JOURNAL OF VIROLOGY, Feb. 2007, p. 1433–1443 Vol. 81, No. 3
0022-538X/07/$08.00+0 doi:10.1128/JVI.02206-06

Vaccinia Virus A6L Encodes a Virion Core Protein Required for Formation of Mature Virion▽

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Received 7 October 2006/Accepted 9 November 2006

Vaccinia virus A6L is a previously uncharacterized gene that is conserved in all sequenced vertebrate poxviruses. Here, we constructed a recombinant vaccinia virus encoding A6 with an epitope tag and showed that A6 was expressed in infected cells after viral DNA replication and packaged in the core of the mature virion. Furthermore, we showed that A6 was essential for vaccinia virus replication by performing clustered charge-to-alanine mutagenesis on A6, which resulted in two vaccinia virus mutants (vA6L-mut1 and vA6L-mut2) that displayed a temperature-sensitive phenotype. At 31°C, both mutants replicated efficiently; however, at 40°C, vA6L-mut1 grew to a low titer, while vA6L-mut2 failed to replicate. The A6 protein expressed by vA6L-mut2 exhibited temperature-dependent instability. At the nonpermissive temperature, vA6L-mut2 was normal at viral gene expression and viral factory formation, but it was defective for proteolytic processing of the precursors of several major virion proteins, a defect that is characteristic of a block in virion morphogenesis. Electron microscopy further showed that the morphogenesis of vA6L-mut2 was arrested before the formation of immature virion with nucleoid and mature virion. Taken together, our data show that A6 is a virion core protein that plays an essential role in virion morphogenesis.

Poxviruses are a family of large, complex, double-stranded DNA viruses that replicate entirely in the cytoplasm of infected cells (16). The most intensively studied family member is the WR strain of vaccinia virus (VACV), which encodes 218 open reading frames in a 190-kb linear genome. A set of 91 open reading frames of WR are conserved in all sequenced vertebrate poxviruses, including 49 open reading frames that are also conserved in invertebrate poxviruses (23). Among the conserved open reading frames are genes that are known to play essential roles in virus entry, viral gene transcription, genome replication, and virion morphogenesis (23). VACV A6L (VACWR125) is one of about 20 conserved open reading frames of WR that has not been previously characterized. The amino acid sequence of A6 gives no hint to its function as it has no recognizable motif or any homolog outside the poxvirus family. To determine the role that A6 plays in viral life cycle, we constructed recombinant VACV encoding A6 with an epitope tag and temperature-sensitive (*ts*) mutants with a lesion in A6. We characterized these recombinant viruses and report here that A6 is a virion core protein playing an essential role in virion morphogenesis.

The morphogenesis of the VACV virion goes through a series of stages that can be distinguished by electron microscopy (recently reviewed in reference 2). Electron-dense virosomes consisting of viral proteins appear first. Then, crescent-shaped precursors of the virion membrane develop at the periphery of the virosome and subsequently circularize to form the spherical immature virions (IVs). As IVs encapsidate the viral genome, they appear as IVs with an electron-dense nucleoid (IVNs). Eventually, IVNs evolve into the brick-shaped intracellular mature virions (MVs), which represent the majority of infectious particles produced during VACV infection. Concomitant with this change, precursor forms of several virion proteins, including p4a and p4b, were proteolytically processed into mature proteins (12, 21). We present data here suggesting that A6 is essential for the transition from IV to IVN and MV.

MATERIALS AND METHODS

Cells and viruses. BS-C-1 cells were maintained in minimum essential medium with Earle's salts supplemented with 10% fetal bovine serum (FBS). HeLa 229 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Wild-type (WT) and mutant WR viruses were propagated on BS-C-1 cells. Temperature-sensitive mutants were propagated at 31°C.

Plasmids construction. The primers A5R-left (5′-AA*GAATTC*ATCTTAAA CATATAGGGAATCATAT-3′) and A6L-right (5′-GTTTG*GTATAC*GGAAT CAGATGCG-3′) were designed to PCR amplify sequences flanking the 3′ end of A6L (the EcoRI site in A5R-left and the AccI site in A6L-right are shown in italics).

The transfer plasmid pLJ2 for appending the V5 epitope tag to the C terminus of A6 was constructed as follows. The coding sequences for the C-terminal half of A6 with a V5 epitope tag and part of A5 were assembled by recombinant PCR with WR genomic DNA as the template and the primer pairs A5R-left (shown above) and 5′-CTAACCCTCTCCTCGGTCTCGATTCTACGTAACTAATAT CGTTTTTGAAGTTTC-3′ and 5′-GAGACCGAGGAGAGGGTTAGGGATA GGCTTACCGAATTTATACGAATATCG-3′ and A6L-right (shown above). The V5 coding sequences in the primers are underlined. The recombinant PCR product was cloned into the EcoRI and AccI sites of pYW31 (14) flanking the green fluorescent protein (*gfp*) and xanthine-guanine phosphoribosyltransferase (*gpt*) cassettes.

The transfer plasmids for introducing the A6L-mut1, A6L-mut2, or A6L-mut3 mutation into VACV were derived from pLJ2 with the recombinant PCR technique. For each A6L mutation, a pair of mutagenesis primers with complementary sequences was used together with either primer, A5R-left or A6L-right, in two separate PCRs in which pLJ2 was used as the template. The resulting PCR products were then assembled together by recombinant PCR and substituted into the A6 coding sequence of pLJ2 by using the XbaI and AccI sites. Each mutation also introduced a unique restriction site in A6 coding sequence to allow

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▽ Published ahead of print on 15 November 2006.

the mutant and WT alleles to be distinguished. The mutagenesis primer pairs were as follows, with the engineered restriction site in parentheses: 5′-GAATA TGCTGCAGCAAATGTCACAATCAAGTC-3′ and 5′-CATTTGCTGCAGC ATATTCGTATAAATTCGGT-3′ (A6L-mut1 and PstI), 5′-GCGTACGCAAT TGCGTGTGCATTAACGATATCTTGCAA-3′ and 5′-CACACGCAATTGC GTACGCCAATAAATTGCTGAACATG-3′ (A6L-mut2 and MfeI), and 5′-T GATTGCCGCGGCTGCCGATAGATATTCTTCAGAG-3′ and 5′-ATCGGC AGCCGCGGCAATCATTACTATTTTGCAAG-3′ (A6L-mut3 and SacII).

The transfer plasmid for introducing the A6L-mut4 mutation to VACV was constructed in a manner similar to that described above, but an additional sequence flanking the mutation was added to the transfer plasmid to increase the chance of recombination. The first PCR product was made with WR genomic DNA as the template and the primer pair 5′-GCATATGCTGCCGCGTTAGC CGATATGAATTTGATAAG-3′ and 5′-TGCTCGAG ATCTGGTGCCGTTG TGAAATCG-3′. The second PCR product was made with pLJ2 as the template and the primer pair A5R-left and 5′-CTAACGCGGCAGCATATGCGACAA TAATCTCTGAAGAA-3′. The two PCR products were assembled together by recombinant PCR and cloned into the XhoI and XbaI sites of pLJ2. The mutation introduced an NdeI site in the A6L coding sequence.

The sequences of the plasmids were confirmed by DNA sequencing with an ABI 3100 genetic analyzer.

Recombinant VACV construction. vA6L-V5, the recombinant VACV expressing A6 with a C-terminal V5 tag, was constructed with transfer plasmid pLJ2 and the transient dominant selection method in a manner similar to one that was previously described (6, 14). Briefly, the transfer plasmid pLJ2 was transfected into BS-C-1 cells infected with WT WR. The cell lysates were applied to BS-C-1 cells that were treated with mycophenolic acid, xanthine and hypoxanthine at concentrations of 25, 250, and 15 μg/ml, respectively. Recombinant viruses expressing GFP were picked under a fluorescence microscope and purified by three rounds of plaque purification. Subsequently, the recombinant viruses were propagated in the absence of mycophenolic acid, and GFP-negative plaques containing a double-crossover mutant were purified by three additional rounds of plaque purification. The insertion of V5 epitope tag in the recombinant virus was confirmed by PCR amplification of the A6L region of the viral genome and by Western blot analysis of infected cell lysates with anti-V5 antibody.

VACV mutants expressing A6 with alanine substitutions were constructed in a manner essentially the same as that for the construction of vA6L-V5, except that the parental virus was vA6L-V5 and the transfer plasmids contained mutations in A6. In addition, double-crossover recombinant viruses were isolated at 31°C. To assess whether the isolated viruses contained the desired A6L mutation, the A6L region of the viral genome was PCR amplified and digested with the restriction enzyme that specifically recognized the mutant allele. Approximately half of the viruses that were analyzed for the presence of the A6L-mut1 or A6L-mut2 mutation contained the desired mutation. Viruses from 12 different plaques were analyzed for the presence of the A6L-mut3 or A6L-mut4 mutation, but they were all found to contain the WT allele.

Marker rescue. The A6L open reading frame and approximately 500 bp of its left and right flanking sequences were PCR amplified from genomic DNA of vA6L-mut2 and WT WR with the primers 5′-CGGGAGCTCATCTTAAACAT ATAGGGAATCATAT-3′ and 5′-CCCGATAAGCTTTACGAACTACATCT GATATTATT-3′. The PCR products were TA cloned into the pCR2.1 vector (Invitrogen), yielding pA6L-mut2 and pA6L. BS-C-1 cells in 35-mm dishes were transfected with 2 μg of pcDNA3.1 (Invitrogen), pA6L-mut2, or pA6L and incubated at 37°C. At 6 h after the transfection, the cells were infected with vA6L-mut2 at a multiplicity of infection of 0.1 and transferred to 40°C. The monolayers were stained with crystal violet after 3 days.

Western blot analysis. The Western blot analysis was performed as previously described (15). Briefly, virions or the whole-cell lysates were solubilized in sodium dodecyl sulfate (SDS) sample buffer, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), 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 antibodies and analyzed with chemiluminescence reagent (Pierce). Mouse monoclonal antibody to V5 epitope was purchased from Sigma-Aldrich. The rabbit polyclonal antisera against L1, A27, and D8 were kindly provided by Shan Lu, University of Massachusetts (22).

Preparation and analysis of virion extracts. Intracellular MV particles of vA6L-V5 were purified by sucrose gradient centrifugation according to the standard protocol (5) and analyzed as previously described by Garcia and Moss (7). Briefly, BS-C-1 cells infected with vA6L-V5 were harvested and Dounce homogenized in 10 mM Tris-HCl (pH 9.0). MV particles were purified from the cytoplasmic fraction by sucrose cushion sedimentation and two sucrose gradient sedimentations. The purified virion was incubated with 0.5% Nonidet P-40 (NP-40) in 50 mM Tris-HCl (pH 8.0) with or without 50 mM dithiothreitol (DTT) for 30 min at 37°C. The soluble membrane-associated proteins and insoluble core protein fractions were separated by centrifugation at 20,000 × *g* for 30 min. The insoluble virion cores were resuspended in 0.2% deoxycholate, 10 mM DTT, 100 mM Tris-HCl, and 250 mM NaCl and incubated on ice for 30 min. The soluble and insoluble core proteins were separated by centrifugation. All virion fractions were analyzed by SDS-PAGE followed by Western blotting or silver staining.

Plaque morphology and one-step growth curve analysis. For examining plaque morphology, BS-C-1 cells in six-well plates were infected with 10-fold serial dilutions of different viruses and kept at either 31 or 40°C. At 48 h postinfection (p.i.), the cell monolayers were stained with crystal violet to visualize the plaques. For one-step growth analysis, BS-C-1 cells in 12-well plates were incubated with 10 PFU per cell of different mutants for 2 h at room temperature. Following adsorption, the cells were washed twice with phosphate-buffered saline (PBS) and moved to a 31°C or 40°C incubator to initiate viral entry and replication. The cells were harvested at 0, 12, and 48 h p.i. The viral titers in the cell lysates were determined by duplicate plaque assays on BS-C-1 cells at 31°C.

Metabolic labeling. BS-C-1 cells in 35-mm dishes were infected at 10 PFU/cell and maintained at 31 or 40°C. For all experiments at 40°C, care was taken to maintain the temperature during the experiments by using prewarmed medium.

For pulse-chase analysis of A6 protein stability, the cells were starved with methionine- and cysteine-free DMEM (Invitrogen) for 30 min at 12 h p.i. and then incubated with methionine- and cysteine-free DMEM plus 100 μCi of [^{35}S]methionine and -cysteine per ml for 30 min. One set of the cells was harvested immediately, while the other set was replenished with DMEM supplemented with 2.5% fetal calf serum and harvested at 16 h p.i. The harvested cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl,1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with protease inhibitor cocktail (Roche Molecular Biochemicals). Ten microliters of the cleared cell lysates was saved while the rest was rotated with 30 μl of 50% (vol/vol) anti-V5-agarose beads (Sigma-Aldrich) for 4 h at 4°C. The beads were washed four times with wash buffer (0.1% [wt/vol] Triton X-100, 50 mM Tris [pH 7.4], 300 mM NaCl, 5 mM EDTA) and resuspended in SDS sample buffer.

For analysis of viral gene expression, the infected cells were pulse labeled for 30 min at various times after infection. The cells were then harvested, washed with PBS, and lysed in SDS sample buffer. The samples were heated to 100°C for 5 min and sonicated for 1 min before SDS-PAGE analysis.

For pulse-chase analysis of virion protein processing, the infected cells were pulse labeled at 8 h p.i. and chased for 12 h. The cells were harvested, washed with PBS, and lysed in SDS sample buffer. The samples were heated to 100°C for 5 min and sonicated for 1 min before SDS-PAGE analysis. The gel was dried before exposure to film for autoradiography.

Fluorescent microscopy. HeLa cells or BS-C-1 cells grown on coverslips were infected at 0.1 PFU/cell. At 12 h p.i., the cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 5 min, blocked with 10% FBS for 60 min, and stained with monoclonal antibody to V5 or polyclonal antibody to VACV (Fitzgerald) for 1 h and a Cy2-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) for an additional hour. The DNA was stained with Hoechst (Molecular Probes). The images under an AX-70 Olympus fluorescence microscope were acquired with a Hamamatsu charge-coupled device camera.

Electron microscopy. For transmission electron microscopy, BS-C-1 cells in 60-mm-diameter dishes were infected with 3 PFU/cell of vA6L-mut2 and kept at either 31 or 40°C. At 24 h p.i., the cells were fixed with 4% formaldehyde and 1% glutaraldehyde for 1 h at 4°C. The cells were then scraped off and prepared for transmission electron microscopy by the Electron Microscopy Core Laboratory at the University of Texas HSC at San Antonio. The thin sections were examined with a JEOL 1230 transmission electron microscope.

RESULTS

A6 is expressed postreplicatively in vaccinia virus-infected cells. To allow the detection of the A6L gene product, we constructed a recombinant vaccinia virus in which the coding sequence for the V5 epitope was inserted in frame in the 3′ end of the A6L open reading frame. The recombinant virus, designated vA6L-V5, was constructed by the strategy of transient dominant selection (6), so no selective marker was left in the virus and only the epitope tag sequence was

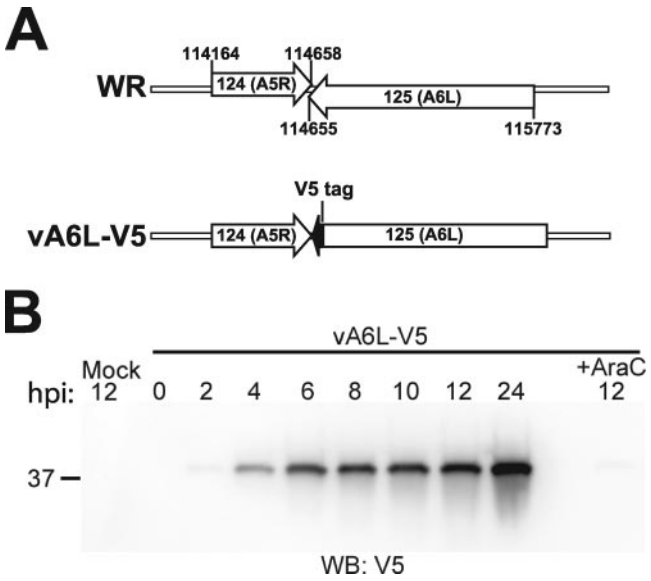

FIG. 1. Temporal expression of A6L during VACV infection. (A) Schematic representation of vA6L-V5, a recombinant VACV encoding A6 with a C-terminal V5 epitope tag. The relevant portion of the genome of vA6L-V5 with the inserted V5 tag (filled arrow) is depicted. For comparison, the corresponding portion of the WT WR genome is depicted with the positions of the open reading frames (open boxes with arrows) indicated by the nucleotide numbers in the complete WR genomic sequence (accession no. AY243312). (B) The kinetics of A6L expression. BS-C-1 cells were mock infected or infected with 10 PFU per cell of vA6L-V5. AraC was added to one of the infections at 40 μg/ml to inhibit DNA replication. The cells were harvested at the indicated time (hours p.i. [hpi]) and analyzed by Western blotting (WB) with a MAb to V5 epitope as described previously (15). The same amount of total proteins as determined by Bradford protein assay were analyzed.

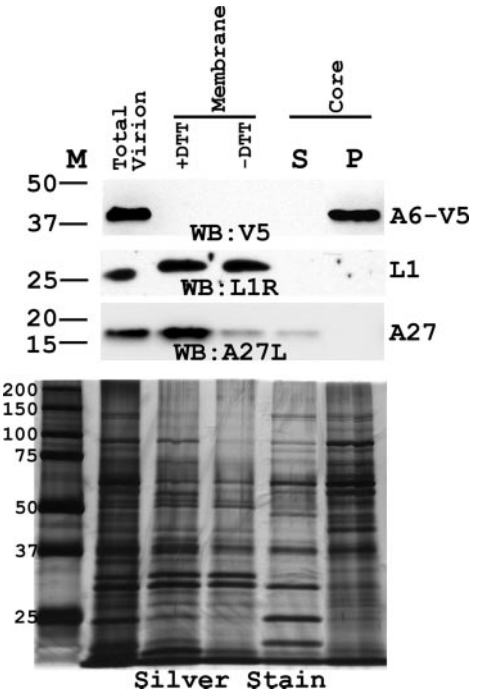

FIG. 2. A6 is packaged in virion core. Intracellular mature virions were purified from vA6L-V5-infected cells by means of a sucrose gradient according to the standard protocol (5). Membrane-associated virion proteins (Membrane) were extracted by incubating the purified virions with 0.5% NP-40 in the absence (−) or presence (+) of 50 mM DTT. The insoluble virion cores (Core) were then incubated with 0.2% deoxycholate and 10 mM DTT and separated by centrifugation into supernatant (S) and pellet (P) fractions. The fractions were analyzed by SDS-PAGE and Western blotting (WB) with antibodies to V5 epitope and to two different virion membrane proteins (L1 and A27). The proteins in the fractions were also visualized after silver staining. Protein standards are indicated on the left, with their masses shown in kilodaltons.

inserted between A6L and A5R (Fig. 1A). In BS-C-1 cells, vA6L-V5 formed plaques with sizes similar to those formed by the WT WR (data not shown), indicating that the insertion of the epitope tag did not affect viral replication. To follow the temporal expression of A6L, BS-C-1 cells were infected with vA6L-V5, harvested at different times after infection, and immunoblotted with a monoclonal antibody (MAb) to the V5 epitope (Fig. 1B). A protein with a size that is consistent with the predicted molecular mass of A6 protein (43 kDa) was barely detectable at 2 h p.i. Its level became substantial after 4 h p.i. and continued to increase for the remainder of the 24-h infection. The suppression of VACV DNA replication with cytosine arabinoside (AraC) reduced A6 expression to a barely detectable level, indicating that A6 was expressed postreplicatively after viral DNA synthesis. The very small amount of A6 protein at 2 h p.i. and in the presence of AraC might come from the viruses added initially to the cells to start the infection, as A6 is a virion component (shown below).

A6 is packaged in the core of mature virion. A recent mass spectrometry analysis of purified VACV virion identified A6 as a minor virion component (1), but a similar study did not detect A6 in mature virion (25). To ascertain whether A6 is a virion component, we purified intracellular mature virions from vA6L-V5-infected cells through sucrose gradients and performed Western blot analysis (Fig. 2). A6 was detected in the total virion, along with virion membrane proteins L1 and A27 (19, 20). However, unlike the membrane proteins, A6 could not be extracted from the virions by the nonionic detergent NP-40 and the reducing agent DTT. Further extraction of the virion core with deoxycholate and DTT also failed to release any A6, indicating that A6 is tightly packaged in the virion core. The profiles of the proteins in different virion fractions, as indicated by band patterns on a silver-stained SDS-PAGE gel, were similar to those previously reported (7).

Construction of temperature-sensitive mutant viruses with a lesion in A6. Our attempt to delete A6L from WR was unsuccessful, suggesting that A6L might play an essential role in viral replication. To determine the role A6 plays in the viral life cycle, we initially tried to construct a recombinant VACV in which the expression of A6L is regulated by a chemical inducer. Although we were able to construct an intermediate virus by inserting an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible copy of A6L gene into the hemagglutinin locus of vT7lacOI (24), we failed to delete the endogenous A6L gene from the intermediate virus in the presence of the inducer for unknown reasons (data not shown). Therefore, we attempted to create a temperature-sensitive (*ts*), conditionally lethal, A6L

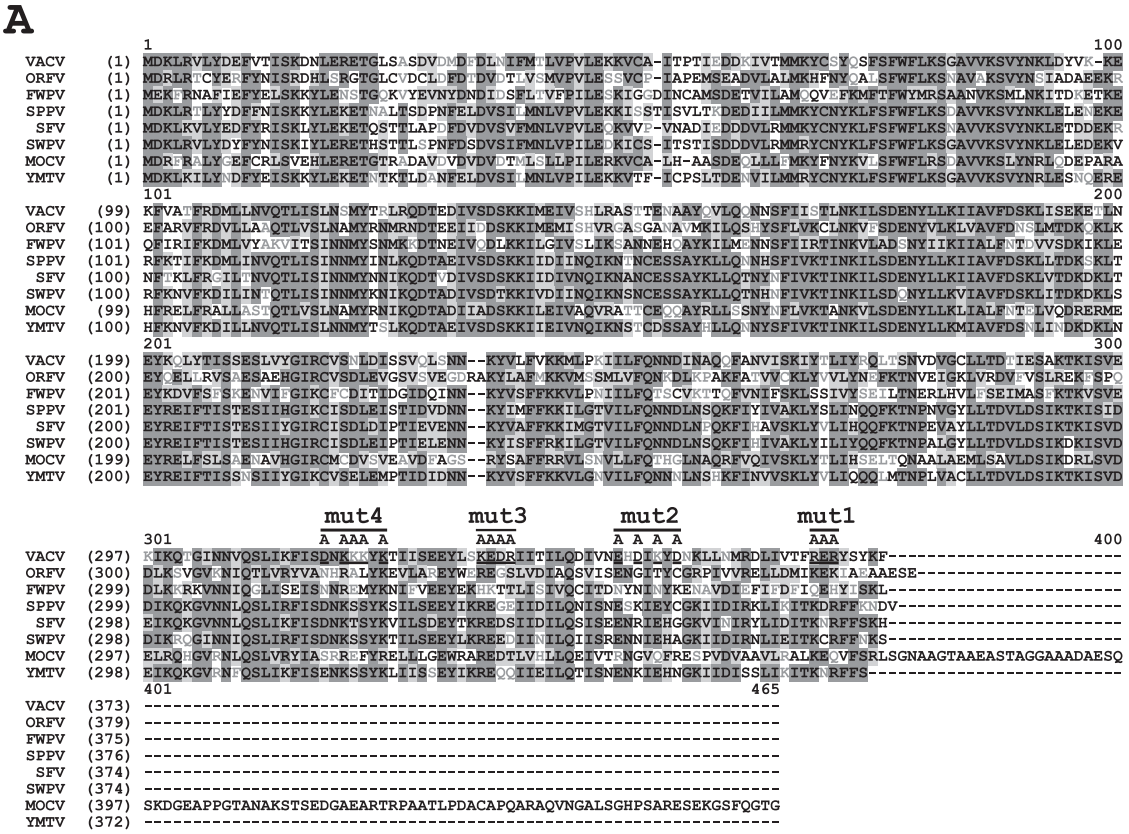

FIG. 3. Construction of temperature-sensitive VACV mutants with a lesion in A6. (A) A multiple-sequence alignment of A6 orthologs in vertebrate poxviruses. Amino acids that are identical or similar in four or more of the eight proteins are highlighted. The dark and light shading indicate amino acids that are identical or similar, respectively. Four clustered charge-to-alanine mutations of VACV A6 (mut1, -2, -3, and -4) are also indicated on the alignment. The transient dominant selection method was used to introduce the four A6 mutations into vA6L-V5, but only viruses containing mut1 or mut2 were successfully isolated (designated vA6L-mut1 or vA6L-mut2). The A6 orthologs are from VACV, (*Orthopoxvirus*), ORF virus (ORFV) (*Parapoxvirus*), fowlpox virus (FWPV) (*Avipoxvirus*), sheeppox virus (SPPV) (*Capripoxvirus*), Shope fibroma virus (SFV) (*Leporipoxvirus*), swinepox virus (SWPV) (*Suipoxvirus*), molluscum contagiosum (MOCV) (*Molluscipoxvirus*), and Yaba monkey tumor virus (YMTV) (*Yatapoxvirus*). (B) Plaque morphology of A6L mutant viruses at different temperatures. BS-C-1 cells were infected with the indicated numbers of PFU of vA6L-mut1, vA6L-mut2, or vA6L-V5. The cells were kept at 31 or 40°C for 48 h, and then the plaques were visualized following crystal violet staining. (C) One-step growth curve of A6L mutant viruses. BS-C-1 cells were infected with 10 PFU per cell of the indicated viruses and kept at either 31 or 40°C. At the indicated time after infection (hours p.i. [hpi]), the cells were harvested and their titers were determined by duplicate plaque assays on BS-C-1 cells at 31°C. (D) Marker rescue of vA6L-mut2. BS-C-1 cells were transfected either with pcDNA3.1 or with the plasmid containing the A6L open reading frame and about 500 bp of flanking DNA from vA6L-mut2 (pA6L-mut2) or WT WR (pA6L). At 6 h after the transfection, the cells were infected with vA6L-mut2 at a multiplicity of infection of 0.1 and incubated at 40°C. The monolayers were stained with crystal violet after 3 days.

mutant with the clustered charge-to-alanine mutagenesis method (9).

Four clusters of charged amino acids located near the C terminus of A6 were targeted for alanine substitution (Fig. 3A). Some of the charged amino acids were not completely conserved in all vertebrate poxviruses, suggesting that they may be replaced without causing a lethal phenotype at low temperature. The transient dominant selection method was used to introduce the mutations into vA6L-V5. For all four mutations, single-crossover recombinant viruses containing a wild-type and a mutant copy of A6L were isolated. However, only double-crossover recombinant viruses containing mut1 or mut2 substitutions in A6 were isolated at 31°C and they were designated vA6L-mut1 or vA6L-mut2, respectively. Repeated efforts to isolate double-crossover recombinant viruses containing mut3 or mut4 substitutions in A6 failed, suggesting that the mutations may cause lethal phenotypes at even the low temperature. At 31°C, both vA6L-mut1 and vA6L-mut2 formed plaques that are similar in size to the parental vA6L-V5 virus (Fig. 3B) and they grew to titers similar to those of vA6L-V5 in one-step growth analysis (Fig. 3C). At 40°C, however, vA6L-mut2 failed to form plaques, while vA6L-mut1 formed tiny plaques at high multiplicities of infection. One-step growth analysis confirmed that vA6L-mut2 did not grow at 40°C and that the growth of vA6L-mut1 was severely impaired and delayed at 40°C. As vA6L-mut2 displayed a more stringent *ts* phenotype than did vA6L-mut1, it was analyzed further in the subsequent studies.

To ensure that the *ts* phenotype of vA6L-mut2 was caused by only the A6L mutation, we tested whether a plasmid containing the WT A6L gene could rescue vA6L-mut2 at the nonpermissive temperature. BS-C-1 cells were transfected either with a plasmid containing WT A6L or with control plasmids and then infected with vA6L-mut2 at 40°C (Fig. 3D). While the

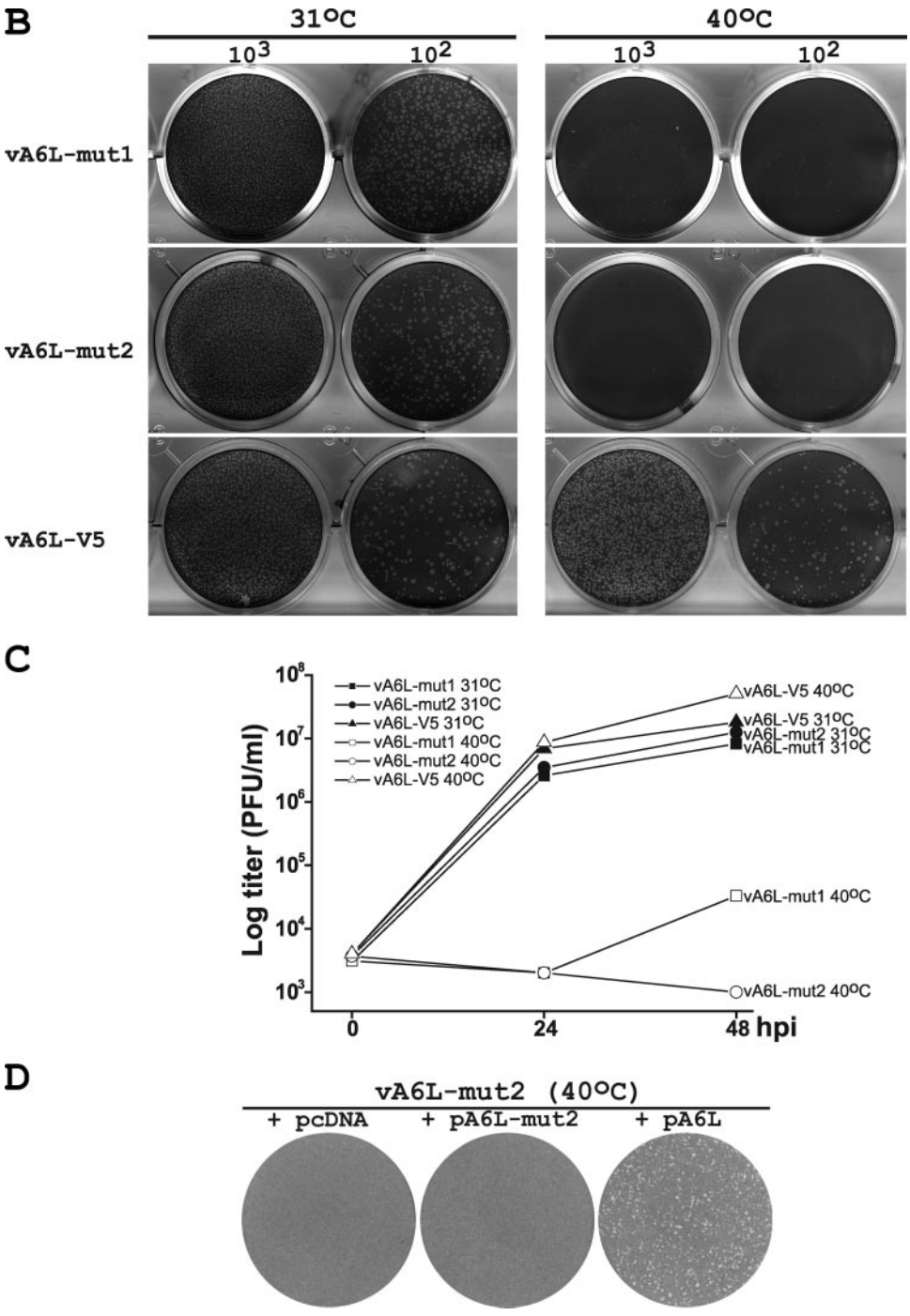

FIG. 3—*Continued.*

infection of the cells transfected with an empty plasmid vector or with a plasmid containing A6L from vA6L-mut2 yielded no plaques, the infection of the cells transfected with a plasmid containing A6L from WT WR resulted in numerous plaques, indicating that the mutation in the A6L open reading frame was solely responsible for the *ts* phenotype of vA6L-mut2.

A6 protein expressed by vA6L-mut2 is unstable at the nonpermissive temperature. Clustered charge-to-alanine mutagenesis may result in a *ts* phenotype by reducing protein stability at high temperatures. We therefore examined the production of A6 by vA6L-mut2 at different temperatures. At 40°C, the A6 protein level in vA6L-mut2-infected cells, as determined by Western blot analysis with anti-V5 antibody, was much lower than that in vA6L-V5-infected cells (Fig. 4A). As a control, the same blot was probed with an antibody to VACV late protein D8 (17). The levels of D8 protein were similar in cells infected by either virus, demonstrating that vA6L-mut2 has a specific defect in A6 protein accumulation.

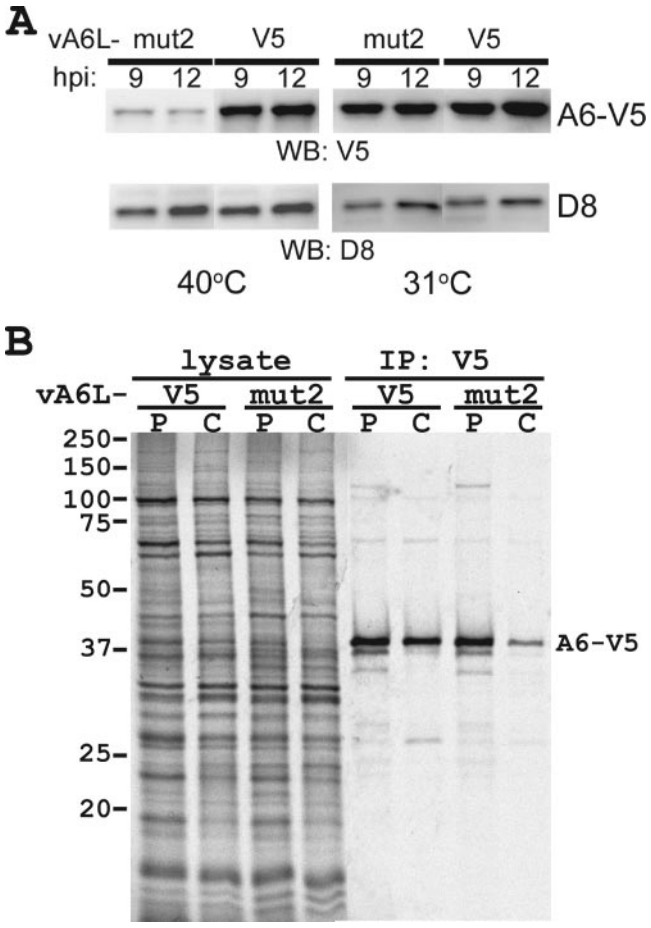

FIG. 4. A6 protein expressed by vA6L-mut2 has a reduced stability at 40°C. (A) The A6 protein level in cells infected by vA6L-mut2 or vA6L-V5. BS-C-1 cells were infected with 10 PFU per cell of vA6L-mut2 or vA6L-V5 and maintained at 31 or 40°C. The cells were harvested at 9 or 12 h p.i. and analyzed with SDS-PAGE and Western blotting (WB) with antibodies to the V5 epitope and VACV late protein D8. (B) Pulse-chase analysis of A6 protein. BS-C-1 cells were infected with vA6L-mut2 or vA6L-V5 at 40°C. At 12 h p.i., the cells were metabolically labeled with [^{35}S]methionine-cysteine for 30 min. The cells were either harvested immediately (P) or replenished with normal medium and harvested 4 h later (C). The cell lysates as well as proteins precipitated from the cell lysates with anti-V5 antibody were analyzed with SDS-PAGE and autoradiography. IP, immunoprecipitate.

At 31°C, the level of A6 protein in vA6L-mut2-infected cells was only slightly reduced compared to that in vA6L-V5-infected cells, indicating that the effect of the mutation on A6 protein accumulation was temperature dependent.

To determine whether the low level of A6 protein in vA6L-mut2-infected cells resulted from reduced protein stability, a pulse-chase experiment was performed. Cells were infected for 12 h at 40°C and then labeled with [^{35}S]methionine-cysteine for 30 min. One set of the cells was harvested immediately (pulse), while the other set was replenished with fresh medium and harvested at 16 h p.i. (chase). In vA6L-mut2-infected cells, the general profiles of labeled proteins before and after the chase were very similar, but a protein with the expected molecular weight of A6 disappeared after the 4-h chase (Fig. 4B). In contrast, this protein remained in vA6L-V5-infected cells. The amount of A6 protein in the cells was further examined by immunoprecipitation with an anti-V5 antibody. Prior to the chase, approximately equal amounts of A6 protein were precipitated from the cells infected by either virus. After the chase, however, the amount of A6 protein that was precipitated from vA6L-mut2-infected cells was significantly lower than that from vA6L-V5-infected cells. Thus, the A6 mutation in vA6L-mut2 caused a loss of function of A6 at the nonpermissive temperature by reducing A6 protein stability.

A6 is required at a late time of infection. To determine the time frame when A6 is required for viral replication, we performed a series of temperature shift-up and shift-down experiments with vA6L-mut2 (Fig. 5). When infected cells were maintained at 31°C for 3 or 6 h and then shifted to 40°C, the 24-h viral yields were similar to those obtained from cells maintained at 40°C for the entire 24 h. Delaying the time of temperature shift-up to 12 h p.i. increased the 24-h viral yield 20-fold, but the yield was still 5-fold less than that obtained from cells maintained at 31°C for the entire 24 h, suggesting that A6 is required for viral replication from 6 h to 24 h p.i. This was also supported by results of the temperature shift-down experiments. Keeping the infected cells at 40°C for 3 or 6 h and then shifting down to 31°C only modestly reduced the 24-h viral yields from that obtained from cells maintained at 31°C for the entire 24 h. Delaying the time of temperature shift-down to 9 or 12 h p.i. resulted in a more substantial decrease in the 24-h viral yield.

vA6L-mut2 is not defective for formation of viral DNA-containing factories. As A6 is expressed postreplicatively, it is not expected to play a role in viral DNA replication. Nevertheless, we examined whether vA6L-mut2 was defective for the formation of viral DNA-containing factories by immunofluorescence microscopy. First, we examined the localization of A6 protein in infected cells (Fig. 6A). At a low multiplicity of infection by vA6L-V5, only some of the HeLa cells were infected. In the infected cells, viral DNA-containing factories were detected as distinct areas outside the nucleus that were positive for DNA staining. A6 protein was found in the entire cytoplasm and was not preferentially localized to viral factories (Fig. 6A). In uninfected cells where there was no DNA staining outside the nuclei, there was no staining by MAb to the V5-epitope, demonstrating the specificity of the antibody.

To assess whether vA6L-mut2 was able to form viral DNA-containing factories at a nonpermissive temperature, BS-C-1 cells were infected at 40°C by either vA6L-mut2 or control vA6L-V5 for 12 h at low multiplicities of infection. Viral DNA-containing factories were clearly visible in all cells that were positively stained by polyclonal antibody to VACV (Fig. 6B), indicating that vA6L-mut2 is not defective for the formation of viral DNA factories.

vA6L-mut2 is not defective for viral protein synthesis at the nonpermissive temperature. Results of Western blot analysis with the antibody to VACV late protein D8 suggested that vA6L-mut2 was not defective for viral late protein synthesis at the nonpermissive temperature. To further assess whether A6 is required for viral gene expression, we examined viral protein synthesis during vA6L-mut2 infection with metabolic labeling. Cells were infected with either vA6L-V5 or vA6L-mut2 and, at various time p.i., were pulse labeled with [^{35}S]methi-

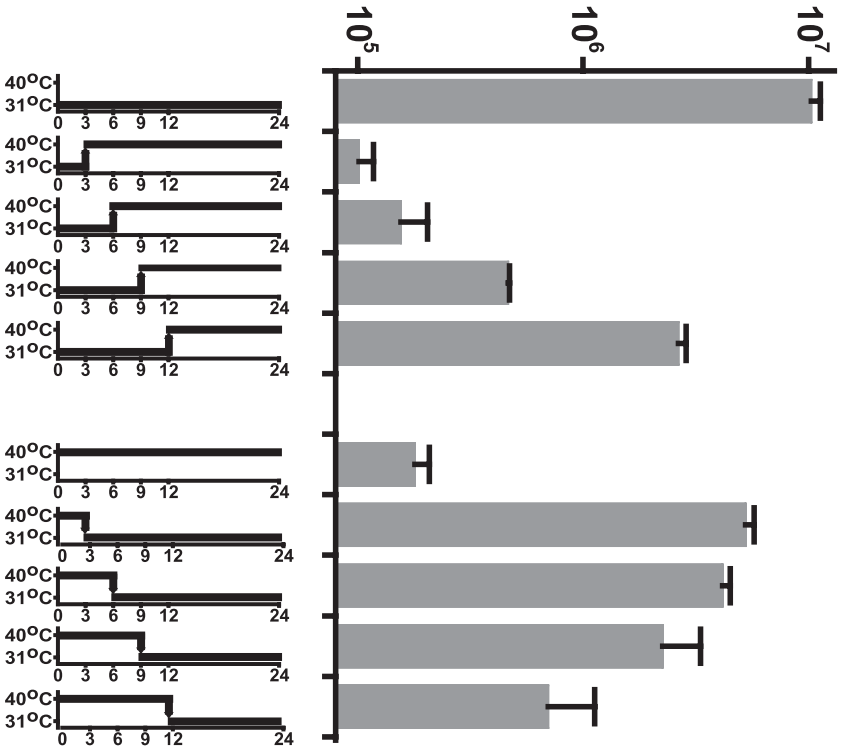

FIG. 5. A6 is required at late time of infection. BS-C-1 cells were infected with 2 PFU per cell of vA6L-mut2 and maintained initially at either 31 or 40°C. At various times after infection, the temperatures of the cells were either shifted up to 40°C or shifted down to 31°C. At 24 h p.i., the cells were harvested and the viral yields were determined by duplicate plaque assay on BS-C-1 cells at 31°C. The schemes of the temperature shift and the 24-h viral yields with the standard deviations of the two plaque assays are shown.

onine-cysteine for 30 min. Cell extracts were then examined by SDS-PAGE and autoradiography (Fig. 7). At both 31 and 40°C, the shut-off of host protein synthesis and the switch to viral protein synthesis were evident at 3 h p.i. The synthesis of late viral proteins was detected at 6 h p.i. and continued to 24 h p.i. The overall viral protein expression pattern at 40°C was similar to that seen at 31°C, except more labeled proteins were present at the higher temperature. At both 31°C and 40°C, the profile of viral proteins expressed by vA6L-mut2 was very similar to that of vA6L-V5, suggesting that vA6L-mut2 is not defective for viral gene expression at the nonpermissive temperature.

vA6L-mut2 is defective for proteolytic processing of several major virion proteins at the nonpermissive temperature. We were unable to purify mature virions from cells infected with vA6L-mut2 at the nonpermissive temperature (data not shown), suggesting that vA6L-mut2 might be defective for virion morphogenesis. Therefore, we examined whether vA6L-mut2 was defective for proteolytic processing of the major virion proteins 4a and 4b, which is a hallmark associated with the formation of intracellular mature viruses (2). Pulse-chase experiments were performed to examine the processing of 4a and 4b. The cells were infected for 8 h and then pulse labeled with [^{35}S]methionine-cysteine for 30 min and chased for 12 h. At 31°C, both vA6L-mut2 and vA6L-V5 were able to process pulse-labeled precursors of 4a and 4b (p4a and p4b) into their mature forms (Fig. 8). As a control, rifampin was used to block virion morphogenesis, resulting in the inhibition of 4a and 4b production. At 40°C, more labeled proteins were present in all samples, but it is still very clear that the amount of mature 4a and 4b proteins in vA6L-V5-infected cells increased significantly after the chase. In contrast, there was little change in the amount of 4a and 4b proteins in vA6L-mut2-infected cells, indicating that vA6L-mut2 might be defective for virion morphogenesis.

Morphogenesis of vA6L-mut2 was blocked before the formation of the mature virions at the nonpermissive temperature. To directly assess whether vA6L-mut2 is defective for virion morphogenesis, we examined thin sections of cells infected with vA6L-mut2 by transmission electron microscopy (Fig. 9). A low magnification of the sections showed that many immature virions and mature virions were present in cells infected at 31°C for 24 h (Fig. 9A). A higher magnification of the same cell showed virion morphogenesis at different stages, including crescents, immature virions, immature virions with nucleoid, and mature virions (Fig. 9B). Extracellular virions were also observed in other areas of the cells (data not shown). In cells infected at 40°C for 24 h, viral factories were readily observed as areas of the cells that are uniform in density and lack cellular organelles (Fig. 9C). Immature virions were present in the viral factories, but their number was lower than that at 31°C. Crescents associated with virosomes were also observed in the viral factory (Fig. 9D). No mature virions or immature virions with nucleoid were found, indicating that the morphogenesis of vA6L-mut2 was blocked before the formation of mature virions at the nonpermissive temperature.

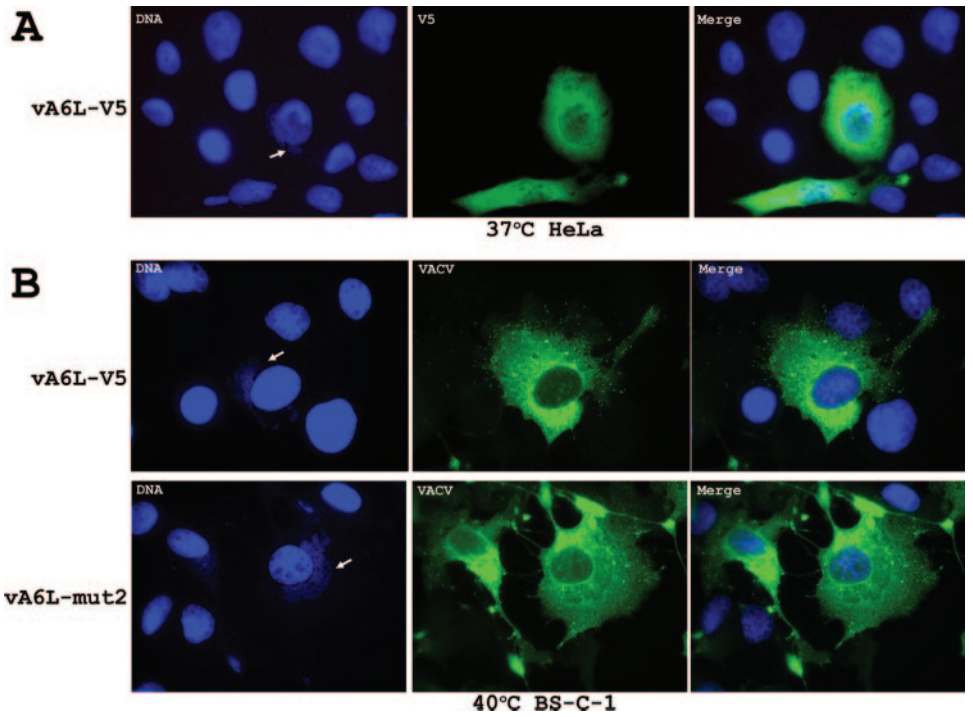

FIG. 6. vA6L-mut2 is not defective for forming viral DNA-containing factories. (A). Localization of A6 protein in infected cells. HeLa cells grown on coverslips were infected with 0.1 PFU/cell of vA6L-V5 at 37°C. At 12 h p.i., the cells were fixed, permeabilized, blocked, and stained with a monoclonal antibody to V5 and a Cy2-conjugated secondary antibody. The DNA was stained with Hoechst dye. The images were acquired with an AX-70 Olympus fluorescence microscope. (B). BS-C-1 cells grown on coverslips were infected with 0.1 PFU/cell of vA6L-V5 or vA6L-mut2 at 40°C. At 12 h p.i., the cells were fixed, permeabilized, blocked, and stained with polyclonal antibody to VACV. The DNA was stained with Hoechst dye. The arrows point to typical DNA-containing viral factories.

DISCUSSION

A set of 91 open reading frames that are conserved in all sequenced vertebrate poxviruses presumably play essential roles in poxvirus replication. However, approximately 20 of these open reading frames, including VACV A6L, have not been characterized previously. We reported here the first genetic analysis of VACV A6L. We showed that A6 is expressed postreplicatively and packaged tightly in virion core. Furthermore, we showed that A6 plays an essential role in virion morphogenesis.

We determined the temporal and spatial expression of A6 by constructing and analyzing a recombinant VACV that ex-

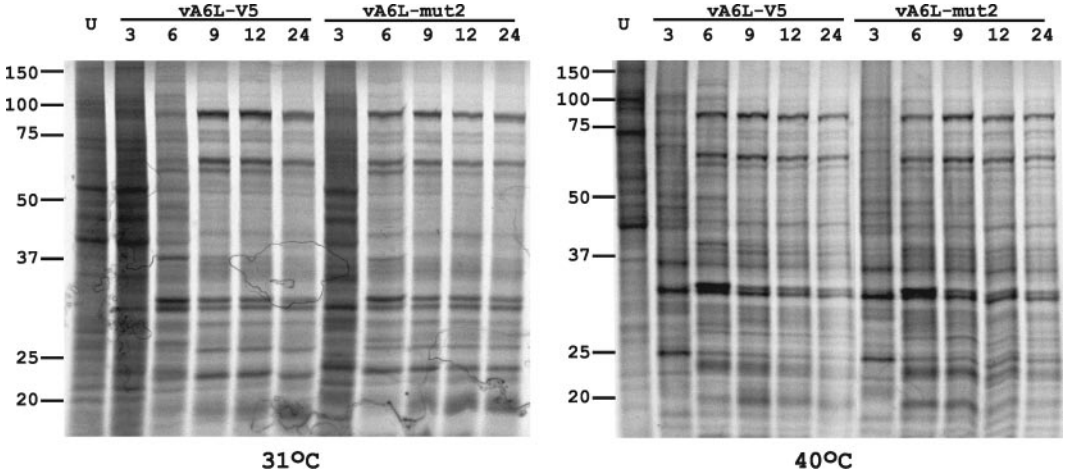

FIG. 7. vA6L-mut2 is not defective for viral gene expression. BS-C-1 cells were infected with 10 PFU/cell of vA6L-V5 or vA6L-mut2 and maintained at 31 or 40°C. At the indicated time points (3, 6, 9, 12, and 24 h p.i.), the cells were metabolically labeled with [^{35}S]methionine-cysteine for 30 min. Uninfected cells were similarly labeled as a control. The labeled cells were harvested, and the cell lysates were analyzed by SDS-PAGE and autoradiography.

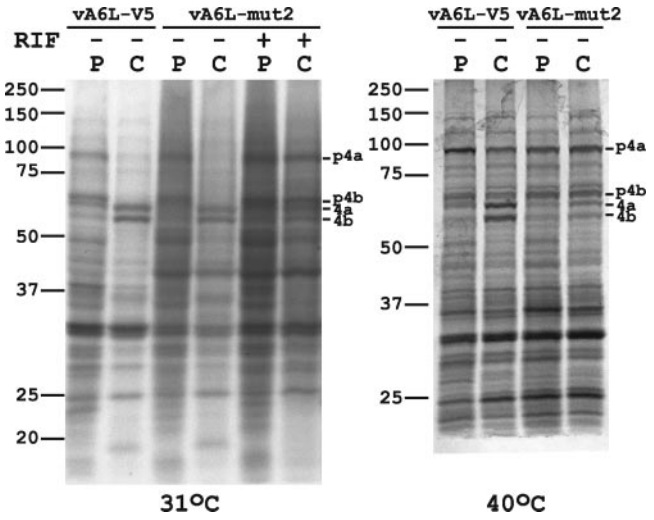

FIG. 8. vA6L-mut2 is defective for proteolytic processing of major virion proteins at nonpermissive temperature. BS-C-1 cells were infected with 10 PFU/cell of vA6L-V5 or vA6L-mut2 and maintained at 31 or 40°C. At 8 h p.i., the cells were pulse-labeled with [^{35}S]methionine-cysteine for 30 min (P) and chased for 12 h (C). As a control, cells were infected with vA6L-mut2 at 31°C in the presence of 100 μg/ml of rifampin (RIF). The cells were analyzed as described in the legend for Fig. 7. The precursor (p4a and p4b) and mature (4a and 4b) forms of the virion proteins are indicated. −, without; +, with.

presses A6 with an epitope tag. A6 expression was shown to be suppressed by the DNA replication inhibitor AraC; thus, by definition, A6L is not an early gene. This is consistent with the presence of an early gene transcription termination signal (TTTTTNT) at the 5′ A6L coding sequence. A6 expression increased continuously at the later time of infection, indicating that the A6L promoter acts as a late stage promoter. The possibility that the A6L promoter also acts as an intermediate stage promoter cannot be ruled out, as A6 expression was evident by 4 h p.i. At the 5′ end of the A6L gene, there are TAAAT and A/T-rich sequences that could serve as the initiator and core of either an intermediate- or a late-stage promoter.

Under mass spectrometry, A6 was previously identified by Chung et al. as a minor virion component (0.15% of total weight) (1). However, a similar study by Yoder et al. did not detect A6 in the virion (25). By performing Western blot analysis of purified virion, we found that A6 was indeed a virion component. Furthermore, we showed that A6 was not a virion membrane protein, which is consistent with the lack of a predicted transmembrane domain in A6. Our conclusion that A6 is tightly packaged in the virion core correlates with a recent mass spectrometry analysis of the myxoma virus virion, which showed that M095L, the A6 ortholog in myxoma virus, was part of the virion core (26). Although A6 is packaged in the virion core, we did not find A6 protein to be preferentially localized to the viral factories, where immature virions are assembled. This is rather unusual for a virion core protein, raising the possibility that A6 is not specifically targeted for virion packaging. A6 may be required outside the viral factories for an additional role in the viral life cycle.

When studying essential VACV late genes, an inducible mutant in which the expression of the specific gene is controlled by a chemical inducer is often constructed. However, our repeated attempts to construct an inducible A6 mutant with vT7LacOI failed. It is unclear why we could not replace the A6L promoter with an inducible promoter or replace the A6L gene with a GFP cassette after inserting an inducible copy of A6L in the HA locus. One possibility is that A6 expression induced by IPTG cannot meet the temporal requirement for the A6 protein in VACV replication. Another possibility is that the insertion of a GFP cassette into the A6L locus may affect the transcription of flanking essential genes. A similar situation was previously reported for F11L (11). While F11L expression can be abolished by introducing a premature stop codon in the F11L locus, the disruption of F11L by the insertion of a selectable cassette resulted exclusively in recombinant viruses containing both the WT and disrupted alleles.

We showed that A6 is essential for VACV replication by constructing and analyzing *ts* mutants with a lesion in A6. The *ts* mutants were created with clustered charge-to-alanine mutagenesis, which was previously used on several VACV genes (3, 4, 8–10, 18). While the substitution of a cluster of charged amino acids often renders the targeted protein nonfunctional by weakening protein interactions at high temperature, it caused a loss of function of A6 in vA6L-mut2 by reducing A6 protein stability in a temperature-dependent manner. As a result, at the high temperature, the steady-state level of A6 was low and vA6L-mut2 was unable to produce infectious progeny, demonstrating that A6 is essential for replication.

Further characterization of the *ts* A6L mutant suggests that A6 is required for the formation of MV. At the nonpermissive temperature, the *ts* mutant was able to synthesize all classes of viral proteins and form viral DNA factories, but it was defective for proteolytic processing of the precursors of major virion proteins 4a and 4b. This suggests that the *ts* mutant is defective for forming MV, as defective 4a and 4b processing is often observed when morphogenesis is blocked at or before the IV stage (12). Transmission electron microscopy of cells infected by the *ts* mutant further showed that the virion morphogenesis was arrested at the IV stage before the formation of IVN and MV. The number of IV produced by the *ts* mutant at the nonpermissive temperature was lower than that at the permissive temperature, so it is also possible that A6 might be required for IV formation, and the presence of a low level of A6 protein at the nonpermissive temperature might allow the formation of some IV.

The exact role that A6 plays in virion morphogenesis remains to be determined. A6 is tightly packaged in virion core, so it may help with the assembly of a proper core consisting of various viral proteins and the viral genome. A6 may do so by interacting with other viral proteins, as multiple proteins were coprecipitated with A6 from pulse-labeled cells (Fig. 4B). After the 4-h chase, however, the profile of coprecipitated proteins changed, even though the profile of labeled proteins in the cell lysate remained essentially the same (Fig. 4B). Several proteins disappeared from the precipitates, but a protein that migrated above the 25-kDa molecular mass marker became more prominent. This may reflect a change in interaction partners for A6 during the process of virion morphogenesis. Alternatively, some of the proteins that interact with A6 may undergo proteolytic processing. A6 protein itself is

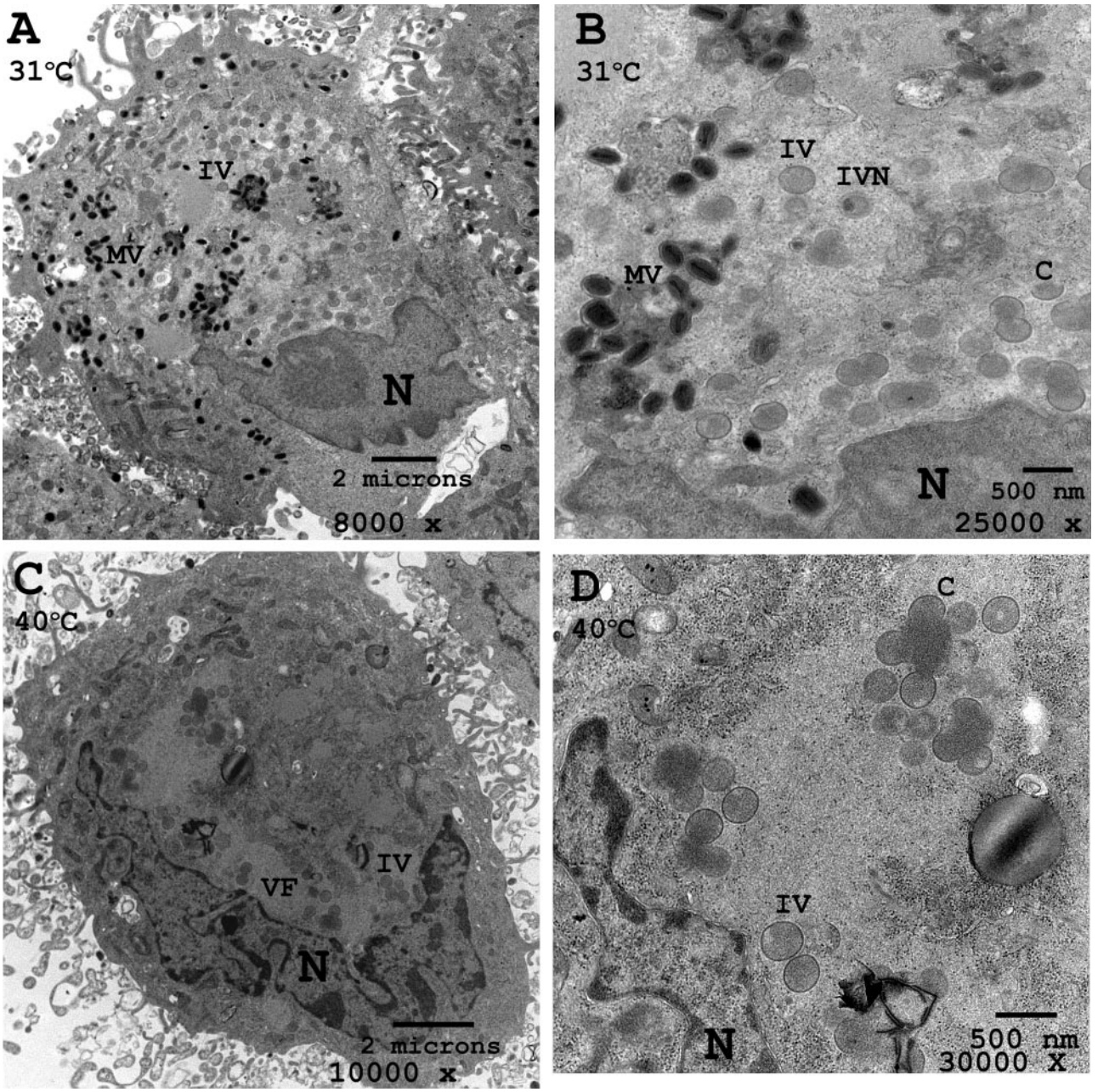

FIG. 9. vA6L-mut2 is defective for virion morphogenesis at the nonpermissive temperature. BS-C-1 cells were infected with 3 PFU per cell of vA6L-mut2 and kept at 31°C (A and B) or 40°C (C and D). At 24 h p.i., the cells were fixed and prepared for transmission electron microscopy. Higher magnification of areas in panels A and C are shown in panels B and D, respectively. Abbreviations: N, nucleus; C, crescent; IV, immature virion; IVN, IV with nucleoid; MV, intracellular mature virion; VF, viral factory.

probably not proteolytically processed, as no C-terminal fragment of A6 was detected in Western blot analysis with anti-V5 antibody. The proteins that coprecipitated with A6 did not include A21, which is a 13.6-kDa protein known to interact with A6 in the yeast two-hybrid analysis (13). We were also unable to coprecipitate epitope-tagged A21 with A6 when both proteins were overexpressed (data not shown). A21 is a virion membrane protein involved in viral entry, while A6 is shown here as a virion core protein involved in virion morphogenesis. It is likely that A6 does not interact with A21 during viral replication. The determination of the identities of A6 interacting proteins and an understanding of the dynamics of A6 protein complex during virion morphogenesis will help to elucidate the complex process of virion morphogenesis.

ACKNOWLEDGMENTS

We thank Shan Lu of the University of Massachusetts for providing rabbit antisera to L1, A27, and D8 and Jianping Liu and Ming Li for providing technical assistance.

This work was partly supported by a grant from the NIAID to Y. Xiang (K22 AI053230).

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