombinant VACV with an inducible A21L gene and demonstrated that the virus has a conditional lethal phenotype. Further analysis indicated that the A21 protein was not required for virus assembly but was essential for virus entry into cells and cell-cell fusion. Therefore, A21, like A28 and H2 (37, 39), forms part of the poxvirus fusion appa-

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FIG. 1. Conservation of the A21L gene in *Poxviridae.* Multiple alignment of A21 orthologs, including a representative from each sequenced genus of the *Chordopoxvirinae* and *Entomopoxvirinae* subfamilies. Invariant cysteines are denoted by white text upon a black background. Other identical or similar residues are indicated by shading. VAC, vaccinia virus (*Orthopoxvirus*); MYX, myxoma virus (*Leporipoxvirus*); LSD, lumpy skin disease virus (*Capripoxvirus*); SWP, swinepox virus (*Suipoxvirus*); YLD, Yaba-like disease virus (*Yatapoxvirus*); ORF, ORF virus (*Parapoxvirus*); MOC, molluscum contagiosum virus (*Molluscipoxvirus*); FWP, fowlpox virus (*Avipoxvirus*); AME, *Amsacta moorei* entomopoxvirus (*Entomopoxvirus B*); MSE, *Melanoplus sanguinipes* entomopoxvirus (*Entomopoxvirus B*).

ratus required for cell entry of intracellular and extracellular virions.

MATERIALS AND METHODS

Propagation and purification of VACV. All studies were carried out with the WR strain of VACV (ATCC VR-1354) or recombinant viruses derived from this strain. Virus amplification and titration were performed as previously described (12) with the addition of 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium for vA21Li unless otherwise indicated. IMVs were purified from HeLa S3 cells by sedimentation twice through a 36% sucrose cushion and banded once or twice on a 25 to 40% sucrose gradient essentially as described previously (12).

Recombinant virus construction. vA21i was constructed from vT7LacOI, a recombinant VACV that possesses an IPTG-inducible T7 RNA polymerase gene and an *Escherichia coli lac* repressor gene situated within the dispensable thymidine kinase locus (2), essentially as described for vE10i (40). The final PCR product used for transfection to generate vA21i consisted of the entire A21L open reading frame (ORF) (nucleotides 127905 to 128258) placed under the control of a *lac* operator-regulated T7 promoter containing a consensus sequence for initiation of translation (CGAAATTAATACGACTCACTATAGG GAATTGTGAGCGCTCACAATTCCCGCCGCCACCATG), adjacent to a copy of the enhanced green fluorescent protein gene (EGFP) (accession number AAG27429) placed under the control of a VACV synthetic early-late promoter (AAAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAATG). Initiation codons are underlined. In addition, flanking sequences of approximately 650 bp were incorporated into the recombinant PCR product to permit homologous recombination. The final product was assembled by recombinant PCR using Accuprime *Pfx* (Invitrogen) as previously described (2). BS-C-1 cells in 24-well plates were infected with 1 PFU per cell of vT7LacOI in Optimem medium (Invitrogen) and 2 h later were transfected with 0.3 μg of purified PCR product and Lipofectamine 2000 (Invitrogen). Infected cells were harvested 24 h after infection and subjected to three freeze-thaw cycles, and dilutions were plated on BS-C-1 monolayers. Virus exhibiting green fluorescence was purified by five rounds of plaque isolation in the presence of 50 μM IPTG.

vA21-V5 was constructed using essentially the same protocol as described for the generation of vA21i. The recombinant PCR product comprised a copy of the A21L gene encoding a C-terminal V5 epitope tag (GKPIPNPLLGLDST) under the control of the native A21L promoter as well as a copy of the EGFP gene under the control of a VACV late promoter and VACV flanking sequences to allow homologous recombination. Following transfection of VACV-infected cells, recombinant virus was identified by green fluorescence and clonally purified during three rounds of plaque isolation. All gene insertions and modifications were confirmed by PCR and sequencing of viral DNA.

One-step virus growth curve. BS-C-1 monolayers in 24-well plates were incubated with 5 PFU per cell of vA21i or vT7LacOI for 1 h at 4°C. Following adsorption, the cells were washed three times before incubation at 37°C in medium with or without 50 μM IPTG. Cells were harvested and subjected to three freeze-thaw cycles and sonicated three times for 30 sec each. Titers were determined by plaque assay as previously described (12).

Antibodies. The following mouse monoclonal antibodies (MAbs) were used: 7D11 to the L1 protein (53), C3 to the A27 protein (31), anti-V5 epitope (Invitrogen), and anti-protein disulfide isomerase (Affinity BioReagents, Golden, CO). Rabbit polyclonal antibodies to the following VACV proteins were used: L1 (28), A4 (10), A10 (R. Doms and B. Moss, unpublished), A3 (3), H4 (1), A28 (G. Nelson and B. Moss, unpublished). For detection of CEV, rat anti-B5R MAb (19C2) was used (35). Cy5-conjugated donkey anti-rat antibody, fluorescein isothiocyanate-conjugated goat anti-mouse antibody, and rhodamine red-X-conjugated goat anti-rabbit antibody were purchased from Jackson Immunoresearch and used according to the manufacturer's recommendations.

Western blot analysis. Virus- or mock-infected BS-C-1 cells were harvested, washed in phosphate-buffered saline (PBS), incubated in cell lysis buffer containing 0.5% NP-40, 10 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$, 10 mM NaCl, 8 μg/ml micrococcal nuclease, and protease inhibitor cocktail (complete mini EDTA-free; Roche) with or without 20 mM *N*-ethylmaleimide (NEM; Sigma) on ice for 30 min, and then solubilized in lithium dodecyl sulfate sample buffer (Invitrogen) with or without NuPage sample reducing agent (Invitrogen). For some experiments, cell pellets were washed with PBS and treated with 20 mM NEM or 20 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; Invitrogen) prior to cell lysis. In other cases, cell lysates were treated with the reducing agent Tris-(2-carboxyethyl) phosphine (TCEP; Sigma) prior to alkylation with NEM or AMS. All lysates were incubated at 70°C for 10 min and

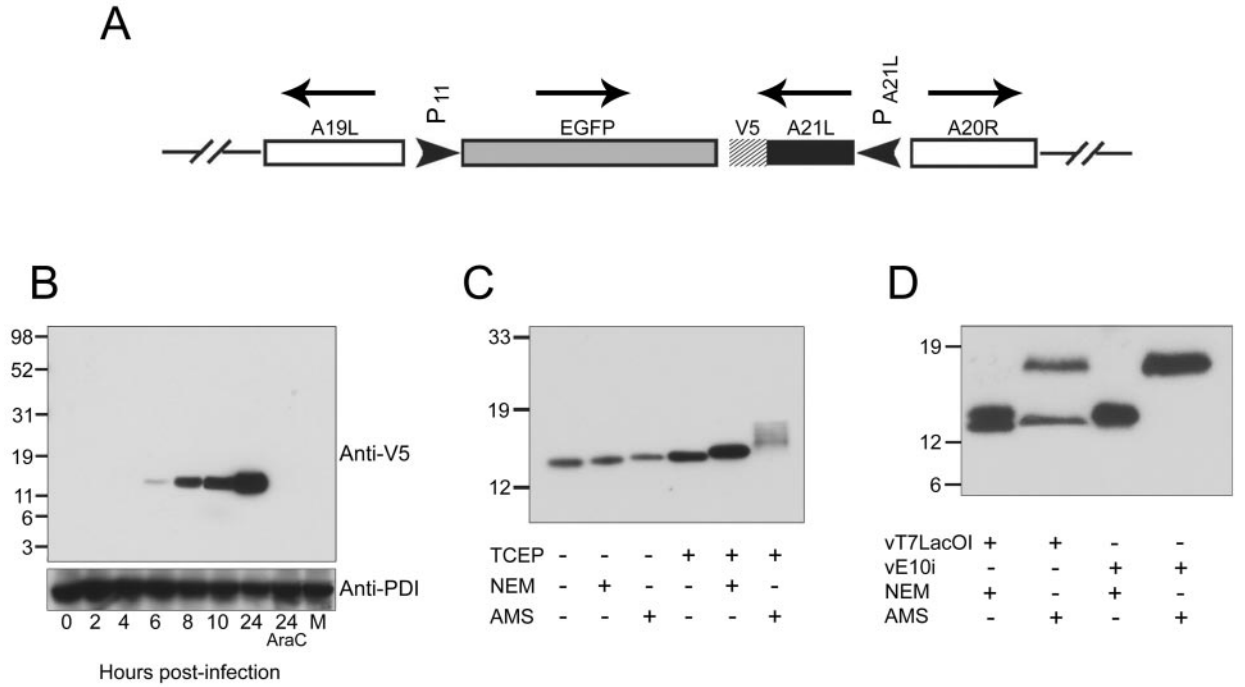

FIG. 2. Expression of A21 and formation of intramolecular disulfide bonds. (A) Diagram of a portion of the genome of recombinant vA21-V5 containing a V5 epitope encoded at the end of the A21L gene. P_{A21L} and P_{11} refer to the natural promoter of the A21L gene and the promoter for the gene encoding the 11K protein, respectively. Arrows over ORFs indicate the direction of transcription. (B) Western blot showing A21 expression. BS-C-1 cells were either mock-infected (M) or infected with 5 PFU of vA21-V5 per cell in the absence or presence of AraC and disrupted in SDS-PAGE loading buffer at indicated hours postinfection. Cell lysates were analyzed by SDS-PAGE and Western blotting with an anti-V5 MAb and anti-protein disulfide isomerase (anti-PDI) as a loading control. (C) Analysis of intramolecular disulfide bonds. BS-C-1 cells were infected with 5 PFU per cell of vA21-V5 for 18 h and then disrupted in SDS-PAGE loading buffer containing NEM or AMS. Some cells were treated with the reducing agent TCEP prior to alkylation with either NEM or AMS. SDS-PAGE and Western blotting were performed using an anti-V5 MAb. (D) Dependence of disulfide bond formation on expression of E10 protein. BS-C-1 cells were infected with vE10i or vT7LacOI in the absence of IPTG. Two hours later, the cells were transfected with a plasmid encoding A21 with a C-terminal V5-epitope tag under the control of the natural A21 promoter. At 18 h postinfection, cells were disrupted in SDS-PAGE loading buffer containing NEM or AMS. SDS-PAGE and Western blotting were carried out with an anti-V5 MAb. The positions of molecular mass standards in kDa are shown on the left axis of each panel.

resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10%, 12%, or 4 to 12% NuPage Bis-Tris gels (Invitrogen). For Western blot analysis of purified virions, indicated amounts were solubilized in lithium dodecyl sulfate sample buffer with or without the NuPage sample reducing agent. Lysates were transferred to nitrocellulose membranes and blocked with Tris-buffered saline supplemented with 5% nonfat dried milk and 0.05% Tween-20 for 1 h at room temperature. Subsequently, membranes were incubated with the appropriate primary antibody, washed, incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce), and analyzed with chemiluminescence reagents (Pierce).

Confocal microscopy. Infected HeLa cells were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature, washed three times with PBS, and quenched with 2% glycine. Subsequently, cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature and washed three times with PBS. Cells were incubated with the appropriate primary antibody or Alexa Fluor 568 phalloidin (Invitrogen) diluted in 10% complement-inactivated fetal bovine serum in PBS for 1 h at 37°C. Cells were washed three times with PBS and incubated with the appropriate fluorophore-conjugated secondary antibody for 1 h at 37°C. DNA was visualized with diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) according to the manufacturer's directions. Images were obtained using a Leica TCS-NT/SP2 inverted confocal microscope with attached argon laser (Coherent Inc.).

Northern blot analysis. Infected cells were washed in PBS, and total RNA was isolated with an RNeasy Minikit (QIAGEN). Total RNA (5 μg) was fractionated in glyoxal-agarose gels prepared according to the manufacturer's directions (Northernmax-Gly; Ambion). RNA species were transferred to Hybond N+ membranes in the presence of 20 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate). [α-^{32}P]dCTP-labeled double-stranded DNA probes were generated from purified PCR products by using Ready-to-go DNA labeling beads (Amersham). Hybridization was performed in Ultrahyb (Ambion) according to the manufacturer's directions.

In vitro RNA synthesis. Purified virions were incubated at 37°C for 1 h in 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 0.05% NP-40, 5 mM ATP, 1 mM GTP, 1 mM CTP, 0.02 mM UTP, and 1 μCi of [α-^{32}P]UTP (3,000 Ci/mmol). Reaction mixtures were spotted onto DE81 filter papers, air dried, and washed five times in ice-cold 10% trichloroacetic acid followed by three washes in 96% ethanol. The incorporation of [α-^{32}P]UMP into trichloroacetic acid-insoluble material was determined by scintillation counting.

Biotinylation of purified virions. Sucrose gradient-purified virions were biotinylated with 1 mg of EZ-Link sulfo-NHS-SS-biotin [sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate; Pierce] per ml for 30 min at 4°C. Excess labeling reagent was quenched according to the manufacturer's directions, and virions were pelleted by centrifugation at 20,000 × *g* for 30 min at 4°C. Virions were solubilized with SDS-PAGE sample buffer, and the resulting lysates were incubated with neutravidin agarose beads (Pierce) for 1 h at 4°C. The supernatant fraction was removed, and the biotinylated proteins were recovered by incubation of the beads with SDS-PAGE sample buffer containing 50 mM DTT for 1 h at 4°C. The pellet and supernatant fractions were analyzed by reducing SDS-PAGE and Western blotting.

Transient expression of A21. A PCR product of the A21L gene fused to DNA encoding a C-terminal V5 tag under the control of its native promoter was generated with the following primer pair: ATGTTCCCCATTCTACCAAAGA ATTATAC (VACV nucleotides 128346 to 128374) and GGGGTCACGTAGA ATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCGGTAGTAAA

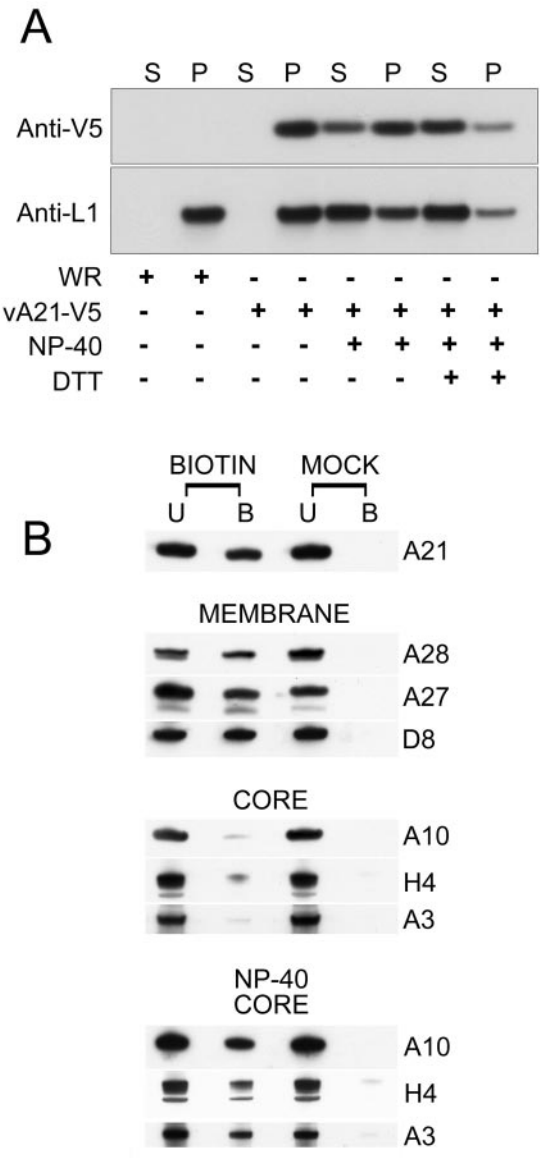

FIG. 3. A21 is a membrane protein exposed on the surface of IMV. (A) Extraction of A21 with a nonionic detergent. Purified VACV (WR) or vA21-V5 virions were suspended in Tris buffer, pH 7.4, and incubated at 37°C for 30 min with or without 1% NP-40 or 50 mM DTT. The virus suspensions were then centrifuged at 20,000 × *g* for 30 min at 4°C and separated into pellet (P) and supernatant (S) fractions. The fractions were analyzed by SDS-PAGE and Western blotting with anti-V5 and anti-L1 MAbs. (B) Biotinylation of IMV surface proteins. Untreated or NP-40-treated purified virions were reacted with 1 mg of sulfo-NHS-SS-biotin per ml for 30 min at 4°C (BIOTIN) or mock-labeled with PBS (MOCK). Excess reagent was quenched and virions were solubilized with SDS-PAGE sample buffer and incubated with neutravidin gel for 1 h at 4°C. The unbound (U) and the bound (B) proteins were treated with SDS-PAGE sample buffer containing 50 mM DTT and analyzed by SDS-PAGE and Western blotting with antibodies to membrane proteins A28, A27, and D8 and to core proteins A10, H4, and A3.

AAATAAGTCAGAATATGCCC (VACV nucleotides 127908 to 127936). Nucleotides noncomplementary to VACV sequences are underlined. The PCR product was amplified using Accuprime *Pfx* polymerase and VACV DNA as a template, purified with a QIAGEN PCR purification kit, and subcloned into pCR-Blunt II-TOPO (Invitrogen). Constructs were verified by PCR and sequencing. For transfections, BS-C-1 cells in 24-well dishes were infected with vE10i or vT7LacOI at a multiplicity of 5. After 2 h, the infected cells were transfected with 0.25 μg of vector DNA and Lipofectamine 2000. At 18 h after infection, cells were collected by centrifugation, washed in PBS, and treated with NEM or AMS.

Low pH-induced cell-cell fusion. For "fusion from without," BS-C-1 monolayers were incubated for 1 h at room temperature with 200 PFU per cell of purified virions from cells infected with vA21i in the presence of IPTG or the corresponding optical density at 260 nm (OD_{260}) of virions from cells infected in the absence of IPTG. Following this, cell monolayers were washed extensively and incubated with either pH 5.3 or pH 7.4 buffer for 2 min at room temperature before the buffer was replaced with medium containing 300 μg of cycloheximide (Sigma) per ml for 3 h to allow cell-cell fusion. Cells were subsequently fixed and stained with Alexa Fluor 568 phalloidin and DAPI for visualization by confocal microscopy. For "fusion from within," BS-C-1 monolayers were infected with 5 PFU per cell of vA21i in the presence or absence of 50 μM IPTG for 18 h and treated with either pH 5.3 or pH 7.4 buffer for 2 min at room temperature before the buffer was replaced with complete Eagle's minimal essential medium. Cells were stained with DAPI and visualized by phase and fluorescence microscopy.

RESULTS

Expression of the A21L gene product. The A21L (VACWR140) gene was selected for study because it belongs to the subset of ORFs that are present in all sequenced poxviruses and encode proteins with putative transmembrane domains. In addition, the gene has been retained in poxviruses such as modified vaccinia virus Ankara (4), which have undergone numerous deletion mutations while propagated in vitro. A multiple alignment of representative A21L orthologs from each sequenced genus of vertebrate and insect poxviruses is shown in Fig. 1. Overall, the N-terminal halves of these proteins are more highly conserved than the C-terminal halves. Four cysteine residues are notable among the completely conserved amino acids, and there is an additional conserved cysteine in the transmembrane domain of chordopoxvirus orthologs. The degree of amino acid identity of the predicted N-terminal transmembrane domains of the vertebrate poxvirus A21L orthologs is relatively high, suggesting some specific interactions in addition to membrane insertion.

Late promoter motifs were discerned upstream of orthologous A21L ORFs, leading to a suggestion that the proteins are expressed at late times of infection (36). To demonstrate that the predicted 13.6-kDa A21 protein is expressed during VACV infection, we generated the recombinant vA21-V5 in which the A21L gene remained under the control of its putative promoter but was modified by addition of codons specifying a C-terminal V5 epitope tag. Selection of the recombinant VACV was facilitated by coinsertion of the EGFP ORF under the control of a late promoter adjacent to the modified A21L gene (Fig. 2A). vA21-V5 was clonally purified, and the predicted genomic modifications were verified by DNA sequencing. The epitope tag had no discernible adverse effect on virus replication as determined by plaque size or virus yield (data not shown). In lysates of BS-C-1 cells infected with vA21-V5, a protein with an apparent mass of approximately 14 kDa was detected by Western blotting with antibody to the V5 tag (Fig. 2B). The 14-kDa protein was detected at 6 h postinfection and increased in amount until 24 h. The protein was not detected even at 24 h when viral DNA replication was prevented by the addition of AraC (Fig. 2B). Western blotting with antibody to protein disulfide isomerase was performed as a loading con-

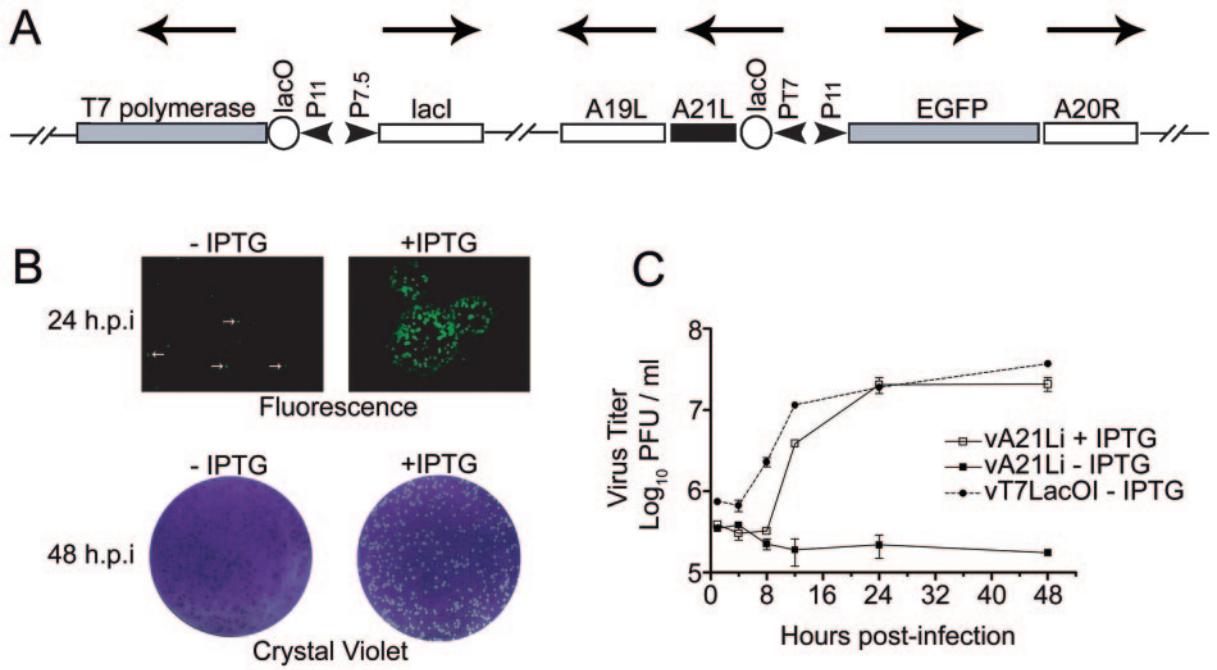

FIG. 4. Construction of vA21i, a conditional lethal VACV with an inducible A21L gene. (A) Diagram of a portion of the genome of vA21i. Arrows over ORFs indicate the direction of transcription. Abbreviations: lacO, *E. coli lac* operator; P_{11}, VACV late promoter; $P_{7.5}$, VACV early/late promoter; lacI, *E. coli lac* repressor gene; P_{T7}, bacteriophage T7 promoter. (B) Plaque formation by vA21i in the presence and absence of IPTG. BS-C-1 monolayers were infected with vA21i in the presence or absence of IPTG. At 24 h postinfection, cells were observed by fluorescence microscopy for the presence of EGFP. At 48 h postinfection, monolayers were fixed and stained with crystal violet. (C) Yields of vA21i in the presence or absence of IPTG. BS-C-1 monolayers were infected with vA21i or vT7LacOI at a multiplicity of 5 PFU per cell in the absence or presence of IPTG. The cells were harvested at indicated hours after infection, and virus titers were determined by plaque assay in the presence of 50 μM IPTG. Experiments were performed in duplicate, and data points represent the mean ± standard error.

trol. Both the kinetics of expression and the requirement for DNA replication correlated well with the presence of a late stage promoter, as predicted from sequence analysis.

A21 contains intramolecular disulfide bonds. The VACV A21 protein contains 5 cysteine residues that could potentially form inter- and intramolecular disulfide bonds. Intermolecular disulfides seemed unlikely, as there was only a small difference in the mobility of unreduced and TCEP-reduced A21 (Fig. 2C). The slightly more rapid mobility of the unreduced species, however, suggested the presence of intramolecular disulfide bonds, which can cause a more compact structure in the presence of SDS. To investigate the presence of intramolecular disulfide bonds, cells infected with vA21-V5 were lysed in the presence of the alkylating agents NEM or AMS. Alkylation of a single sulfhydryl group with NEM or AMS increases the molecular mass of a protein by 0.125 or 0.536 kDa, respectively. The slight retardation of mobilities observed when A21 was alkylated by NEM or AMS was most consistent with a single reactive cysteine and two intramolecular disulfides (Fig. 2C). The presence of intramolecular disulfide bonds was supported by an additional increase in mobility when A21 was first reduced with TCEP and then alkylated with NEM or AMS (Fig. 2C). Under the latter conditions, a ladder of bands extending to the maximal 2.7-kDa increase predicted by alkylation of all five cysteines with AMS was partially resolved.

Formation of intramolecular disulfide bonds in A21 is dependent on the VACV-specific redox pathway. The VACV genome encodes three oxidoreductases (E10, A2.5, and G4) that act sequentially to catalyze the formation of intramolecular disulfide bonds within the cytoplasmic domains of certain IMV membrane proteins (38, 41, 42, 50). Repression of any one of the redox proteins prevents the formation of disulfide bonds in the target protein. The following experiment was designed to establish whether formation of the intramolecular disulfides of A21 depends on the VACV-encoded redox pathway. A plasmid, comprising the entire A21L ORF with a C-terminal V5 epitope tag under the control of its native promoter, was transfected into BS-C-1 cells infected with a conditional lethal VACV mutant vE10i (42) in the absence of inducer so that expression of the E10L gene product did not occur. As a control, cells infected with the vE10i parental virus vT7LacOI were transfected in parallel; vT7LacOI expresses the E10 protein at wild-type levels in the absence of inducer. The alkylation procedure described in the previous section was used to discriminate between free reactive cysteines and unreactive disulfides. Cells were disrupted in the presence of NEM or AMS, and the resulting cell lysates were analyzed by Western blotting. A21 was completely reduced in cells infected with vE10i in the absence of inducer as shown by the complete mobility shift caused by alkylation with AMS (Fig. 2D). In contrast, there was a mixture of oxidized and reduced A21 in

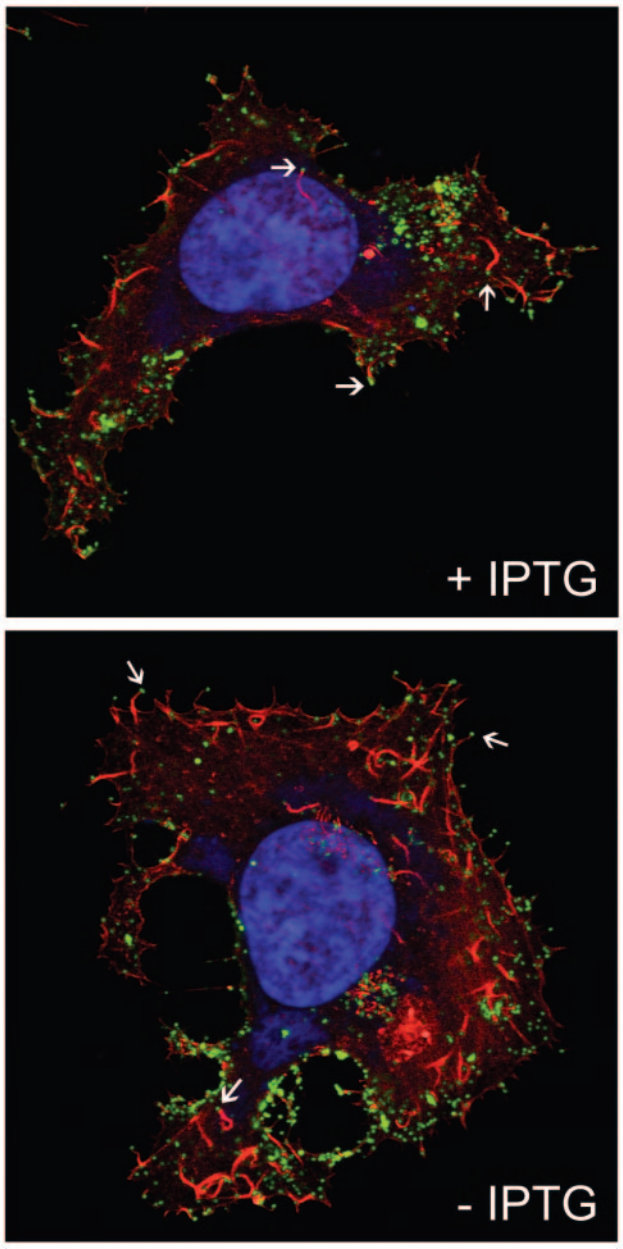

FIG. 5. Visualization of CEVs with actin tails. HeLa cell monolayers were infected at a multiplicity of 5 PFU per cell in the absence or presence of 50 μM IPTG for 20 h, fixed with 3% paraformaldehyde, and quenched with 2% glycine. Cell surface CEVs were immunolabeled with anti-B5R MAb and Cy5-conjugated goat anti-rat secondary antibody. Following this, cells were permeabilized by the addition of 0.1% Triton X-100 and stained with DAPI to visualize cellular and viral DNA and with Alexa Fluor 568 phalloidin to visualize filamentous actin. Arrows point to representative CEVs at the tips of actin tails.

cells infected with the vT7LacOI control virus as seen by the double bands after alkylation with NEM or AMS. In vT7LacOI-infected cells, the unoxidized A21 probably resulted from over expression, as was previously reported when other genes were expressed by transfection (38). Thus, the VACV redox system is required for the formation of two intramolecular disulfide bonds in A21.

A21 is associated with the IMV membrane of mature virions. The association of A21-V5 with sucrose gradient-purified virions was demonstrated by Western blotting (Fig. 3A). The release of some A21-V5 into the supernatant upon addition of the nonionic detergent NP-40 and the release of the majority in the presence of NP-40 and DTT paralleled that of L1, a well-characterized IMV membrane protein (Fig. 3A). To determine whether A21 is on the surface of IMV, purified virions were treated or mock-treated with the membrane-impermeant biotinylating agent sulfo-NHS-SS-biotin, which selectively reacts with exposed primary amines. After excess reagent was quenched, the proteins were dissociated with SDS and incubated with neutravidin beads, which bind biotin. The bound and unbound proteins were subjected to SDS-PAGE and Western blot analysis. Binding of proteins to neutravidin was specific as no proteins of the mock-treated virions bound (Fig. 3B). The proportion of biotinylated A21 was similar to that of the other IMV surface proteins A27 (48), A28 (38), and D8 (24) and considerably more than the three core proteins tested (Fig. 3B). The core proteins were not intrinsically resistant to biotinylation, as this occurred after the membrane was removed by NP-40 treatment (Fig. 3B).

A21 expression is required for plaque formation and the production of infectious virus. The conservation of A21 among vertebrate and invertebrate poxviruses suggested that A21 is essential for poxvirus replication. To test this hypothesis, we constructed vA21i, which requires IPTG for expression of A21. As diagrammed in Fig. 4A, vA21i contains a constitutively expressed *E. coli lac* repressor, bacteriophage T7 RNA polymerase regulated by an *E. coli lac* operator, and the A21L gene regulated by a T7 promoter and *lac* operator. Consequently, in the presence of IPTG, the *lac* repressor is inactivated and T7 RNA polymerase transcribes A21. In the absence of IPTG, the *lac* repressor prevents expression of T7 RNA polymerase, which is needed for transcription of the A21L ORF. For additional stringency, a *lac* operator also regulates A21 transcription. The EGFP gene was coinserted adjacent to the A21L ORF to distinguish vA21i from parental vT7lacOI (2) and aid in picking plaques with a fluorescence microscope. vA21i formed large plaques at the optimal IPTG concentration (50 μM), as shown by fluorescence or crystal violet staining (Fig. 4B). In contrast, plaque formation was completely abolished in the absence of IPTG. Upon close inspection, single EGFP-positive cells were detectable by fluorescence microscopy in cells infected without IPTG (Fig. 4B), demonstrating that vA21i has a defect in virus replication or spread. To determine the nature of the defect, viral replication was assessed under single-step growth conditions in the absence or presence of IPTG. As illustrated in Fig. 4C, the formation of infectious virus was dependent on IPTG, as no significant increase in virus titer was evident 24 h postinfection in the absence of inducer. In contrast, the titer of vA21i increased nearly 100-fold over the same period in the presence of 50 μM IPTG.

A21 expression is not required for morphogenesis and formation of IMV and CEV. To determine if vA21i has an assembly defect under nonpermissive conditions, BS-C-1 cells were

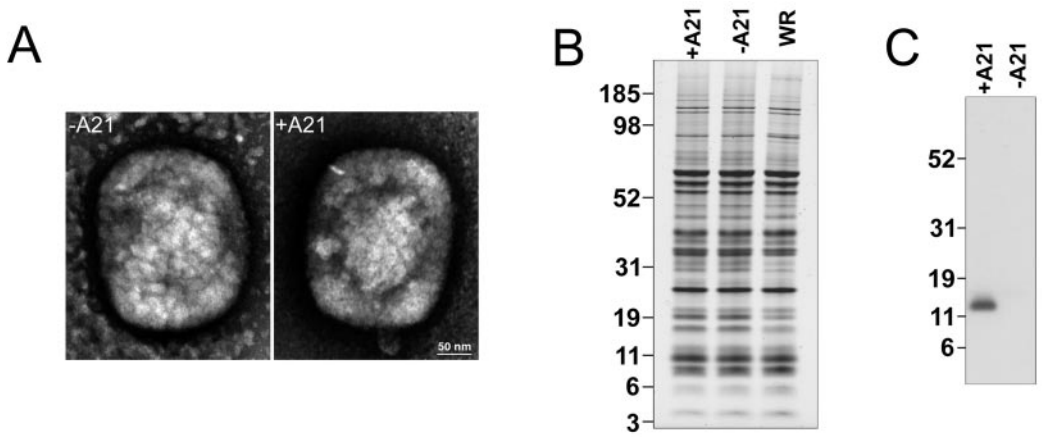

FIG. 6. Electron microscopy and SDS-PAGE of A21$^-$ and A21$^+$ IMVs. (A) Electron micrographs of negatively stained sucrose gradient-purified A21$^-$ and A21$^+$ IMVs. Virions were deposited on grids, washed with water, and stained with 7% uranyl acetate in 50% ethanol for 30 sec. (B) SDS-PAGE of sucrose gradient-purified A21$^-$, A21$^+$, and VACV WR IMVs. The numbers of particles were determined from the optical density and equal amounts of the three types of virions were analyzed by SDS-PAGE and silver staining. The positions and molecular masses in kDa of marker proteins are shown on the left. (C) Western blot of A21$^-$ and A21$^+$ virions. Following SDS-PAGE, Western blotting was performed with rabbit polyclonal antibody to the A21 peptide DKDRDDIPGFARSC.

infected with vA21i in the presence or absence of IPTG and analyzed by transmission electron microscopy. Morphogenesis appeared entirely normal under both conditions; e.g., immature virions, IMVs, intracellular enveloped virions, and CEVs were abundant (data not shown). Confocal microscopy was used to determine whether the CEVs were associated with actin tails, which are important for cell-to-cell spread (33, 34, 51, 54). HeLa cells were infected with vA21i in the presence or absence of IPTG and stained with a MAb to the B5 protein, an outer membrane component of CEVs. The cells were then permeabilized and stained with phalloidin to detect actin. CEVs were present at the tips of actin-containing microvilli in the presence or absence of IPTG (Fig. 5), indicating that the defect in spread of vA21i could not be attributed to a failure at this step or a preceding one.

Comparison of the structure and protein composition of A21$^+$ and A21$^-$ virions. Until now, all preparations of vA21i were made in the presence of IPTG and then used to infect cells in the presence or absence of inducer. Thus, the infecting virus particles always contained the A21 protein, though no further A21 protein synthesis occurred in the absence of IPTG. In the next experiments, we purified IMVs from cells infected with vA21i in the absence or presence of IPTG to produce A21$^-$ or A21$^+$ virions, respectively. The virions were indistinguishable by electron microscopy (Fig. 6A) and SDS-PAGE electrophoresis (Fig. 6B), except for the absence of the A21 protein in A21$^-$ virions (Fig. 6C). The particle/PFU ratio of A21$^-$ virions, however, was >50-fold higher than that of A21$^+$ virions, indicating that most A21$^-$ virions were noninfectious. This low infectivity, which may have been due to traces of the residual A21$^+$ inoculum used to prepare the A21$^-$ virions, was sufficient to account for the decrease in virus yield during one-step growth experiments (Fig. 4C).

A21 virions are unable to penetrate cells and mediate early gene expression. The low infectivity of A21$^-$ virions could have many different causes, manifested at various stages of infection. However, a block at binding or entry seemed most likely, as A21 is an IMV membrane protein. Because poxviruses incorporate the enzymes and factors needed for transcription of early genes, viral RNA synthesis is a good indicator of the presence of cores in the cytoplasm. Early transcription was analyzed at 2 h after infection in the presence of AraC by Northern blotting using ^{32}P-labeled DNA probes complementary to the viral growth factor (C11R) or DNA polymerase (E9L) genes. Prominent bands corresponding to C11R and E9L transcripts were detected in extracts of cells infected with A21$^+$ virus and wild-type VACV but were barely detectable in cells infected with A21$^-$ virus (Fig. 7A). Actin mRNA served as a loading control (Fig. 7A). We also noted that cells infected with A21$^-$ virions exhibited little to no cytopathic effect in comparison to cells infected with A21$^+$ virions; this observation is consistent with a reduction in early gene expression since VACV-induced cell rounding is dependent upon viral early protein synthesis (5).

Although a primary defect in transcription seemed unlikely, it was necessary to demonstrate that A21$^-$ virions are capable of synthesizing RNA. We used an in vitro system in which virions are permeabilized with NP-40 detergent and RNA synthesis is measured by incorporation of ^{32}P-labeled ribonucleoside triphosphates (20). Little difference was found in the ability of permeabilized A21$^-$ and A21$^+$ virions to incorporate radiolabeled ribonucleoside triphosphates into RNA (Fig. 7B). In both cases, RNA synthesis was proportionate to the amount of virions added to the reaction.

The data thus far suggested that the A21 protein might be required for virus binding or entry. To investigate this, we used a procedure adapted from Vanderplasschen et al. (46), which depends on the inability of antibodies to bind core proteins even after permeabilization unless the envelope is removed during entry. We incubated equivalent physical amounts of purified A21$^-$ or A21$^+$ virions with HeLa cells at 4°C for 1 h to allow cell attachment but not penetration. Unattached virus

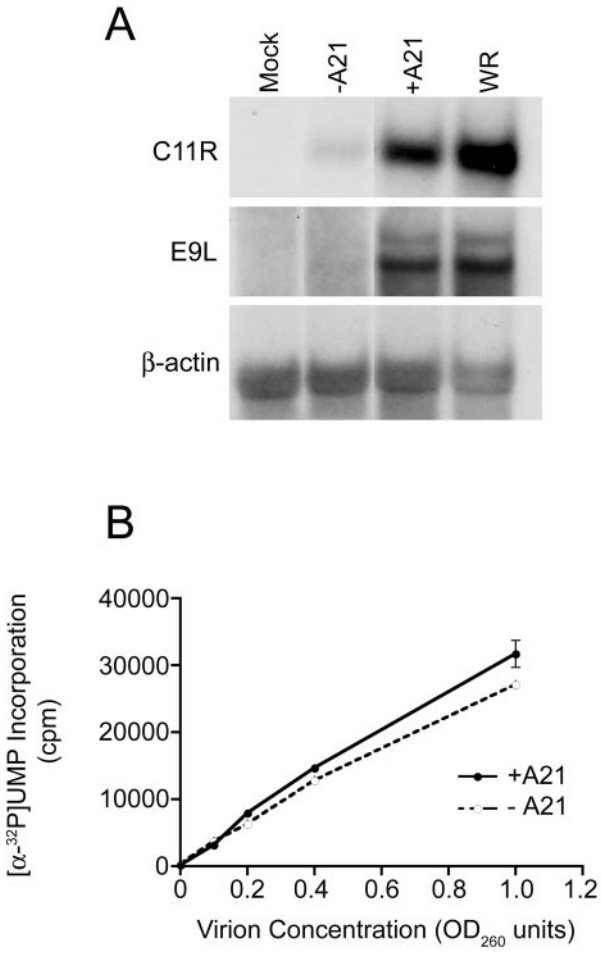

FIG. 7. In vivo and in vitro transcription mediated by $A21^-$ and $A21^+$ virions. (A) Northern blot analysis. BS-C-1 cell monolayers were infected with 5 PFU per cell of wild type VACV WR, $A21^+$ virions, or the corresponding OD_{260} of $A21^-$ virions. Total RNA was harvested at 3 h after infection and analyzed by gel electrophoresis and Northern blotting with [α-^{32}P]dCTP-labeled double-stranded DNA probes to C11R, E9L, and β-actin transcripts. (B) In vitro transcription. Purified virions were incubated in reaction buffer containing NP-40, DTT, Mg^{++}, ribonucleoside triphosphates, and [α-^{32}P]UTP. Incorporation of [α-^{32}P]UMP into trichloroacetic acid-insoluble material was determined by scintillation counting and is expressed as counts per minute (cpm).

was removed by washing the cell monolayer, and the cells were incubated for 2 h at 37°C in the presence of cycloheximide, an inhibitor of protein synthesis. Under the latter conditions, cores accumulate in the cytoplasm but are not further uncoated. Cells were fixed and permeabilized following the 4°C and 37°C incubations and allowed to bind antibodies to the L1 membrane protein and A4 core protein. L1 staining indicated similar numbers of $A21^-$ and $A21^+$ virions attached to cells after the 4°C adsorption period, demonstrating no obvious defect in cell attachment (Fig. 8). At this temperature, no cores were detected in the cytoplasm, consistent with the temperature dependence of this process (11, 27). However, considerable cytoplasmic A4 staining was evident in $A21^+$-infected cells after incubation at 37°C, whereas only rare staining occurred in $A21^-$-infected cells. Thus, $A21^-$ virions are defective in a postadsorption step necessary for the entry of viral cores into the cytoplasm.

A21 virions are unable to induce low pH-triggered cell-cell fusion. VACV is able to induce cells to undergo two types of low pH-triggered syncytium formation, termed fusion from within and fusion from without (11, 14). The terms are somewhat misleading because there is abundant evidence to indicate that in each case fusion is mediated by virions on the cell surface. The difference is that fusion from within is triggered by briefly lowering the pH at late times of infection when progeny CEVs are on the surface, whereas fusion from without is accomplished by adding large amounts of purified IMVs to cells and then briefly lowering the pH. To analyze fusion from without, BS-C-1 cells were incubated with 200 PFU per cell of $A21^+$ virions or a corresponding physical amount of $A21^-$ virions, treated briefly with pH 5.3 buffer, and incubated at 37°C for 3 h in the presence of cycloheximide to prevent viral protein synthesis and cytopathic effects. Cells were then fixed and stained with DAPI to visualize nuclear DNA and with Alexa Fluor 568 phalloidin to visualize filamentous actin, since syncytia formation is accompanied by extensive actin rearrangement. BS-C-1 cell monolayers inoculated with $A21^+$ virions exhibited extensive cell-cell fusion after low pH triggering, whereas cell monolayers that had been inoculated with $A21^-$ virions did not (Fig. 9A). To analyze fusion from within, cells were infected with 5 PFU per cell of vA21i in the presence or absence of IPTG, and a brief low pH treatment was administered at 20 h postinfection. Cells were returned to neutral pH, incubated for 3 h, fixed, and viewed by phase and fluorescence microscopy. As vA21i constitutively expresses EGFP, content mixing in syncytia was detected as uniform green fluorescence throughout the cytoplasm of cells that were infected in the presence of IPTG (Fig. 9B). In contrast, fusion of cells infected in the absence of IPTG was not observed. Thus, A21 was required for entry of viruses into cells and for low pH-triggered cell-cell fusion.

DISCUSSION

We have taken a genetic approach to identify and characterize proteins that are essential for poxvirus replication. Our strategy has been to identify genes with orthologs in all sequenced poxviruses and then to construct inducible mutants. This approach successfully led to the identification of the A28 and H2 entry/fusion proteins (37, 38). We noted that the previously uncharacterized A21L gene is conserved in all poxviruses and that the predicted product closely resembles A28 and H2 in having an N-terminal hydrophobic domain and four invariant cysteine residues. Our studies indicated further similarities in that A21 was expressed late in infection, contained two intramolecular disulfide bonds formed by the VACV-encoded redox system (41), and was incorporated into the IMV membrane.

A functional relationship between A21, A28, and H2 was established by making an inducible A21L mutant. Replication and spread of the latter mutant was entirely dependent on the addition of an inducer. Under nonpermissive conditions, many mutants with defects in expression of IMV membrane proteins exhibit blocks in virus assembly or maturation. Examples in-

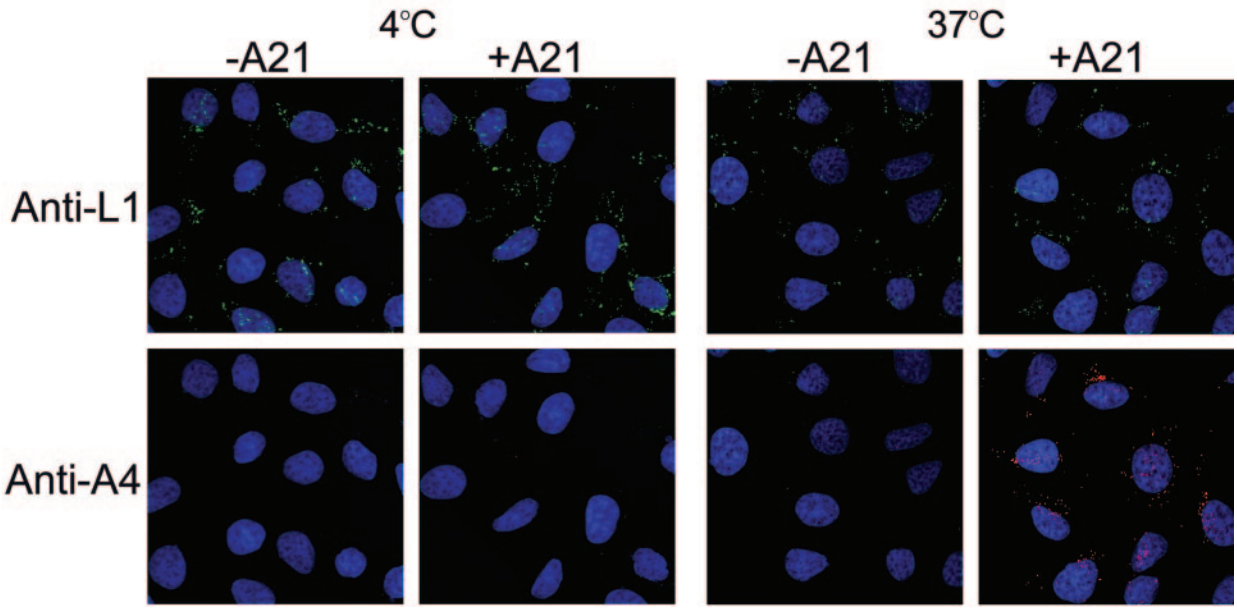

FIG. 8. Inability of A21$^-$ virions to release cores into the cytosol. HeLa cell monolayers were incubated with 20 PFU per cell of A21$^+$ virions or the corresponding OD_{260} of A21$^-$ virions at 4°C for 1 h. Cells were washed extensively and fixed or incubated for a further 2 h at 37°C in the presence of cycloheximide before fixation. Autofluorescence was quenched with 2% glycine, and cells were permeabilized with 0.1% Triton X-100. Cells were incubated with mouse anti-L1 MAb to detect IMVs on the cell surface and rabbit anti-L4 antibody to detect cytoplasmic cores, followed by fluorescein isothiocyanate-conjugated goat anti-mouse (green) and rhodamine Red-X-conjugated goat anti-rabbit (red) antibody, respectively. DNA was visualized by staining with DAPI. Optical sections were obtained by confocal microscopy and are displayed as maximum intensity projections.

clude the A9 (55), A14 (32, 45), A17 (30, 52), and L1 (29) proteins. A28 and H2 mutants, however, are notable exceptions as morphologically normal but noninfectious virions are formed (37, 38). Similarly, we found that the A21 mutant exhibited no obvious defect in morphogenesis but produced noninfectious IMV, intracellular enveloped virion, and CEV progeny in the absence of inducer. Virions lacking A21 could bind to cells, but cores were not detected in the cytosol and viral RNA was not synthesized. Furthermore, virions lacking A21 did not induce low pH-triggered cell-cell fusion. Thus, the phenotype of the A21 null mutant was indistinguishable from that of the previously described A28 and H2 null mutants (37, 38), suggesting that all three are part of the poxvirus entry/fusion apparatus.

Another interesting finding is that each of the entry/fusion proteins identified so far has intramolecular disulfide bonds that are formed by the poxvirus cytoplasmic redox pathway (41). An ancient relationship between the fusion and redox proteins is suggested by their conservation in all poxviruses. The redox pathway was discovered during efforts to explain how the intramolecular disulfide bonds of the L1 IMV membrane protein are formed (42). Antibodies to L1 block virus entry (25, 53), but we do not know whether the L1 protein is directly involved in entry/fusion because null mutants are impaired in virus assembly (29).

Our finding of a third poxvirus entry/fusion protein located in the IMV membrane reinforces our contention that only this membrane is fusogenic (37, 38). Evidently, the outer membrane of extracellular virions is disrupted prior to fusion of IMVs with the cell membrane. Nevertheless, how A28, H2, and A21 mediate fusion is not yet understood. The physical interaction of A28 and H2 was demonstrated previously (37), and preliminary data indicate that A21 is also a part of the complex (T. G. Senkevich, unpublished). A requirement for three or more viral gene products for entry and fusion is unusual but not unprecedented, as this occurs with herpesviruses. For example, cooperative interaction between gB, gD, gH, and gL is required for herpes simplex virus entry though not for cell attachment (44). We presume that the poxvirus fusion complex exists in a metastable prefusion state, as do fusion complexes of other viruses (13). There are two well-recognized methods of activation: exposure to low pH and specific interaction with target cell receptors at neutral pH. Low pH triggers VACV-induced syncytia formation (11, 14) and accelerates virus entry (17; A. C. Townsley, unpublished). Nevertheless, Vanderplasschen et al. (46) presented evidence that IMV entry is not dependent on a low pH pathway, although EEV entry is. These data suggest that poxviruses may use both low pH and receptor-activated mechanisms. However, cellular receptors for poxviruses have not yet been identified. The result of activation is insertion of a viral fusion peptide into the cell membrane (13). Whether the putative poxvirus fusion peptide exists in one of the poxvirus fusion proteins identified thus far or in another protein yet to be discovered remains to be determined.

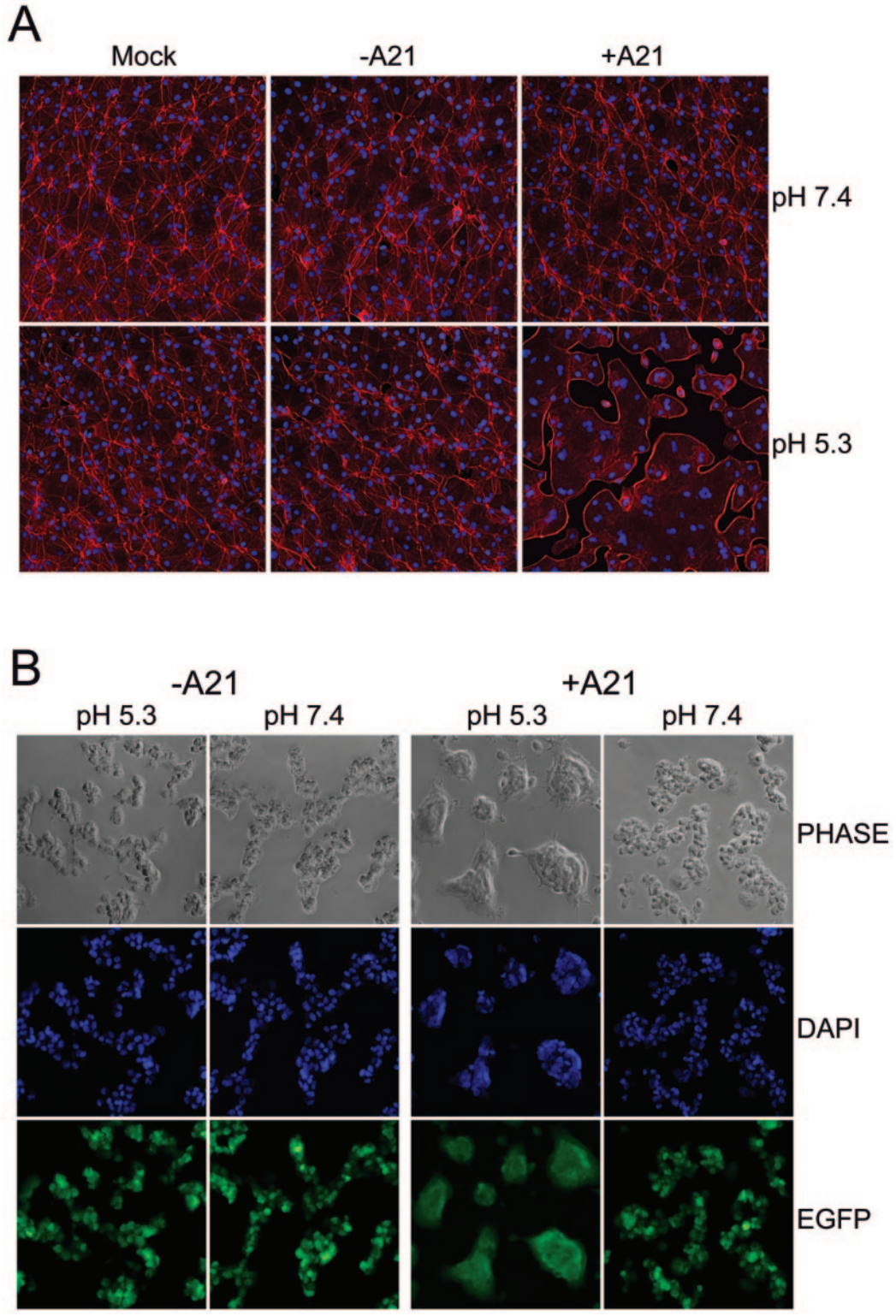

FIG. 9. Inability of virions lacking A21 to mediate low pH-triggered cell-cell fusion. (A) Fusion from without. B-SC-1 monolayers were infected with either 200 PFU per cell of $A21^+$ virions or the corresponding OD_{260} of $A21^-$ virions. Following this, a transient low pH treatment (pH 5.3) or control treatment (pH 7.4) was administered, and then the buffer was replaced with regular medium containing 300 μg of cycloheximide per ml. After 3 h, the cells were fixed and stained with Alexa Fluor 568 phalloidin and DAPI before visualization by confocal microscopy. (B) Fusion from within. BS-C-1 monolayers were infected with 5 PFU per cell of vA21i in the presence or absence of 50 μM IPTG for 18 h and transiently treated with either pH 5.3 or pH 7.4 buffer. The buffer was replaced with regular medium, and after 3 h the cells were stained with DAPI and visualized by phase microscopy and fluorescence microscopy for DNA and constitutively expressed EGFP.

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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. **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.
3. **Ansarah-Sobrinho, C., and B. Moss.** 2004. Vaccinia virus G1 protein, a predicted metalloprotease, is essential for morphogenesis of infectious virions but not for cleavage of major core proteins. J. Virol. **78:**6855–6863.
4. **Antoine, G., F. Scheiflinger, F. Dorner, and F. G. Falkner.** 1998. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. Virology **244:**365–396.
5. **Bablanian, R., B. Baxt, J. A. Sonnabend, and M. Esteban.** 1978. Studies on the mechanisms of vaccinia virus cytopathic effects. II. Early cell rounding is associated with virus polypeptide synthesis. J. Gen. Virol. **39:**403–413.
6. **Blasco, R., and B. Moss.** 1992. Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. J. Virol. **66:**4170–4179.
7. **Boulter, E. A., and G. Appleyard.** 1973. Differences between extracellular and intracellular forms of poxvirus and their implications. Prog. Med. Virol. **16:**86–108.
8. **Chang, A., and D. H. Metz.** 1976. Further investigations on the mode of entry of vaccinia virus into cells. J. Gen. Virol. **32:**275–282.
9. **Chung, C.-S., J.-C. Hsiao, Y.-S. Chang, and W. Chang.** 1998. A27L protein mediates vaccinia virus interaction with cell surface heparin sulfate. J. Virol. **72:**1577–1585.
10. **Demkowicz, W. E., J. S. Maa, and M. Esteban.** 1992. Identification and characterization of vaccinia virus genes encoding proteins that are highly antigenic in animals and are immunodominant in vaccinated humans. J. Virol. **66:**386–398.
11. **Doms, R. W., R. Blumenthal, and B. Moss.** 1990. Fusion of intra- and extracellular forms of vaccinia virus with the cell membrane. J. Virol. **64:** 4884–4892.
12. **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, N.Y.
13. **Earp, L. J., S. E. Delos, H. E. Park, and J. M. White.** 2005. The many mechanisms of viral membrane fusion proteins. Curr. Top. Microbiol. Immunol. **285:**25–66.
14. **Gong, S. C., C. F. Lai, and M. Esteban.** 1990. Vaccinia virus induces cell fusion at acid pH and this activity is mediated by the N-terminus of the 14-kDa virus envelope protein. Virology **178:**81–91.
15. **Hsiao, J. C., C. S. Chung, and W. Chang.** 1998. Cell surface proteoglycans are necessary for A27L protein-mediated cell fusion: identification of the N-terminal region of A27L protein as the glycosaminoglycan-binding domain. J. Virol. **72:**8374–8379.
16. **Hsiao, J. C., C. S. Chung, and W. Chang.** 1999. Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells. J. Virol. **73:**8750–8761.
17. **Ichihashi, Y.** 1996. Extracellular enveloped vaccinia virus escapes neutralization. Virology **217:**478–485.
18. **Janeczko, R. A., J. F. Rodriguez, and M. Esteban.** 1987. Studies on the mechanism of entry of vaccinia virus into animal cells. Arch. Virol. **92:**135–150.
19. **Jensen, O. N., T. Houthaeve, A. Shevchenko, S. Cudmore, T. Ashford, M. Mann, G. Griffiths, and J. K. Locker.** 1996. Identification of the major membrane and core proteins of vaccinia virus by two-dimensional electrophoresis. J. Virol. **70:**7485–7497.
20. **Kates, J. R., and B. R. McAuslan.** 1967. Poxvirus DNA-dependent RNA polymerase. Proc. Natl. Acad. Sci. USA **58:**134–141.
21. **Lin, C. L., C. S. Chung, H. G. Heine, and W. Chang.** 2000. Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection in vitro and in vivo. J. Virol. **74:**3353–3365.
22. **Locker, J. K., A. Kuehn, S. Schleich, G. Rutter, H. Hohenberg, R. Wepf, and G. Griffiths.** 2000. Entry of the two infectious forms of vaccinia virus at the plasma membrane is signaling-dependent for the IMV but not the EEV. Mol. Biol. Cell **11:**2497–2511.
23. **Moss, B.** 2001. *Poxviridae*: the viruses and their replication, p. 2849–2883. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, Pa.
24. **Niles, E. G., and J. Seto.** 1988. Vaccinia virus gene D8 encodes a virion transmembrane protein. J. Virol. **62:**3772–3778.
25. **Oie, M., and Y. Ichihashi.** 1987. Modification of vaccinia virus penetration proteins analyzed by monoclonal antibodies. Virology **157:**449–459.
26. **Payne, L. G.** 1980. Significance of extracellular virus in the in vitro and in vivo dissemination of vaccinia virus. J. Gen. Virol. **50:**89–100.
27. **Payne, L. G., and E. Norrby.** 1978. Adsorption and penetration of enveloped and naked vaccinia virus particles. J. Virol. **27:**19–27.
28. **Ravanello, M. P., C. A. Franke, and D. E. Hruby.** 1993. An NH2-terminal peptide from the vaccinia virus L1R protein directs the myristylation and virion envelope localization of a heterologous fusion protein. J. Biol. Chem. **268:**7585–7593.
29. **Ravanello, M. P., and D. E. Hruby.** 1994. Conditional lethal expression of the vaccinia virus L1R myristylated protein reveals a role in virus assembly. J. Virol. **68:**6401–6410.
30. **Rodríguez, D., M. Esteban, and J. R. Rodríguez.** 1995. Vaccinia virus A17L gene product is essential for an early step in virion morphogenesis. J. Virol. **69:**4640–4648.
31. **Rodriguez, J. F., R. Janeczko, and M. Esteban.** 1985. Isolation and characterization of neutralizing monoclonal antibodies to vaccinia virus. J. Virol. **56:**482–488.
32. **Rodriguez, J. R., C. Risco, J. L. Carrascosa, M. Esteban, and D. Rodriguez.** 1998. Vaccinia virus 15-kilodalton (A14L) protein is essential for assembly and attachment of viral crescents to virosomes. J. Virol. **72:**1287–1296.
33. **Roper, R., E. J. Wolffe, A. Weisberg, and B. Moss.** 1998. The envelope protein encoded by the A33R gene is required for formation of actin-containing microvilli and efficient cell-to-cell spread of vaccinia virus. J. Virol. **72:**4192–4204.
34. **Sanderson, C. M., F. Frischknecht, M. Way, M. Hollinshead, and G. L. Smith.** 1998. Roles of vaccinia virus EEV-specific proteins in intracellular actin tail formation and low pH-induced cell-cell fusion. J. Gen. Virol. **79:**1415–1425.
35. **Schmelz, M., B. Sodeik, M. Ericsson, E. J. Wolffe, H. Shida, G. Hiller, and G. Griffiths.** 1994. Assembly of vaccinia virus: the second wrapping cisterna is derived from the *trans* Golgi network. J. Virol. **68:**130–147.
36. **Senkevich, T. G., E. V. Koonin, J. J. Bugert, G. Darai, and B. Moss.** 1997. The genome of molluscum contagiosum virus: analysis and comparison with other poxviruses. Virology **233:**19–42.
37. **Senkevich, T. G., and B. Moss.** 2005. Vaccinia virus H2 protein is an essential component of a complex involved in virus entry and cell-cell fusion. J. Virol. **79:**4744–4754.
38. **Senkevich, T. G., B. M. Ward, and B. Moss.** 2004. Vaccinia virus A28L gene encodes an essential protein component of the virion membrane with intramolecular disulfide bonds formed by the viral cytoplasmic redox pathway. J. Virol. **78:**2348–2356.
39. **Senkevich, T. G., B. M. Ward, and B. Moss.** 2004. Vaccinia virus entry into cells is dependent on a virion surface protein encoded by the A28L gene. J. Virol. **78:**2357–2366.
40. **Senkevich, T. G., A. Weisberg, and B. Moss.** 2000. Vaccinia virus E10R protein is associated with the membranes of intracellular mature virions and has a role in morphogenesis. Virology **278:**244–252.
41. **Senkevich, T. G., C. L. White, E. V. Koonin, and B. Moss.** 2002. Complete pathway for protein disulfide bond formation encoded by poxviruses. Proc. Natl. Acad. Sci. USA **99:**6667–6672.
42. **Senkevich, T. G., C. L. White, E. V. Koonin, and B. Moss.** 2000. A viral member of the ERV1/ALR protein family participates in a cytoplasmic pathway of disulfide bond formation. Proc. Natl. Acad. Sci. USA **97:**12068–12073.
43. **Smith, G. L., A. Vanderplasschen, and M. Law.** 2002. The formation and function of extracellular enveloped vaccinia virus. J. Gen. Virol. **83:**2915–2931.
44. **Spear, P. G., and R. Longnecker.** 2003. Herpesvirus entry: an update. J. Virol. **77:**10179–10185.
45. **Traktman, P., K. Liu, J. DeMasi, R. Rollins, S. Jesty, and B. Unger.** 2000. Elucidating the essential role of the A14 phosphoprotein in vaccinia virus morphogenesis: construction and characterization of a tetracycline-inducible recombinant. J. Virol. **74:**3682–3695.
46. **Vanderplasschen, A., M. Hollinshead, and G. L. Smith.** 1998. Intracellular and extracellular vaccinia virions enter cells by different mechanisms. J. Gen. Virol. **79:**877–887.
47. **Vazquez, M. I., and M. Esteban.** 1999. Identification of functional domains in the 14-kilodalton envelope protein (A27L) of vaccinia virus. J. Virol. **73:**9098–9109.
48. **Vazquez, M. I., G. Rivas, D. Cregut, L. Serrano, and M. Esteban.** 1998. The vaccinia virus 14-kilodalton (A27L) fusion protein forms a triple coiled-coil structure and interacts with the 21-kilodalton (A17L) virus membrane protein through a C-terminal α-helix. J. Virol. **72:**10126–10137.
49. **Ward, B. M.** 2005. Visualization and characterization of the intracellular movement of vaccinia virus intracellular mature virions. J. Virol. **79:**4755–4763.
50. **White, C. L., T. G. Senkevich, and B. Moss.** 2002. Vaccinia virus G4L glutaredoxin is an essential intermediate of a cytoplasmic disulfide bond pathway required for virion assembly. J. Virol. **76:**467–472.
51. **Wolffe, E. J., E. Katz, A. Weisberg, and B. Moss.** 1997. The A34R glyco-

protein gene is required for induction of specialized actin-containing microvilli and efficient cell-to-cell transmission of vaccinia virus. J. Virol. **71:** 3904–3915.

52. **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.
53. **Wolffe, E. J., S. Vijaya, and B. Moss.** 1995. A myristylated membrane protein encoded by the vaccinia virus L1R open reading frame is the target of potent neutralizing monoclonal antibodies. Virology **211:**53–63.
54. **Wolffe, E. J., A. S. Weisberg, and B. Moss.** 1998. Role for the vaccinia virus A36R outer envelope protein in the formation of virus-tipped actin-containing microvilli and cell-to-cell virus spread. Virology **244:**20–26.
55. **Yeh, W. W., B. Moss, and E. J. Wolffe.** 2000. The vaccinia virus A9L gene encodes a membrane protein required for an early step in virion morphogenesis. J. Virol. **74:**9701–9711.