

## Vaccinia Virus A30L Protein Is Required for Association of Viral Membranes with Dense Viroplasm To Form Immature Virions

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The previously uncharacterized A30L gene of vaccinia virus has orthologs in all vertebrate poxviruses but no recognizable nonpoxvirus homologs or functional motifs. We determined that the A30L gene was regulated by a late promoter and encoded a protein of approximately 9 kDa. Immunoelectron microscopy of infected cells indicated that the A30L protein was associated with viroplasm enclosed by crescent and immature virion membranes. The A30L protein was also present in mature virions and was partially released by treatment with a nonionic detergent and reducing agent, consistent with a location in the matrix between the core and envelope. To determine the role of the A30L protein, we constructed a stringent conditional lethal mutant with an inducible A30L gene. In the absence of inducer, synthesis of viral early and late proteins occurred but the proteolytic processing of certain core proteins was inhibited, suggesting an assembly block. Inhibition of virus maturation was confirmed by electron microscopy. Under nonpermissive conditions, we observed aberrant large, dense, granular masses of viroplasm with clearly defined margins; viral crescent membranes that appeared normal except for their location at a distance from viroplasm; empty immature virions; and an absence of mature virions. The data indicated that the A30L protein is needed for vaccinia virus morphogenesis, specifically the association of the dense viroplasm with viral membranes.

Poxviruses comprise a large family of complex, double-stranded DNA viruses that replicate in the cytoplasm of vertebrate or invertebrate cells (17). Vaccinia virus (VV), the best-characterized member of the family, has a genome of approximately 190 kbp that encodes nearly 200 proteins. Viral transcription, DNA replication, and progeny assembly occur in discrete areas called viral factories that are typically located near the nucleus of the infected cell. Morphological studies have shown that the assembly of VV virions proceeds through a series of intermediate stages (8, 16). The first characteristic viral structure discernible by electron microscopy is a crescent-shaped membrane with spicules on the convex surface and electron-dense granular viroplasm in the concavity. The membrane eventually encloses the granular material to form a spherical, immature virion (IV) that appears circular in thin section. The IV undergoes further maturation, including condensation of the viral genome and proteolytic processing of viral core proteins, to form the infectious brick-shaped intracellular mature virion (IMV). A double membrane, derived from the *trans*-Golgi network or endosomal cisternae (14, 23, 24), wraps some of the IMV to form the intracellular enveloped virions (IEV). The IEV are transported to the periphery of the cell, where they can fuse with the plasma membrane, a process that results in the loss of the outermost membrane and the formation of extracellular cell-associated enveloped virions (CEV). Some CEV detach from the cell to form extracellular

enveloped virions (EEV). CEV and EEV are thought to be responsible for cell-to-cell transmission and long-range viral spread, respectively (1, 4, 5, 20).

The generation of temperature-sensitive, drug-resistant, and inducer-dependent conditional lethal mutants has enabled the identification and characterization of many viral proteins involved in virion assembly. Putative roles for these proteins were deduced from the stage at which virion assembly was blocked. Thus, temperature-sensitive mutants with lesions in the F10L serine/threonine kinase (25, 27) or the H5R phosphoprotein (11) did not form recognizable viral membranes under nonpermissive conditions, although viral protein synthesis occurred. When expression of the membrane protein encoded by the A17L open reading frame (ORF) was repressed, large granular masses of viroplasm surrounded by small vesicles or tubules accumulated (21, 30), and neither the characteristic viral membranes nor the IV formed. Inhibition of A14L expression also resulted in the accumulation of small vesicles, but in this case there were also aberrant crescent-like membranes that were detached from the masses of viroplasm (21, 26). Inhibition of D13L expression (31) or addition of the drug rifampin (13, 18, 19) led to an arrest of morphogenesis with the accumulation of masses of viroplasm coated with irregular membranes that lacked spicules. Using similar methods, proteins required for later steps in morphogenesis, e.g., conversion of IV to IMV and IMV to IEV, were identified.

Transfection experiments, carried out in our laboratory to identify additional viral proteins required for early steps in virion assembly, suggested the involvement of a protein encoded by the A30L ORF (unpublished data of J. Granek, E. J. Wolffe, and B. Moss). In the present study, we provide the

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initial characterization of the A30L protein and evidence that it is involved in the association of the dense granular viroplasm with viral membranes.

#### MATERIALS AND METHODS

**Cells and viruses.** BS-C-1 (ATCC CCL6) and HeLa S3 (ATCC CCL2.2) cells were grown in Eagle's minimal essential medium (EMEM) and Dulbecco's minimal essential medium, respectively, each obtained from Quality Biologicals Inc. and supplemented with 10% fetal bovine serum (FBS). VV strain WR and the recombinant VV (rVV) vT7LacOI were propagated in HeLa cells as previously described (12). The rVV vA30Li was replicated in the continuous presence of 50  $\mu$ M isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) and 2.5% FBS. All virus stocks were stored at -70°C.

**Antibodies.** Antiserum to a peptide corresponding to the C-terminal 11 amino acids of the predicted A30L ORF preceded by a cysteine residue (CAASAG REFNRR) was produced in rabbits (Spring Valley, Woodbine, Md.). The murine monoclonal antibody (MAb) MHA.11 (BAbCo/Covance, Berkeley, Calif.) recognizes the nine-amino-acid influenza virus hemagglutinin (HA) epitope tag (YPYDVPDYA).

**Nuclease protection assays.** BS-C-1 cells were infected with VV strain WR at a multiplicity of infection of 10 and harvested at various times. Total RNA was purified from infected cells using TRIzol reagent as directed by Life Technologies, Inc. Polyadenylated mRNA was isolated from total RNA using a MicroPoly (A) Pure kit according to the protocol supplied by Ambion Inc. (Austin, Tex.). A DNA fragment containing a bacteriophage T7 promoter attached to a segment encompassing 89 bp upstream and 112 bp downstream of the A30L putative transcription initiator element was generated from VV genomic DNA and the oligonucleotide primers 5'-GGTAATACGACTCACTATAAGGGCAA TTCATGTACCACGGATAATGTAG-3' and 5'-CGGACAAAGTGTGTAAT TGCAGCTTTAC-3' (the T7 promoter sequence is underlined). A radiolabeled riboprobe complementary to the A30L coding strand was generated by in vitro transcription using T7 RNA polymerase and [ $\alpha$ -<sup>32</sup>P]UTP (3,000 Ci/mmol, 10 mCi/ml). A radiolabeled riboprobe complementary to the F18R gene of VV strain WR (F17R in VV strain Copenhagen) was also generated by in vitro transcription, using the previously described pGEM11K plasmid (2) as the template. The radiolabeled riboprobes were gel purified and eluted in 350  $\mu$ l of 0.5 M ammonium acetate-1 mM EDTA-0.2% sodium dodecyl sulfate (SDS). A 60- $\mu$ g sample of polyadenylated mRNA was hybridized overnight with excess radiolabeled riboprobe in 80% deionized formamide-100 mM sodium citrate (pH 6.4)-300 mM sodium acetate (pH 6.4)-1 mM EDTA. Remaining single-stranded RNA was digested with a 1:200 dilution of the nuclease mixture containing S1 nuclease, RNase A, and RNase T<sub>1</sub>. Protected fragments were analyzed by electrophoresis in an 8% polyacrylamide-8 M urea gel and visualized by autoradiography.

**Plasmids.** To construct pVOTE.1A30L, a complete copy of the A30L ORF flanked by the *Nco*I and *Bam*HI restriction sites at the 5' and 3' ends, respectively, was generated by PCR using VV genomic DNA as the template and the oligonucleotide primers 5'-CCCATGGAAGACCTTAACGGCAAAC-3' and 5'-CGGATCCAACGACGATTGAAATTCTCTCC-3' (*Nco*I and *Bam*HI restriction sites are underlined). The PCR product was digested with *Bam*HI and *Nco*I and inserted into pVOTE.1 (28).

To construct pVOTE.1A30L-HA, a complete copy of the A30L ORF containing an influenza virus HA epitope tag at the C terminus and flanked by the *Nco*I and *Bam*HI restriction sites at the 3' and 5' ends, respectively, was generated by PCR using VV genomic DNA as the template and the primers 5'-CCCATGGAAGACCTTAACGGCAAAC-3' and 5'-CCGGATCCTCAAGCATAGTCTGGAACATCATATGGATACGACGATTGAAATTCTCTCC-3' (*Nco*I and *Bam*HI restriction sites are underlined; the sequence corresponding to the HA tag is in italics). The PCR product was digested with *Bam*HI and *Nco*I and inserted into pVOTE.1.

To construct pA30(LF)/gus/A30(RF) for deletion of the A30L ORF, we made a PCR product containing (i) a complete copy of the bacterial  $\beta$ -glucuronidase (*gus*) gene under the control of the A30L promoter and (ii) flanking sequences of the A30L ORF, comprising a portion of the A29L ORF (downstream flank) and a complete copy of the A31R ORF together with portion of the A32L ORF (upstream flank). A 902-bp DNA segment corresponding to the downstream-flanking region of the A30L ORF (right flank) was generated by PCR using VV genomic DNA as the template and the oligonucleotide primers 5'-TGAATAAAATATTAAATATAACAAAAAGTCGAAAAAGAATTCC-3' and 5'-CGAACCGATGGTATGATTCTAACCTA-3'. A PCR product containing the complete *gus* gene was generated using plasmid pZippy-NEO/GUS, provided by T. Shors, as the template and the oligonucleotide primers 5'-GTTTATTAAT-

ATTTTATTCATATTGTTGCCTCCCTGC-3' and 5'-ATAATATTAAATGGT-ACGTCCTGTAGAAACC-3', in which the lowercase letter indicates a point mutation to produce a better Kozak translation initiation sequence. An 881-bp DNA segment corresponding to the upstream-flanking region of the A30L ORF was generated by PCR using VV genomic DNA as the template and the oligonucleotide primers 5'-CAGGACGTAcCATTAAATATTATAAACATT GTG-3' and 5'-CACGTACCAATTAGGACGGGC-3'. The final PCR product of 3,597 bp was inserted into the pCR2.1-TOPO vector (Invitrogen) to produce plasmid pA30(LF)/gus/A30(RF).

The inserts of all constructs were sequenced by the fluorescence dideoxy-termination procedure, using an Applied Biosystems model 310A genetic analyzer.

**Generation of rVV.** The rVV vA30Li was constructed in two steps. BS-C-1 cells were infected with vT7LacOI at 1 PFU per cell for 1 h at 37°C. The cells were then washed twice with Opti-MEM I reduced medium (Life Technologies) and transfected with 2.5  $\mu$ g of pVOTE.1A30L, using DOTAP according to the protocol of the manufacturer (Roche Molecular Biochemicals, Indianapolis, Ind.). After 5 h, the transfection mixture was removed and replaced with complete EMEM containing 2.5% FBS. The cells were harvested at 24 h after infection, and diluted lysates were used to infect BS-C-1 monolayers in the presence of mycophenolic acid, xanthine, and hypoxanthine to select for virus expressing xanthine-guanine phosphoribosyltransferase. The infected cells were covered with agar, and mycophenolic acid-resistant plaques were visualized 48 h later with neutral red and picked with a Pasteur pipette. Three successive rounds of plaque purification were performed to isolate the rVV vA30L/A30Li. The presence of the A30L ORF in the VV HA locus was confirmed by PCR and agarose gel electrophoresis. vA30L/A30Li was then used to generate vA30Li. BS-C-1 cells were infected with vA30L/A30Li at a multiplicity of 1 and transfected with 2.5  $\mu$ g of pA29gusA31 as described above. The lysates were used to infect BS-C-1 monolayers in the presence of 50 to 100  $\mu$ M IPTG. The infected cells were overlaid with agar, incubated for 2 days at 37°C, and then overlaid with a second layer of agar containing 200  $\mu$ g of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-Gluc; Clontech Laboratories, Palo Alto, Calif.) per ml. After 2 more days of incubation, blue plaques containing the rVV expressing *gus* were picked and used to infect fresh monolayers of BS-C-1 cells. In this way, VA30Li was isolated by three consecutive rounds of plaque purification.

The rVV vA30LiHA, containing the influenza virus HA tag at the C terminus of A30L, was generated by the procedure described for vA30Li except that pVOTE.1A30L-HA was used instead of pVOTE.1A30L.

**Plaque assay.** BS-C-1 cell monolayers, in six-well tissue culture plates, were infected with 10-fold serial dilutions of virus. After 1 h of adsorption, the inocula were removed and the cell monolayers were washed twice with complete EMEM containing 5% FBS and 0.5% methylcellulose, with or without IPTG as specified. The infected cells were incubated at 37°C for 2 days, stained with crystal violet, and counted.

**One-step virus growth.** BS-C-1 cell monolayers, in six-well tissue culture plates, were infected with 5 PFU of virus per cell. After 1 h of adsorption, the inocula were removed and the cell monolayers were washed twice with complete EMEM containing 2.5% FBS. The cells were then incubated in complete EMEM containing 2.5% FBS with or without 50  $\mu$ M IPTG and harvested at various times after infection. The infected cells were subjected to three freeze-thaw cycles, sonicated, and stored at -70°C. Virus titers were determined by plaque assay in the presence of 50  $\mu$ M IPTG.

**Western blot analysis.** Proteins from infected cell lysates or purified virions were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The resolved proteins were electrophoretically transferred onto nitrocellulose membrane (Osmonics, Inc.), and the membrane was blocked overnight in 10% nonfat dried milk in TTBS (100 mM Tris [pH 7.5], 150 mM NaCl, 0.05% [vol/vol] Tween 20). The membranes were incubated for 1 h with the anti-A30L peptide polyclonal antibody at a 1:250 dilution, the anti-A14L polyclonal antibody at a 1:1,000 dilution, or the anti-A10L polyclonal antibody at a 1:1,000 dilution. The membranes were washed in TTBS and incubated with anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (Amersham) at a 1:5,000 dilution. Bound IgG was detected using the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, Ill.).

**Virion extraction.** Purified virus particles were incubated for 1 h at 37°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5) and 1% (vol/vol) NP-40, with or without 50 mM dithiothreitol (DTT). The insoluble and soluble materials were separated by centrifugation at 20,000  $\times$  g for 30 min at 4°C. Proteins from the pellet and supernatant were analyzed by electrophoresis on an SDS-10 to 20% Tris-Tricine polyacrylamide gel (Invitrogen) followed by Western blotting.

**Analysis of [<sup>35</sup>S]methionine- and [<sup>35</sup>S]cysteine-labeled polypeptides by SDS-PAGE.** BS-C-1 cells were infected with either vA30Li or vT7LacOI virus at a multiplicity of 10 for 1 h at 37°C. The inocula were removed, and the infected

cells were incubated with complete EMEM containing 2.5% FBS, with or without 50  $\mu$ M IPTG in the case of vA30Li and with or without 100  $\mu$ g of rifampin per ml in the case of vT7LacOI. For pulse-labeling, the cells were incubated with methionine- and cysteine-free medium containing 2.5% dialyzed FBS (Life Technologies) and labeled with 100  $\mu$ Ci of [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine per ml for 30 min. The cells were then harvested, washed once with cold phosphate-buffered saline, and incubated with micrococcal nuclease (0.1  $\mu$ g/ $\mu$ l) in 10 mM Tris-HCl (pH 7.5)-10 mM KCl-1 mM CaCl<sub>2</sub>-0.2% (vol/vol) NP-40-20 mM  $\beta$ -mercaptoethanol-0.2 mM phenylmethylsulfonyl fluoride for 30 min on ice. The samples were then diluted 1:1 in 0.125 M Tris HCl (pH, 6.8)-4% SDS-20% (vol/vol) glycerol-10% (vol/vol)  $\beta$ -mercaptoethanol-0.004% bromophenol blue. For pulse-chase experiments, the labeling medium was removed and replaced with complete EMEM containing 2.5% FBS and incubated for 12 h prior to lysis. The samples were analyzed by electrophoresis on SDS-4 to 20% polyacrylamide gels (Invitrogen) in Tris-glycine-SDS buffer.

## RESULTS

**Transcription of the A30L gene.** Early, intermediate, and late poxvirus promoters have distinctive sequences (3, 9, 10). Analysis of the A30L gene revealed the presence of an A+T-rich region and a TAAATG initiator element typical of a late promoter. To prove that A30L is a late gene and to precisely locate the transcription initiation site, a 202-nucleotide RNA probe was made that was complementary to a DNA segment that included part of the A30L ORF, the putative TAAATG initiator element, and 89 nucleotides of upstream sequence. If transcription initiated within the TAAATG motif, then the A30L mRNA from infected cells would hybridize to a 112-nucleotide segment of the riboprobe and protect the latter from nuclease digestion. Initiation of transcription from other sites would result in protected fragments that were either smaller or larger than 112 nucleotides (Fig. 1A). In the experiment depicted in Fig. 1B, the uniformly labeled 202-nucleotide riboprobe was hybridized to polyadenylated mRNA purified from mock-infected cells or VV-infected cells at various times after infection. The RNAs were then treated with nucleases, subjected to gel electrophoresis, and analyzed by autoradiography. Two bands were detected when the RNA came from cells that had been infected with VV for 4 to 24 h (Fig. 1B). The fragment of approximately 112 nucleotides was the size predicted if the RNA initiated within the TAAATG sequence. The 202-nucleotide fragment corresponded to the protected full-length riboprobe, presumably derived from hybridization with transcripts initiated from an upstream gene. No protection of the probe was observed when the RNA was obtained from uninfected cells or from cells infected for only 2 h or infected for 8 h in the presence of the protein synthesis inhibitor cycloheximide. Both the timing of A30L transcription and the absence of RNA synthesis in the presence of cycloheximide, which increases the amounts of early RNAs specifically, were consistent with regulation by a late promoter. For comparison, a second riboprobe complementary to the well-characterized F18R late transcript was hybridized to the same RNA samples. The expected size fragment of 126 nucleotides was produced with RNA isolated at 4 to 24 h postinfection but not at earlier times or from cells infected in the presence of cycloheximide, in a manner similar to that observed for A30L transcription (Fig. 1B). The results obtained with the nuclease protection assay were confirmed by Northern blotting using total RNA extracted from VV-infected cells and a uniformly labeled riboprobe complementary to the coding sequence of the A30L ORF. The riboprobe hybridized to RNAs of differ-

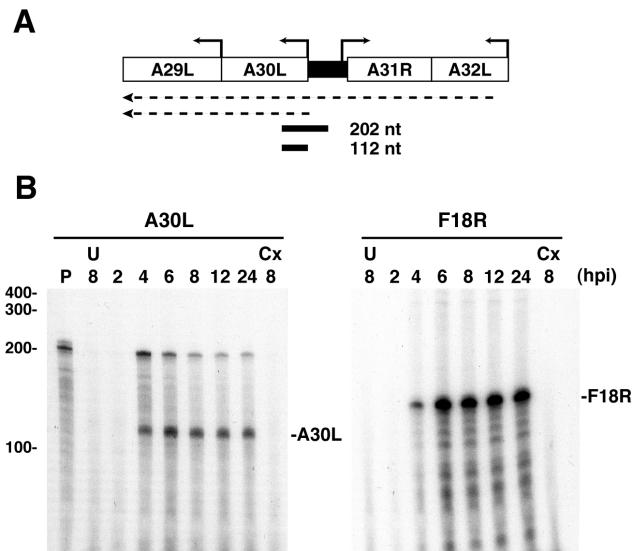


FIG. 1. Transcriptional analysis of the A30L gene. (A) Schematic diagram of the A30L and adjacent ORFs. Arrows above the ORFs indicate the predicted locations of the promoters and the directions of transcription. The dashed arrows below the ORFs represent RNAs that could be initiated from the A30L or the A32L promoter. The solid bars below the dashed arrows represent the full-length 202-nucleotide (nt) riboprobe and the 112-nucleotide segment of the probe that would be protected from nuclease digestion by hybridization to a mRNA that initiated at the putative A30L promoter. (B) RNase protection assays of A30L and F18R transcripts. At the indicated hours postinfection (hpi), total RNA was extracted from uninfected cells or from VV-infected cells incubated for various times in the absence or for 8 h in the presence of cycloheximide (Cx). The polyadenylated RNA was purified from each sample and hybridized to uniformly  $^{32}$ P-labeled RNA probes complementary to the 5' ends and upstream sequences of the A30L or F18R ORFs. Hybridized samples were digested with a mixture of RNase T<sub>1</sub>, RNase A, and S1 nuclease, and the remaining probe fragments were analyzed by PAGE and autoradiography. The numbers on the left represent the sizes in nucleotides of  $^{32}$ P-labeled RNA markers (RNA Century; Ambion). Lane P contained full-length undigested probe; the remaining lanes contained samples derived from cells mock infected for 8 h (U) or from cells harvested at 2 through 24 h after infection. The expected positions of the probe fragments protected by the A30L and F18R transcripts are indicated on the right.

ent lengths producing a diffuse band, as expected for the majority of late mRNAs that do not terminate in a precise manner (data not shown).

**Temporal synthesis of the A30L protein.** The A30L ORF was predicted to encode a protein of 77 amino acids with a predicted mass of 8.7 kDa. To establish that such a protein is made and to determine its time of synthesis, total-cell lysates from VV-infected cells were analyzed by SDS-PAGE followed by Western blotting using an antiserum raised against the C-terminal 11 amino acids of A30L. A protein migrating as expected for a mass of approximately 9 kDa was clearly detected at 8 h after infection and increased in intensity at later times (Fig. 2). An additional faint band migrating between the 14.3- and 21.5-kDa markers at 24 h after infection was noticed but not identified. No difference in the mobility of the A30L was observed when the protein was analyzed under nonreducing conditions using the anti-A30L antiserum (data not shown), consistent with the absence of cysteine residues in the predicted amino acid sequence.

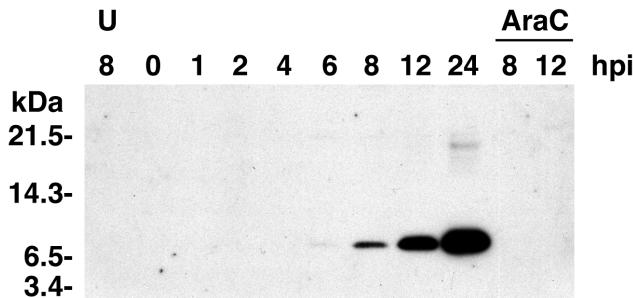


FIG. 2. Temporal synthesis of the A30L protein. BS-C-1 cells were mock infected for 8 h (U) or infected with VV at a multiplicity of 10 in the absence or presence of cytosine arabinoside (AraC) and harvested between 0 and 24 h postinfection (hpi). Proteins from total-cell extracts were resolved by electrophoresis on a 10 to 20% gradient polyacrylamide gel in SDS-Tricine buffer and analyzed by Western blotting using antisera directed to the C-terminal 11 amino acids of the A30L protein. Proteins were detected by chemiluminescence. The positions of migration and molecular masses of marker proteins are indicated on the left.

The A30L protein was not detected when cells were infected with VV in the presence of the DNA synthesis inhibitor cytosine arabinoside (AraC) (Fig. 2), demonstrating a requirement for viral DNA synthesis that was consistent with late expression.

**Association of the A30L protein with virions.** The association of the A30L protein with highly purified virus particles was demonstrated by SDS-PAGE of sucrose gradient fractions. The peak of A30L protein, determined by Western blotting (Fig. 3B), coincided with the fractions containing the most

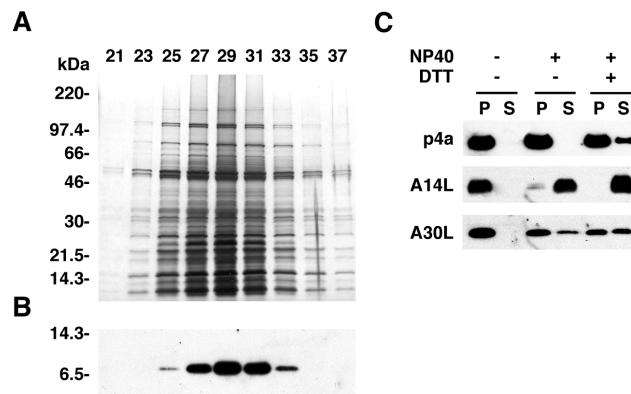


FIG. 3. Association of the A30L protein with purified virions. (A) Purified VV particles were sedimented on a sucrose gradient. Fractions were collected, and the proteins were resolved by electrophoresis on an SDS-4 to 20% gradient polyacrylamide gel. Proteins were visualized by silver staining. The positions of migration and molecular masses of marker proteins are indicated on the left. (B) Proteins from the sucrose gradient fractions analyzed in panel A were separated by electrophoresis on a 10 to 20% gradient polyacrylamide gel in SDS-Tricine buffer, transferred to a nitrocellulose membrane, and probed with rabbit polyclonal A30L peptide antibody. The bands detected by chemiluminescence are shown. (C) Sucrose-gradient purified VV ( $10^8$  PFU) was incubated in Tris buffer containing 1% NP-40 with or without 50 mM DTT. After centrifugation, the soluble (S) and insoluble (P) fractions were analyzed by SDS-PAGE and Western blotting using the rabbit polyclonal A30L peptide antiserum, A14L rabbit polyclonal antibody, or A10L (P4a) rabbit polyclonal antibody as indicated.

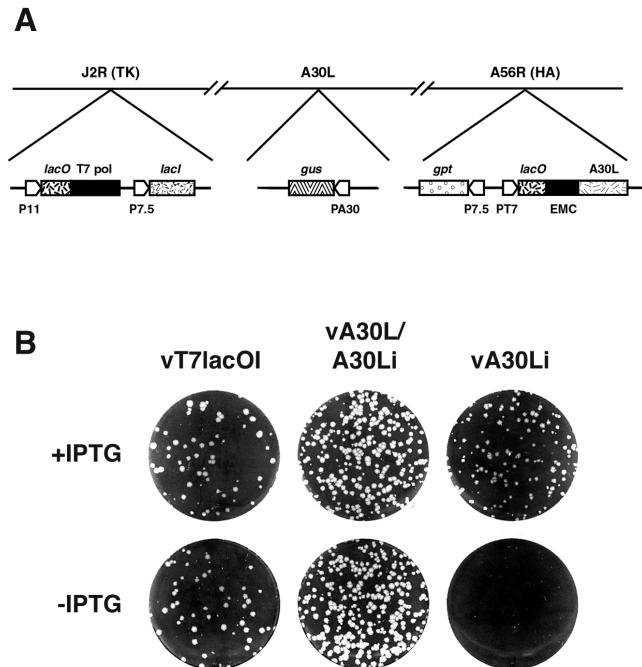


FIG. 4. Construction of an A30L-inducible rVV. (A) Schematic diagram representing the genome of vA30Li. The J2R (thymidine kinase [TK]), A30L, and A56R (HA) loci are depicted. Insertions into these loci are shown below the line. Additional abbreviations: P11, a VV late promoter; P7.5, a VV early-late promoter; *lacO*, *E. coli* *lac* operator; *lacI*, *E. coli* *lac* repressor gene; T7 pol, bacteriophage T7 RNA polymerase gene; PT7, bacteriophage T7 promoter; EMC, encephalomyocarditis virus cap-independent translation enhancer element; *gus*, *E. coli*  $\beta$ -glucuronidase gene; *gpt*, *E. coli* guanine phosphoribosyltransferase gene. (B) Effect of IPTG on virus plaque formation. BS-C-1 cell monolayers were infected with vT7LacOI, vA30L/A30Li, or vA30Li in the presence or absence of 50  $\mu$ M IPTG as indicated. Cells were stained with crystal violet at 48 h after infection.

virus particles as determined by measurement of optical density at 260 nm (data not shown) and by detection of other virion proteins by silver staining (Fig. 3A).

VV membrane and core proteins can be separated by centrifugation after treatment of virions with a nonionic detergent. SDS-PAGE followed by Western blotting, however, demonstrated that the A30L protein was only partially released from the virus particles treated with either NP-40 alone or NP-40 plus DTT (Fig. 3C). As a control for the efficiency of the extraction procedure, the nitrocellulose membrane that had been incubated with the anti-A30L antibody was stripped and reprobed with antibodies to A14L and A10L (P4a) proteins (Fig. 3C). As expected, the A14L membrane protein was completely extracted with NP-40 plus DTT, while most of the A10L core protein remained associated with the insoluble fraction. The partial extraction of the A30L protein with NP-40 and DTT suggested that it might be located in the matrix between the core and the membrane.

**Generation of an rVV expressing an inducible copy of A30L.** To determine the role of A30L in the virus replication cycle, we constructed an rVV in which the expression of A30L was stringently regulated (Fig. 4A). This rVV was constructed in two steps. First, the inducible copy of A30L, containing a bacteriophage T7 promoter, the *Escherichia coli lac* operator,

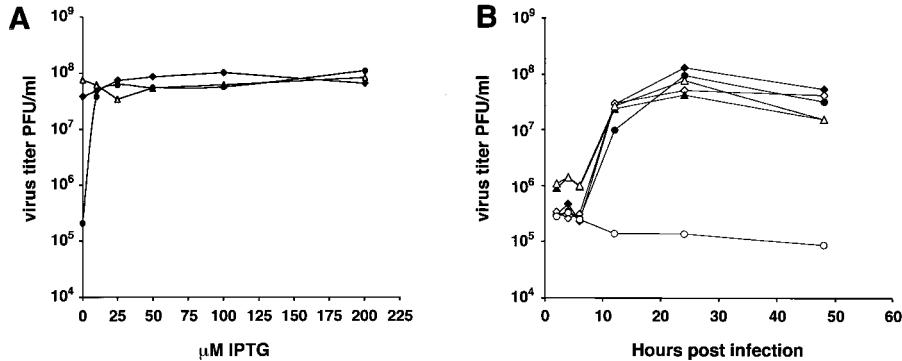


FIG. 5. Effect of IPTG on yields of vA30Li. (A) BS-C-1 cells were infected with vT7LacOI (◆), vA30L/A30Li (△), or vA30Li (●) at a multiplicity of 5 and incubated in the presence of 0 to 200  $\mu$ M IPTG for 24 h. All virus titers were determined by plaque assay in the presence of 50  $\mu$ M IPTG. (B) BS-C-1 cells were infected with vT7LacOI (◆), vA30L/A30Li (▲), or vA30Li (●) in the absence (open symbols) or presence (filled symbols) of 50  $\mu$ M IPTG. Cells were harvested at the indicated times after infection, and the total virus titer of each sample was determined as for panel A.

and part of the untranslated leader sequence of encephalomyocarditis virus RNA, was inserted into the HA locus (A56R ORF) of the previously constructed vT7LacOI recombinant virus (28). vT7LacOI contains an IPTG-inducible copy of the bacteriophage T7 RNA polymerase gene and a continuously expressed *E. coli lac* repressor gene. The resulting intermediate virus, vA30L/A30Li, contained both endogenous and inducible copies of A30L. In the second step, the endogenous A30L gene was deleted from the vA30L/A30Li virus by homologous recombination using a plasmid containing the *gus* gene under the control of the A30L promoter. The final recombinant virus, vA30Li, was isolated in the presence of 50  $\mu$ M IPTG and identified by the expression of the *gus* gene. Another recombinant virus, vA30LiHA, in which the inducible copy of A30L has a C-terminal influenza virus HA tag, was constructed in a similar manner. The genotypes of both vA30Li and vA30LiHA were confirmed by PCR.

**IPTG is required for plaque formation and replication of vA30Li.** To determine the effect of IPTG on plaque formation, BS-C-1 cells were infected with vA30Li in the presence or absence of 50  $\mu$ M IPTG. As controls, additional cells were infected with the vT7LacOI parental or vA30L/A30Li intermediate virus in the presence or absence of 50  $\mu$ M IPTG. The vT7LacOI and vA30L/A30Li viruses, each containing the original copy of A30L, formed plaques in the presence or absence of IPTG. In contrast, vA30Li, which contains only the inducible copy of A30L, required IPTG for plaque formation (Fig. 4B).

To determine if inhibition of plaque formation was due to a defect in viral replication or spread, we analyzed the yields of cell-associated virus in the presence or absence of IPTG under one-step growth conditions. The parental virus vT7LacOI and the intermediate virus vA30L/A30Li replicated in the presence or absence of IPTG, whereas replication of vA30Li virus was entirely dependent on the addition of IPTG (Fig. 5). Similar yields of vA30Li were achieved with IPTG concentrations of 25 to 200  $\mu$ M IPTG (Fig. 5A). At 50  $\mu$ M IPTG, the yield and kinetics of replication of vA30Li were similar to those of the parental vT7LacOI and the intermediate virus vA30L/A30Li (Fig. 5B).

**Inducible synthesis of the A30L protein.** BS-C-1 cells were infected with vA30Li at a multiplicity of 1 or 10 and incubated for 24 h in the presence of 0 to 100  $\mu$ M IPTG. In the absence of IPTG, no A30L protein was detected in cells infected at the low or high multiplicity of infection (Fig. 6). The A30L protein was detected in infected cells incubated in the presence of 10  $\mu$ M IPTG. Higher amounts of A30L were synthesized when cells were incubated in the presence of 25  $\mu$ M IPTG, but no noticeable increase was observed at higher concentrations. Thus, similar concentrations of IPTG were required for maximal induction of A30L protein synthesis (Fig. 6) and virus yield (Fig. 5A).

**Synthesis of viral proteins in the absence of A30L expression.** During a productive VV infection, early, intermediate, and late proteins are synthesized consecutively. The cessation of host protein synthesis makes it particularly easy to label and visualize the abundant late viral proteins by SDS-PAGE. To determine the effects of the repression of A30L expression on

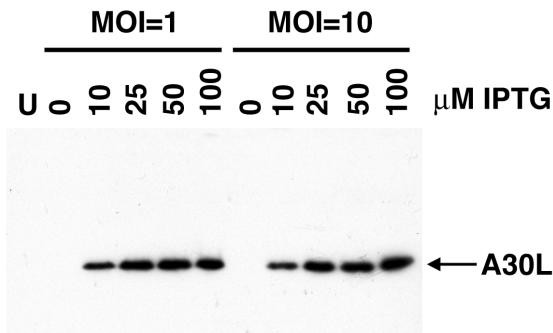


FIG. 6. Effect of IPTG on the synthesis of the A30L protein. BS-C-1 cells were mock infected (U) or infected with vA30Li at a multiplicity of infection (MOI) of 1 or 10 in the presence of 0 to 100  $\mu$ M IPTG. At 24 h after infection, the cells were harvested and the proteins were analyzed by electrophoresis on a 10 to 20% gradient polyacrylamide gel using SDS-Tricine buffer. The proteins were then transferred to a nitrocellulose membrane and incubated with the rabbit polyclonal A30L peptide antibody. The bands detected by chemiluminescence are shown. The arrow points to the A30L protein.

viral protein synthesis, BS-C-1 cells were infected with vA30Li or the parental virus vT7LacOI in the presence or absence of IPTG. At various times, the cells were labeled for 30 min with a mixture of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine. At the end of the labeling periods, whole-cell extracts were analyzed by SDS-PAGE and radiolabeled proteins were visualized by autoradiography. As shown in Fig. 7A, shifts from host to early viral proteins and from early to late viral proteins were observed in vA30Li-infected cells in the presence or absence of IPTG. At 3 and 6 h after infection, the protein synthesis pattern of cells infected with vA30Li in the absence of IPTG was virtually identical to that of cells infected either with vT7LacOI or with vA30Li in the presence of IPTG. The overall patterns of proteins labeled at later times were similar under all conditions. Nevertheless, some differences were noted. In cells infected with vA30Li in the absence of IPTG, a prominent labeled band of approximately 20 kDa was detected at 9 and 12 h but was much less intense at 24 h. A faint band of similar mobility was detected maximally at 6 h after infection with either vT7LacOI or vA30Li in the presence of IPTG (Fig. 7A). In addition, doublet bands of approximately 14 and 25 kDa were resolved from extracts of cells infected with vT7LacOI or vA30Li in the presence of IPTG, but only the lower species of each doublet was detected in extracts of cells infected with vA30Li in the absence of IPTG (Fig. 7A). These distinctive features were reproducible and could reflect subtle differences in the synthesis, degradation, or processing of specific proteins in cells infected with vA30Li in the absence of inducer, but there was no general defect in viral protein synthesis.

**Effects of A30L repression on the processing of viral proteins.** The proteolytic processing of several core proteins is coupled to morphogenesis (15) and can be blocked by the drug rifampin or by infection with certain mutant viruses. Indeed, the absence of proteolytic processing has been used as an indicator of a defect in viral morphogenesis. To investigate the effect of inhibition of A30L expression on the processing of viral proteins, we carried out pulse-chase experiments. BS-C-1 cells were infected with vA30Li in the presence or absence of IPTG. As a control, cells were infected with vT7LacOI in the presence or absence of rifampin. Infected cells were labeled for 30 min with a mixture of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine at 6 h after infection (Fig. 7B). Cells were either harvested immediately after labeling or chased for 12 h in the presence of unlabeled amino acids. Pulse-labeling indicated that the major core precursor proteins P4a and P4b were synthesized in similar amounts by both viruses under permissive and nonpermissive conditions. However, repression of A30L expression resulted in the inhibition of proteolytic processing of P4a and P4b to 4a and 4b during the chase, producing an effect similar to that caused by rifampin (Fig. 7B) and suggesting that the A30L protein is required for assembly or morphogenesis.

**Morphogenesis of vA30Li under nonpermissive conditions.** Electron microscopy was used to determine the stage at which virus replication was blocked in the absence of A30L expression. BS-C-1 cells were infected with vA30Li in the presence or absence of IPTG for 24 h. The cytoplasm of cells infected with vA30Li in the presence of IPTG contained the expected range of viral structures, including crescents, and a large number of IV and mature particles (Fig. 8B). Cells infected with vA30Li in the absence of IPTG, however, showed large electron-dense

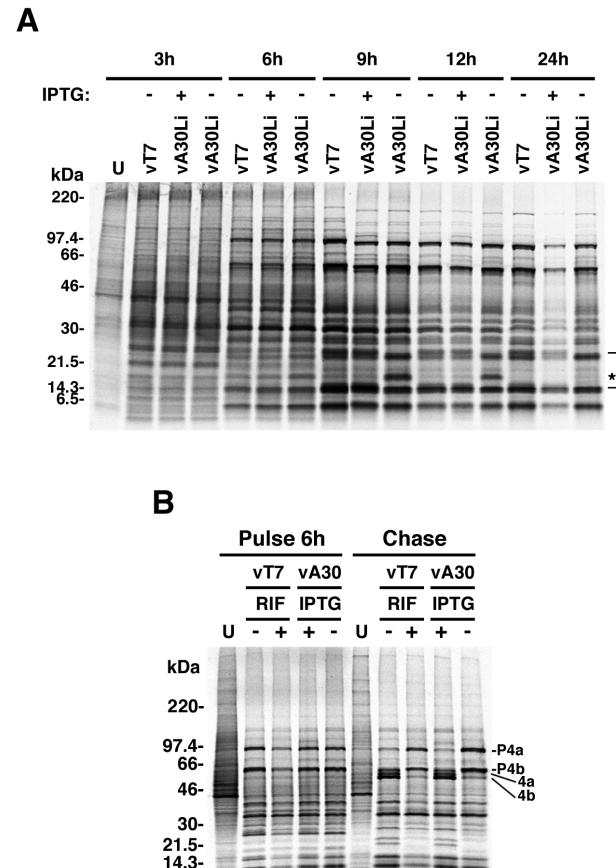


FIG. 7. Synthesis and processing of viral proteins. (A) Pulse-labeling of viral proteins. BS-C-1 cells were infected with vT7LacOI or vA30Li at a multiplicity of 10 in the presence (+) or absence (-) of 50  $\mu$ M IPTG as indicated. Cells were labeled with a mixture of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine for 30-min periods starting at 3, 6, 9, 12, or 24 h after infection or after mock infection (U). Immediately after labeling, the cells were washed and lysed, and the labeled proteins were denatured with SDS and mercaptoethanol and analyzed by electrophoresis on a 4 to 20% gradient polyacrylamide gel. An autoradiograph is shown. Numbers on the left correspond to molecular masses of the marker proteins. The positions of migration of proteins that are over- or underexpressed in cells infected with vA30Li in the absence of IPTG are indicated by an asterisk or a dash, respectively. (B) Proteolytic processing of viral late proteins. BS-C-1 cells were infected either with vT7LacOI in the presence (+) or absence (-) of 100  $\mu$ g of rifampin (RIF) per ml or with vA30Li in the presence (+) or absence (-) of 50  $\mu$ g of IPTG per ml. At 6 h after infection, the cells were pulse-labeled with a mixture of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine for 30 min. Cells were either harvested immediately (pulse) or incubated with excess unlabeled methionine for an additional 12 h (chase). The proteins were denatured with SDS and mercaptoethanol and analyzed by electrophoresis on a 4 to 20% gradient polyacrylamide gel and autoradiography. The positions of migration of the major core precursor protein (P4a and P4b) and their mature, processed forms (4a and 4b) are shown on the right.

masses of viroplasm with clearly demarcated but nonmembraneous borders. Frequently, "holes" were observed, suggesting the trapping of less-dense material within the masses. Crescent membranes appeared normal in size and shape except for the absence of adjacent dense viroplasm (Fig. 8A). Circular membranes characteristic of thin sections of IV were also seen but

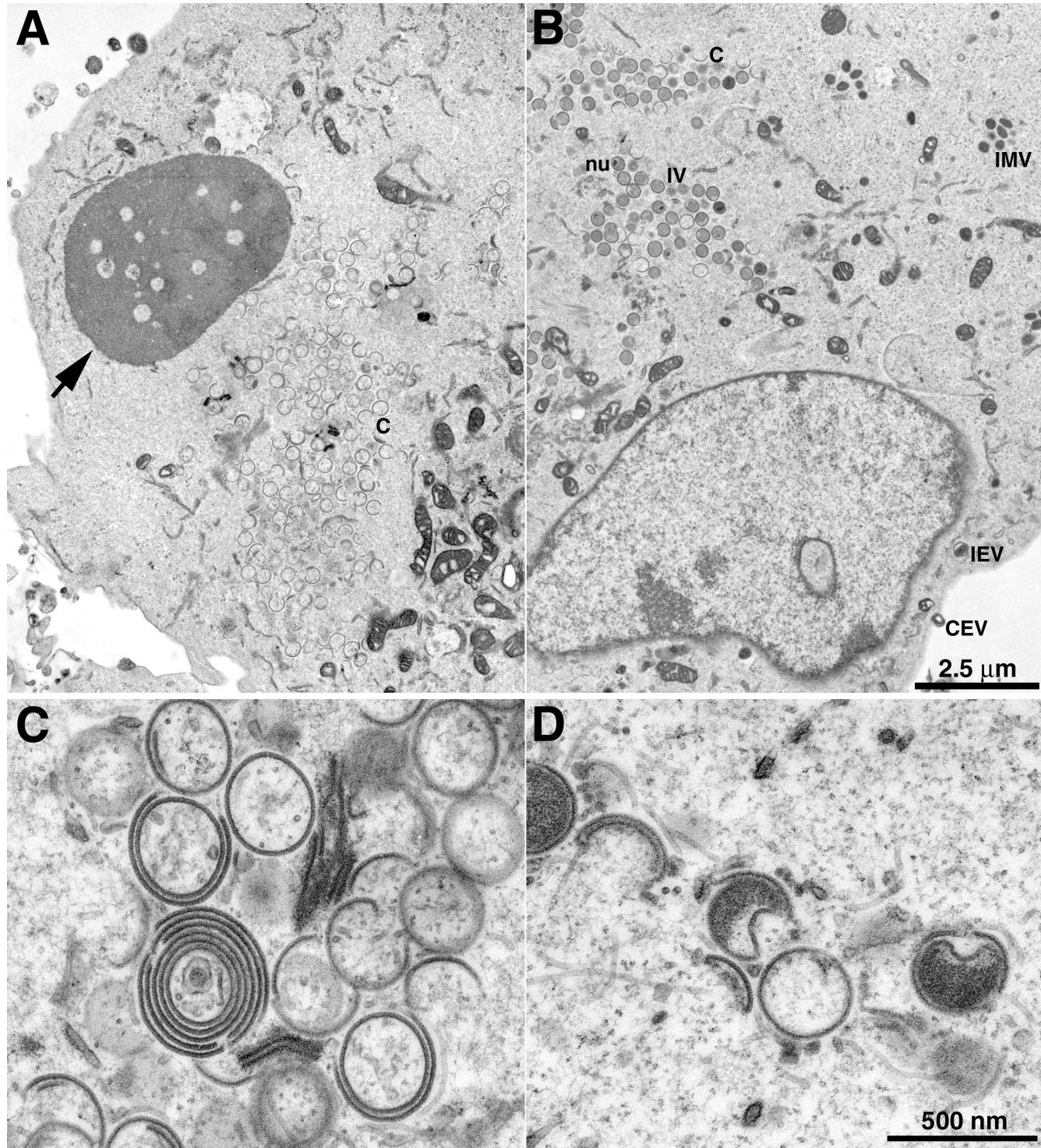


FIG. 8. Electron microscopy of cells infected with vA30Li in the presence or absence of IPTG. BS-C-1 cells were infected with vA30Li at a multiplicity of 10 in the presence (B) or absence (A, C, and D) of 50  $\mu$ M IPTG. At 24 h after infection, the cells were fixed and prepared for transmission electron microscopy. The arrow in panel A points to a large dense granular mass that forms in the absence of IPTG. Abbreviations: C, crescents; nu, nucleoid within an IV.

their centers were electron lucent, indicating that the viral membranes had enclosed little or none of the dense granular viroplasm. Some of these membranes were multilayered, giving an onion-like appearance (Fig. 8C). Although the separation

of membranes and granular viroplasm was the distinctive phenotype of this mutant, a small minority of membrane crescents with dense granular viroplasm and rare IV with nucleoids and aberrant mature virions were found in some cells infected with

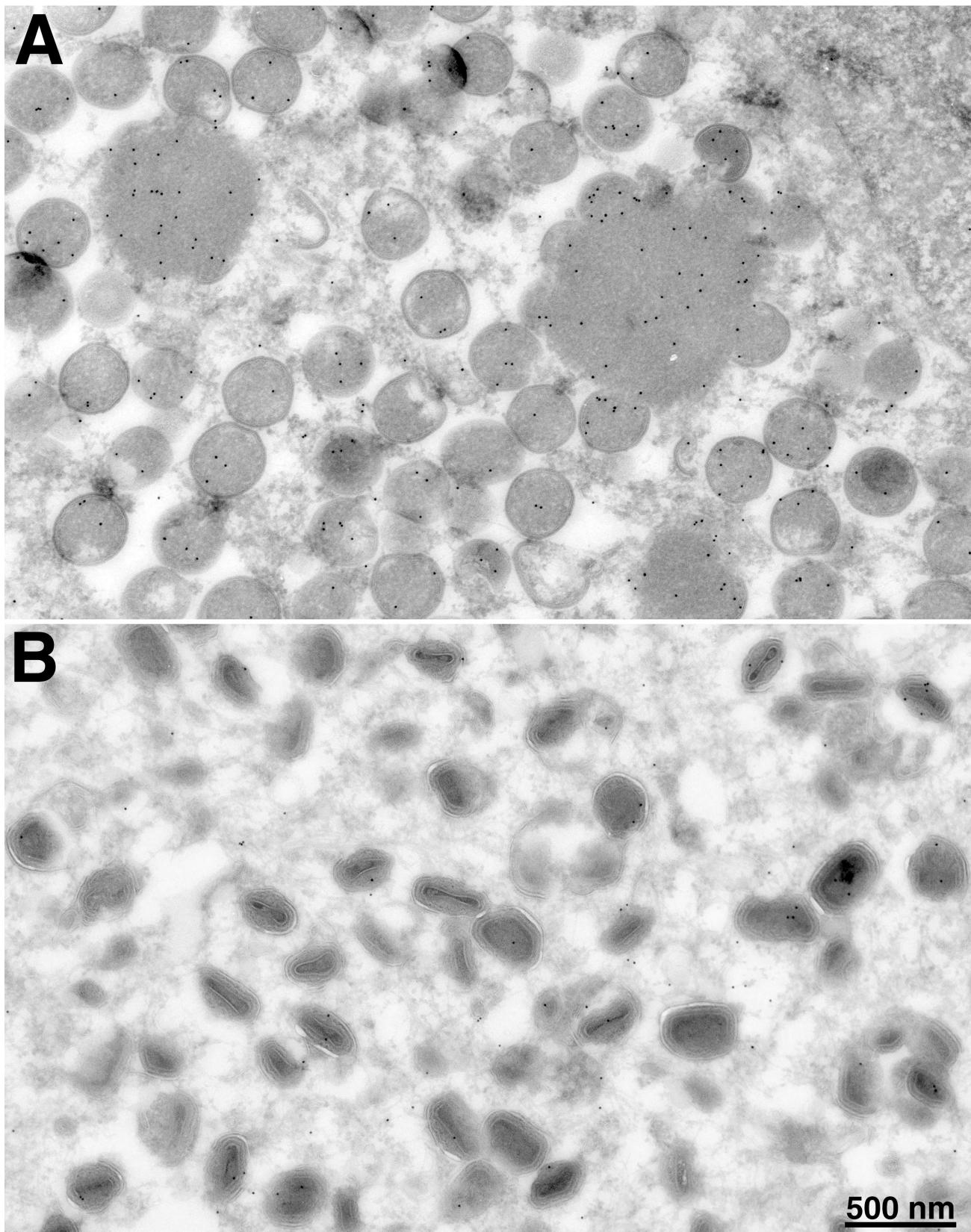


FIG. 9. Localization of the A30L protein by immunoelectron microscopy. BS-C-1 cells were infected with vA30LiHA at a multiplicity of 10 in the presence of 100  $\mu$ g of IPTG, per ml. After 22 h, the cells were fixed in paraformaldehyde, cryosectioned, and incubated with MAb MHA.11 followed by rabbit anti-mouse IgG and protein A-conjugated to colloidal gold. Fields with numerous IV and IEV are shown in panels A and B, respectively.

vA30Li in the absence of IPTG (Fig. 8D). We suspect that the formation of these viral structures was due to incomplete repression of A30L synthesis in some cells.

**Association of A30L protein with viroplasm and immature and mature virus particles.** The biochemical experiments described above indicated that the A30L protein was associated with purified virus particles but was probably not a membrane component. Immunoelectron microscopy was carried out to further investigate the localization of the A30L protein in VV virions and to determine the stage at which it associates with assembling virus particles. Because the polyclonal antibody to the C-terminal peptide of A30L was not optimal for immunoelectron microscopy, we constructed rVV vA30LiHA, in which an influenza virus HA tag was added to the C terminus of the inducible copy of the A30L protein. BS-C-1 cells were infected with vA30LiHA in the presence or absence of IPTG. The HA-tagged A30L protein was visualized by incubating the ultrathin cryosections with an HA MAb followed by protein A conjugated to colloidal gold. In cells infected in the presence of IPTG, gold grains were seen mostly in association with the granular masses of viroplasm and within IV in the factory regions (Fig. 9A). There was no specific labeling of membranes. Outside the factory areas, grains could also be seen in association with IMV and dispersed through the cytoplasm (Fig. 9B). Gold grains were more numerous on the IV than the IMV, possibly due to decreased accessibility of the HA epitope to the antibody after virus maturation. Gold grains were rarely seen in cells infected with vA30LiHA in the absence of IPTG, demonstrating the specificity of the labeling (data not shown).

## DISCUSSION

Sequence comparisons indicated that the A30L ORF is conserved among all members of the chordopoxvirus subfamily analyzed thus far. However, there was no obvious similarity to any other protein present in the available databases. Furthermore, analysis of the deduced amino acid sequence of the A30L protein did not reveal any predicted structural or functional domain that could provide a clue to its function. Examination of the nucleotide sequence of the A30L gene did indicate the presence of a typical late transcription initiator element (TAAAT) overlapping the putative ATG translation initiation site. Nuclease protection assays demonstrated that A30L was transcribed at late times during VV infection and showed that the transcriptional start site was at or near the predicted TAAATG motif. Western blotting using a polyclonal serum raised against the C-terminal amino acids of the A30L protein confirmed that A30L is a late protein with a molecular mass of approximately 9 kDa. The A30L protein was associated with purified IMV and could be only partially released by the detergent NP-40 even when DTT was included, suggesting that it might be a matrix protein located between the core and membrane. A similar location was suggested for the protein encoded by the ORF called A4L in VV strain Copenhagen or A5L in VV strain WR (6, 29). Immunoelectron microscopy revealed that the A30L protein was associated with the dense granular viroplasm in viral factories as well as the interior of IV and IMV, with no apparent membrane localization.

To investigate the role of the A30L protein in the VV replicative cycle, we constructed vA30Li, in which the endogenous

A30L gene was replaced by an inducible copy. Synthesis of the A30L protein was dependent on the concentration of IPTG, with no A30L protein detected in cells infected in the absence of the inducer. Furthermore, replication of the virus was entirely dependent on IPTG and was maximal at IPTG concentrations that fully induced A30L expression. The myxoma virus homolog, which is expressed late and is virion associated, also appears to be essential as efforts to delete that gene were unsuccessful (Jing Xin Cao and Grant McFadden, personal communication). Our metabolic pulse-labeling experiments indicated that early and late VV protein synthesis occurred in the absence of inducer, although some subtle differences were noted. The most prominent of these was a band of 20 kDa that was prominent at 9 and 12 h but was diminished at later times after infection in cells infected with vA30Li in the absence of inducer. A faint band of similar mobility was seen at earlier times under permissive conditions, raising the possibility that this 20-kDa protein is normally made in low amounts. Nevertheless, there seemed to be no perturbation in the regulation of the majority of viral proteins. There was, however, a profound inhibition of the proteolytic processing of certain core proteins including P4a and P4b in the absence of A30L expression. This result is typical of situations in which virus assembly is blocked at or before the stage of formation of IV (15).

As predicted, electron microscopy of cells infected with vA30Li in the absence of IPTG demonstrated a striking defect in morphogenesis. The electron micrographs revealed large masses of granular viroplasm that were not associated with viral membranes. Interestingly, there was a sharp boundary between the dense viroplasm and the less-dense surrounding material, even though no membrane border was evident. Moreover, there were apparent holes in the dense viroplasm that were filled with less-dense material, indicating the absence of mixing. Although crescent and circular membranes, representing thin sections of incomplete and possibly complete spherical particles formed, the majorities of these were devoid of granular viroplasm and appeared empty. Some of the membranes formed concentric layers resembling an onion. This phenotype, in which the foci of viroplasm are not associated with membranes, is similar to that of certain genetically unmapped temperature-sensitive mutants of the IHD-J strain of VV described by Dales et al. (7). Viroplasmic masses and empty crescents have also been described in cells infected with an inducible A14L mutant under nonpermissive conditions (22, 26). However, A14L is a membrane protein, and an additional phenotype of the A14L mutant is the accumulation of vesicles and aberrant membranes. In addition, the normal-looking viral membranes formed in the absence of the A30L protein were usually distant from the masses of viroplasm, whereas they seemed to be closer in the case of the A14L mutant. An intriguing possibility for further investigation is that the A30L matrix protein and the A14L membrane protein interact with each other to allow the association of viroplasm with membranes.

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