

# The Vaccinia Virus 4c and A-Type Inclusion Proteins Are Specific Markers for the Intracellular Mature Virus Particle

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Received 16 January 1996/Accepted 4 March 1996

**Gel analysis of vaccinia virus particles purified by bouyant density demonstrates a protein with an estimated molecular mass of 59 kDa, which is apparently restricted to the intracellular mature virion (IMV) form. Western blotting (immunoblotting) and immunoprecipitation procedures identify the protein as the vaccinia virus 4c protein, which facilitates occlusion of poxvirus particles within cowpox cytoplasmic inclusions. Western blotting procedures also identify the truncated A-type inclusion protein of vaccinia virus as a specific marker for IMV particles. Kinetic analyses of virion maturation and 4c production suggest that peak enveloped virion production occurs before peak IMV production in the virus replication cycle and that 4c production is concomitant with maturation of IMV. The implications for a distinct and evolutionarily conserved function of IMV in viral pathogenesis are discussed.**

Vaccinia virus (VV), the prototype orthopoxvirus, produces two types of infectious virion: enveloped virus (EV), which exits the cell to be released into the extracellular medium (extracellular EV [EEV]) or retained on the outer face of the plasma membrane (cell-associated EV [CEV]), and intracellular mature virus (IMV), which is retained in the cytoplasm of the host cell (3, 29, and reviewed in reference 16). When maturing as EV, cytoplasmic virions are enwrapped with a double membrane derived from the trans-Golgi network (11, 27). In exiting the cell through the plasma membrane one of the trans-Golgi-derived membranes is lost, to give an EV virion which has one more unit membrane than the cytoplasmic IMV form of the virus. EV particles possess a number of virally encoded protein markers found in or on the outer membrane and absent from IMV, including four (P37, gp42, gp22, and A36R) which are involved in some way with EV formation or release (2, 4, 9, 17, 31). EV particles are responsible for dissemination of infection in tissue culture and in vivo (3, 21).

Although IMV particles are infectious, it has not been determined if these particles are a true end product of maturation or merely an infectious precursor of EV. One previous study has demonstrated the presence of IMV-specific proteins; however, these proteins were not identified and their association with IMV particles was dependent on the cell line used to grow the virus, raising doubts about their identity as virally encoded proteins and their relevance to virus maturation (20). Despite the fact that IMV particles constitute the majority of progeny virions in most tissue culture systems, there are as yet no clues to the function of IMV in the virus life cycle and no evidence to demonstrate that the failure of these particles to mature as EV by acquiring a trans-Golgi-derived envelope is not a tissue culture artifact.

The intact A-type inclusion (ATI) protein of orthopoxviruses is approximately 160 kDa in size (10, 18). In cowpox virus (CPV)-infected cells the ATI protein forms large electron-dense cytoplasmic inclusions within which virus particles are occluded. Occlusion within the ATI does not occur by passive entrapment of virions within the matrix of the nascent ATIs,

because strains of CPV which form ATIs but do not occlude virions within them exist (28). Recombination of the VV 4c gene into an occlusion-negative strain of CPV renders the virus occlusion positive, indicating that the 4c gene product plays an essential role in this process (23).

In VV and a number of other orthopoxviruses, including monkeypox virus, camelpox virus, and variola virus (smallpox), the open reading frame (ORF) encoding the ATI protein is interrupted, resulting in the production of a truncated protein with a size of between 92 and 96 kDa depending on the virus (1, 6, 15). These truncated proteins do not form ATIs capable of occluding virus particles, and thus any function that requires occlusion cannot be manifested by these viruses. However, the conservation of production of the truncated protein, with the truncation occurring at similar positions in the ORF, over evolutionary time suggests an important function for the ATI protein which is independent of virus occlusion within intact ATI similar to those produced by CPV. In the study reported here we analyze the distribution of both the ATI and 4c proteins of VV on IMV and EV and describe experiments to examine the hypothesis that IMV particles are an end stage particle rather than an infectious precursor of EV. Data which suggest that production of mature EV particles takes precedence over production of IMV particles during replication are presented.

## MATERIALS AND METHODS

**Cell lines and viruses.** Monolayers of RK13 rabbit epithelial kidney and BSC40 African green monkey kidney epithelial cell lines were maintained in Eagle's minimal essential medium (MEM-E; Sigma, St. Louis, Mo.) supplemented with 10% heat-inactivated fetal bovine serum (Whittaker Bioproducts, Walkersville, Md.), 2 mM L-glutamine, and 50 mM gentamicin sulfate at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. The IHD-J, Copenhagen, and vRB10 strains of VV and the Brighton Red (BR) and occlusion-positive BR (BR<sup>+</sup>) strains of CPV were grown and purified as previously described (12), with the exception that for purification of EEV, EEV particles were collected by centrifugation of the clarified culture supernatant from infected cells prior to further purification procedures. Viruses were quantitated by plaque titration of virus suspensions on confluent monolayers of BSC40 cells. The BR and BR<sup>+</sup> strains of CPV were the generous gift of D. Pickup (Duke University, Durham, N.C.). The BR<sup>+</sup> strain is a recombinant derived by insertion of the 4c gene of VV strain WR into the thymidine kinase locus of BR (23). The P37 knockout virus vRB10 was the generous gift of B. Moss (National Institutes of Health). The vRB10 virus is a recombinant of the IHD-J strain of VV in which most of the coding sequence of the F13L ORF has been deleted (2).

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**Antisera.** Antisera to D8L and 4c were generated by immunization of rabbits with material isolated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels of purified VV particles. Antiserum reactive with the vaccinia and CPV ATI proteins was generated by immunization of a rabbit with the CPV ATI protein isolated from an SDS-PAGE gel of BR-infected BSC40 cells. Antiserum to P37 was generated to an N-terminally histidine-tagged P37 protein generated from a pET16b expression vector (Clontech) and purified on nickel-conjugated agarose beads (Ni-NTA; Quiagen). All antisera failed to recognize proteins in mock-infected cell extracts in Western blots (immunoblots). Antiserum to 4c has a slight cross-reactivity with the VV 4b protein (30a). For Western blotting procedures, this cross-reactivity was eliminated by preincubation of the antiserum with a nitrocellulose filter to which virus of the Copenhagen strain had been Western blotted. The Copenhagen strain of VV does not produce a 4c protein, and this procedure effectively eliminates all anti-4b cross-reactivity.

**Cesium chloride density gradients.** CsCl gradients were prepared with pre-mixed solutions with densities of 1.20, 1.25, and 1.30 g/ml. Each solution (3.5, 4.0, and 3.5 ml, respectively) was underlayered in a 12-ml Beckman ultraclear centrifuge tube. Virus suspensions were overlaid on the gradient in an 800- $\mu$ l volume, and the gradient was centrifuged in a Beckman SW41 rotor at 100,000  $\times g$  for 3 h at 15°C. After centrifugation, fractions were collected from the bottom of the tube under the control of a peristaltic pump (5 drops per fraction).

**Radiolabeling of VV- and CPV-infected cells.** For metabolic labeling of VV and CPV proteins, confluent monolayers of RK13 or BSC40 cells were infected at a multiplicity of infection (MOI) of 5 PFU per cell in 5-cm-diameter dishes. Cultures were maintained in MEM-E containing only 1.5 mg of methionine per liter and supplemented with 5% dialyzed fetal bovine serum and 15  $\mu$ Ci of [<sup>35</sup>S]methionine plus cysteine (EXPRE<sup>35</sup>S<sup>35</sup>S, 1,128 Ci/mmol; New England Nuclear) (denoted as [<sup>35</sup>S]Met hereafter). Cells were released from the monolayer with a rubber policeman. The cells were collected in the culture medium, pelleted by centrifugation at 16,000  $\times g$  for 1 min in a microcentrifuge, and resuspended in 20  $\mu$ l of 10% SDS. Samples were boiled for 5 min and then adjusted to a volume of 0.5 ml with SDS-free radioimmunoprecipitation assay (RIPA) buffer (1% [wt/vol] sodium deoxycholate, 1% [vol/vol] Triton X-100, 150 mM NaCl, 50 mM Tris-HCl [pH 7.4]).

For labeling of virions containing newly synthesized viral DNA, confluent monolayers were infected at an MOI of 5 in 5-cm-diameter tissue culture dishes. At 0 h postinfection (p.i.), 50  $\mu$ Ci of [methyl-<sup>3</sup>H]thymidine ([<sup>3</sup>H]TdR) was added to each dish. At 24 h p.i., cells were harvested in the original culture medium and lysed by Dounce homogenization. Nuclei were removed by centrifugation at 900  $\times g$  for 10 min at 4°C. Virus aggregates were dispersed by Duall homogenization, and virus was separated from the radiolabel-containing culture medium by centrifugation at 40,000  $\times g$  for 80 min over a cushion of 36% sucrose. Virus particles were collected from the pellet.

**Immunoprecipitation.** Immunoprecipitations were performed in 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, 0.2% (wt/vol) SDS, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.4) (RIPA). Samples were either resuspended in RIPA or adjusted to RIPA with a 10 $\times$  stock solution and supplemented with 10 U of benzoylase endonuclease (EM science, Gibbstown, N.J.) per ml. Samples were clarified by microcentrifugation at 16,000  $\times g$  for 1 min. Clarified samples were adjusted to between 5 and 50  $\mu$ l of specific rabbit antiserum per ml. For immunoprecipitation with anti-4c antiserum, the antibody was first added to an unlabeled extract of VV Copenhagen-infected cells in 0.5 ml of RIPA buffer to absorb nonspecific reactivity. For immunoprecipitation with anti-P37 antiserum, an unlabeled extract of vRB10-infected cells was used to absorb nonspecific reactivity. After vortexing and incubation on ice for 1 h, labeled samples were added to a final volume of 1 ml of RIPA buffer. Samples were vortexed briefly and incubated on ice for 2 h. After incubation on ice, 200  $\mu$ l of a 10% (vol/vol) suspension of washed, preswollen protein A-Sepharose beads (Sigma) was added to each reaction mixture. The samples were further incubated at 4°C for 18 h with constant agitation. The beads were washed three times with RIPA and finally resuspended in SDS-PAGE sample buffer.

**SDS-PAGE and fluorography.** Discontinuous SDS-PAGE was performed in 10% polyacrylamide gels under denaturing conditions by using a tricine-buffered gel system (26). For detection of radiolabeled proteins, gels were equilibrated with 22.2% 2,5-diphenyloxazole in dimethyl sulfoxide, dried, and exposed to Kodak Biomax MR X-ray film at -70°C (5).

**Western blotting.** Western blots were performed essentially by the method of Towbin et al. (30). Polyacrylamide gels from SDS-PAGE procedures were equilibrated in 25 mM Tris base-192 mM glycine-20% (vol/vol) methanol. Proteins were electroblotted from gels to nitrocellulose filters in the same buffer. Filters were washed with 20 mM Tris-500 mM NaCl (pH 7.5) (TBS), and excess binding capacity was adsorbed with the same buffer supplemented with 3% gelatin and 1% bovine serum albumin (BSA). Primary and secondary antibodies were applied in TBS supplemented with 0.05% (vol/vol) Tween 20 and 1% (wt/vol) gelatin. The secondary antibody was goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Bio-Rad) and was visualized by using a nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (BCIP) detection system (Bio-Rad).

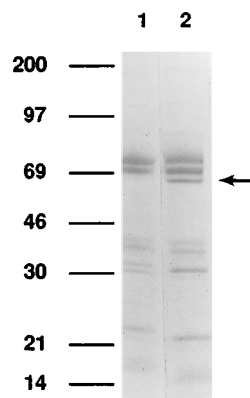


FIG. 1. SDS-PAGE analysis of CsCl-purified EEV and IMV virions of the IHD-J strain. Equivalent numbers of PFUs of EEV (lane 1) and IMV (lane 2) were subjected to electrophoresis in a 10% polyacrylamide gel under reducing conditions, and the gel was subsequently stained with Coomassie brilliant blue. A unique IMV band corresponding to the VV 4c protein is marked with an arrow. Lower-molecular-mass proteins with an apparent IMV or EEV restriction are in fact present in both lanes but in different abundances. Molecular masses (in kilodaltons) are indicated on the left.

## RESULTS

**SDS-PAGE and Western blot analyses of EV and IMV virions.** EEV and IMV particles were purified on CsCl gradients in which the two particles sediment differentially because of the extra membrane possessed by EV. Equivalent quantities of each form, as determined by plaque titration on BSC40 cell monolayers, were subjected to SDS-PAGE by the method of Schägger and von Jagow (26). The major virion proteins, 4a and 4b, were clearly seen in both IMV and EEV preparations (Fig. 1). In the IMV preparation a unique band migrating at approximately 59 kDa was also observed. From its position in the gel, this band is likely to be the 4c protein initially described by Sarov and Joklik (25). The restriction of the protein to IMV particles has not previously been described and was confirmed by Western blot analysis of IMV and EEV virions (Fig. 2). Only CsCl purified preparations of IMV were found to react with rabbit antiserum to the putative 4c protein, while in contrast, only EEV preparations were found to react with antiserum to the previously described EV-restricted protein P37. Both IMV and EEV preparations were recognized by antiserum to the D8L gene product, which is found on both IMV and EEV particles (14, 17). Because the VV 4c protein has been shown by other researchers to be essential for occlusion of CPV particles within CPV ATIs (23), parallel Western blots of VV IMV and EEV particles were probed with a rabbit antiserum reactive with the ATI proteins of both VV and CPV. The ATI protein of VV was detected only in association with IMV virions. In SDS-PAGE and Western blot analyses of VV IMV and EEV virions in which samples were boiled in reducing sample buffer for only 3 min, the antiserum to the ATI protein reacted with an additional protein with an approximate molecular mass of 190 kDa in IMV preparations only (data not shown). This additional band was not observed when samples were boiled for 5 rather than 3 min prior to SDS-PAGE, suggesting that the 190-kDa protein may represent a dimer of 92-kDa ATI proteins.

**Identity of the IMV-restricted putative 4c protein.** The 4c protein of VV was originally identified by SDS-PAGE analysis (25). Further studies on the mechanism of orthopoxvirus occlusion within CPV ATIs demonstrated a VV factor essential for the occlusion mechanism (28). Subsequently, a VV ORF

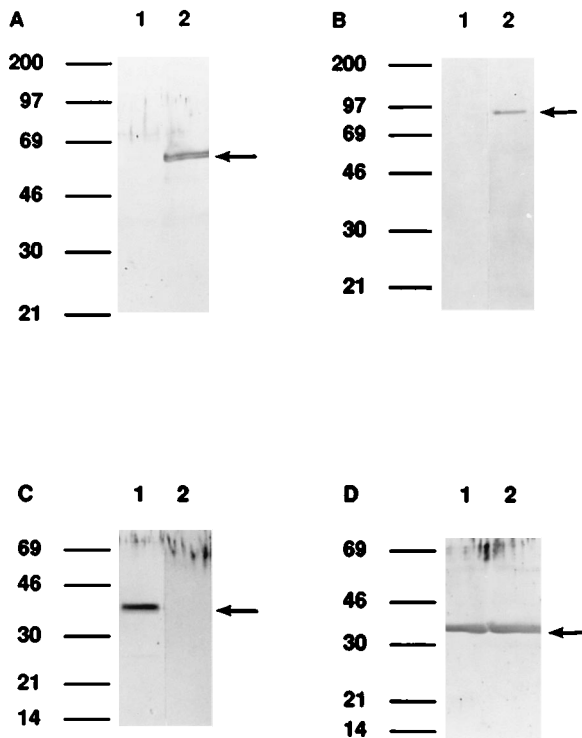


FIG. 2. Western blot analysis of CsCl-purified EEV and IMV virions. Equivalent numbers of PFUs of EEV (lane 1) and IMV (lane 2) were subjected to electrophoresis in parallel 10% polyacrylamide gels under reducing conditions, and the proteins were subsequently electroblotted to nitrocellulose filters by the method of Towbin et al. (30). Nitrocellulose filters were probed with antiserum to 4c (A), ATI protein (B), P37 (C), or D8L (D), as described in Materials and Methods. Molecular masses (in kilodaltons) are indicated on the left. Unique bands are indicated by arrows.

encoding a protein designated as 4c has been recombined into the genome of the occlusion-negative BR strain of CPV, rendering the virus occlusion positive (23). Immunoprecipitation analysis of the parental BR virus and the occlusion-positive recombinant demonstrates the presence of the IMV-restricted 4c protein in total cell extracts of the recombinant virus but not in extracts of the occlusion-negative parent (Fig. 3). This indicates that the IMV-restricted protein which we describe here is indeed the 4c protein that other researchers have discovered to be an essential factor involved in the occlusion mechanism.

**Kinetics of expression of 4c.** In order to further our understanding of how differentiation of nascent virions into IMV and EV is regulated, it is of interest to determine if maturation of IMV and EV occurs simultaneously or if the production of one or the other form is essentially complete before the onset of maturation of the second form. To this end we simultaneously examined the kinetics of production of 4c and the EV-restricted protein P37. VV-infected cultures were incubated with [<sup>35</sup>S]Met at various time periods p.i., and total cell extracts were subjected to immunoprecipitation with antisera to 4c and P37 (Fig. 4). The data show that P37 is detectable by this method at 4 to 6 h p.i., somewhat earlier in the infectious cycle than is 4c, which is first observed at 6 to 9 h p.i. In addition, while P37 appears to reach a maximal rate of synthesis by 9 to 12 h p.i., the apparent maximal rate of 4c synthesis is not reached until 18 to 21 h p.i.

**Kinetics of production of IMV and EV particles.** To determine if the earlier production of P37 relative to 4c is reflected

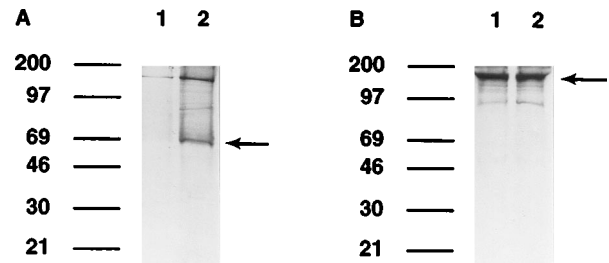


FIG. 3. Immunoprecipitation analysis of CPV strains BR and BR<sup>V+</sup>. Confluent monolayers of BSC40 cells in 5-cm-diameter petri dishes were infected with the BR strain of CPV (lane 1) or the occlusion-positive BR<sup>V+</sup> recombinant expressing the 4c gene of VV strain WR (lane 2) at an MOI of 5. At 21 h p.i. monolayers were released with a rubber policeman, and the cells were disrupted by boiling for 5 min in a 20- $\mu$ l volume of 10% SDS. Samples were then adjusted to a 1-ml volume of RIPA buffer. Each sample (900  $\mu$ l [A] and 100  $\mu$ l [B]) was subjected to immunoprecipitation with antiserum to 4c (A) and to the ATI protein (B), in a final volume of 1 ml. For 4c immunoprecipitation, film was exposed to the gel for 36 h. For ATI precipitation, film was exposed to the gel for 1 h. A background band at approximately 160 kDa in panel A is assumed to be coprecipitating ATI protein. Because of the excess of ATI protein over 4c in the infected cell at 24 h p.i., coprecipitating 4c is not anticipated to be visible in ATI immunoprecipitations in panel B. One representative of two experiments is shown. Molecular masses (in kilodaltons) are indicated on the left.

in earlier production of EV relative to IMV, the relative quantities of EV and IMV were determined in synchronously infected cultures at various times p.i. Cultures were incubated with [<sup>3</sup>H]TdR from 0 h p.i., and total progeny virions were

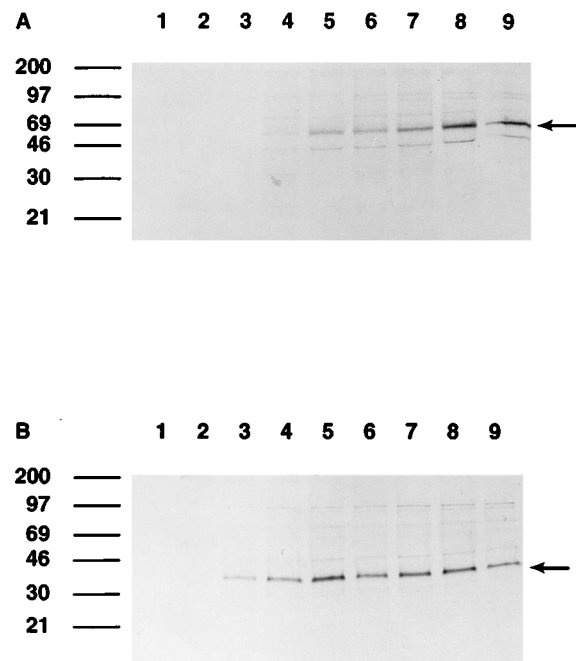


FIG. 4. Kinetics of expression of 4c and P37. Confluent monolayers of BSC40 cells in replicate 5-cm-diameter petri dishes were infected with VV-IHD-J at an MOI of 5. Cultures were radiolabeled with [<sup>35</sup>S]Met as described in Materials and Methods, for various time periods p.i. At the end of each labeling period, monolayers were released with a rubber policeman and cells were prepared for immunoprecipitation as described in the legend to Fig. 3. Each sample (500  $\mu$ l) was subjected to immunoprecipitation with antiserum to 4c (A) and to P37 (B). The labeling periods (in hours p.i.) were 0 to 1 (lane 1), 1 to 4 (lane 2), 4 to 6 (lane 3), 6 to 9 (lane 4), 9 to 12 (lane 5), 12 to 15 (lane 6), 15 to 18 (lane 7), 18 to 21 (lane 8), and 21 to 24 (lane 9). One representative of two experiments is shown. Molecular masses (in kilodaltons) are indicated on the left.

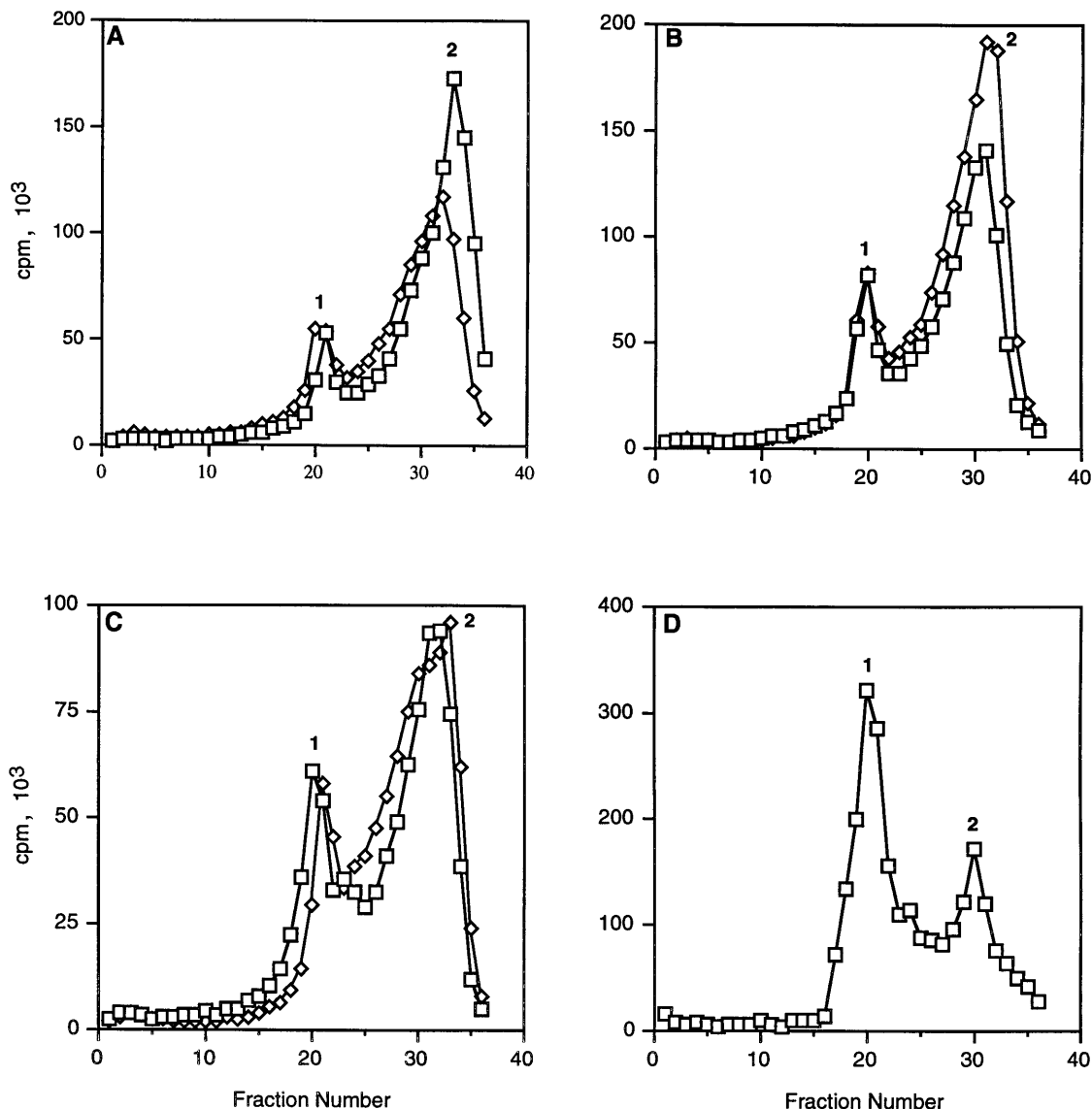


FIG. 5. Kinetics of EV and IMV production. Confluent monolayers of RK13 cells in replicate 5-cm-diameter petri dishes were infected with VV-IHD-J at an MOI of 5 and radiolabeled with [ $^3\text{H}$ ]TdR from 0 h p.i. At various times p.i., monolayers were released with a rubber policeman and cells were harvested in the culture medium. Samples were diluted to 6 ml with 100 mM Tris (pH 7.5), and the cells were disrupted by Dounce homogenization. A postnuclear supernatant was obtained by centrifugation at  $900 \times g$  for 10 min and was subjected to ultracentrifugation through a 6-ml cushion of 36% sucrose at  $40,000 \times g$  for 80 min. The pellet was disrupted by Duall homogenization and layered over a CsCl gradient as described in Materials and Methods. Gradient fractions were precipitated with trichloroacetic acid in the presence of 0.05% BSA and harvested onto glass fiber filters with a Skatron cell harvester prior to liquid scintillation counting. For the 24-h time point, radiolabeled virus from a 5-cm-diameter dish was pooled with unlabeled virus prepared and harvested in a similar fashion from  $5 \times 15$  cm petri dishes, to facilitate Western blot analysis of fractions containing peaks of radioactivity. Cultures were harvested at the following times (in hours) p.i.: (A) 10 ( $\square$ ) and 11 ( $\diamond$ ), (B) 12 ( $\square$ ) and 13 ( $\diamond$ ), (C) 14 ( $\square$ ) and 15 ( $\diamond$ ), and (D) 24 ( $\square$ ). 1, IMV containing fractions; 2, EV containing fractions. One representative of two experiments is shown.

ultracentrifuged through CsCl gradients. Because of the different ratios of protein to lipid, IMV and EV particles sediment with distinctly different densities in CsCl gradients. Fractionation of gradients and liquid-scintillation counting of fractions revealed that EV production predominates over IMV at earlier time points p.i. but that IMV predominates at very late times p.i. (Fig. 5). SDS-PAGE and Western blot analyses of peaks obtained from the 24-h time point demonstrate that the peaks of radioactivity correspond to points in the gradient where particles of exclusively IMV- or EV-density sediment (data not shown).

## DISCUSSION

In recent years information has emerged concerning VV virion proteins restricted to the EV form of the virus (2, 4, 8, 9, 11, 17, 19, 20, 31). These proteins have become markers for EV particles, and studies of their function are likely to yield further information about the life cycle and envelopment mechanisms of poxviruses. In contrast, there is little information concerning similar markers for IMV. The groundbreaking study by Payne (20) described IMV-restricted proteins with approximate molecular masses of 200, 95, 65, and 13 kDa. The relative quantities of these proteins in association with IMV

varied with the cell line used to grow the virus, and in one cell line they were found to be absent altogether. The identities of the proteins described by Payne (20) have not been determined; however, from the data presented here, it seems likely that the 95-kDa protein is the truncated ATI protein of VV (see Fig. 2). It is also likely that the 200-kDa protein described by Payne using a different SDS-PAGE system (20) is the 190-kDa protein recognized by antiserum to the ATI protein when samples were boiled in reducing sample buffer for only 3 rather than 5 min prior to SDS-PAGE and Western blot (data not shown). We assume that this protein represents a dimer of the ATI protein. We have not observed the 65-kDa protein shown by Payne (20) to migrate in SDS-PAGE with less mobility than that of the major core proteins 4a and 4b. The 4c protein which we describe here migrates with an apparent molecular mass of 59 kDa and is observed below 4a and 4b on SDS-PAGE gels (see Fig. 1). The demonstration that the antiserum raised to 4c is reactive with extracts of cells infected with an occlusion-positive recombinant of the BR strain of CPV, but not with the parental BR virus, indicates that the IMV-restricted protein which we have called 4c is encoded by the same VV ORF shown by other researchers to confer an occluding phenotype on the recombinant virus and also referred to as 4c (23).

It thus appears that the function of IMV particles is associated with occlusion in occluding members of the genus and with the ATI protein itself in nonoccluding members. It has been assumed that the function of intact ATIs is to protect virions from environmental degradation during transmission between hosts, in an analogous fashion to that observed in entomopoxviruses and baculoviruses (32; reviewed in reference 13). Clearly, the data presented here suggest that only IMV particles can be protected in this way, and that while the function of EV may be to disseminate an infection through the host, IMV may be the form that is responsible for transmission between hosts.

This hypothesis does not explain why the gene for the ATI protein is truncated in many orthopoxviruses or why the approximate point of truncation is conserved between virus species. The truncation preserves a region of the protein, at the carboxy-terminal portion of the truncated molecule, that is immunodominant for antibody responses in mice, suggesting that the molecule may have an immunomodulatory function (1, 6, 15). Given the likelihood that the common ancestor of orthopoxviruses produced intact ATIs, the ATI itself may have placed considerable constraints on the infectivity of IMV particles. The large size of ATIs leads to speculation that the primary target cell in a new host is a phagocytic cell such as a macrophage. Not only would a macrophage be capable of ingesting the ATI, but it is also likely to be capable of degrading the matrix of the ATI to release the occluded virions. Previous studies have shown that the VV 14-kDa structural antigen has fusogenic activity at low pH (7, 24), which would effectively provide the virus with an escape route from the low-pH late-endocytic or lysosomal compartments where ATI degradation would be most likely to occur.

If this hypothesis is correct, the lack of an intact ATI might impair the ability of IMV particles to infect phagocytic cells. However, if IMV particles display a protein to which the host directs a strong antibody response, we would expect phagocytosis to be enhanced by Fc receptor-mediated recognition of the antibody-coated virion. This fits well with data from other researchers showing that the ATI protein truncations in VV, variola virus, camelpox virus, and monkeypox virus preserve the immunodominant epitopes for the antibody response, found in the carboxy-terminal region of the truncated proteins, and that the ATI protein is an immunodominant antigen for

the antibody response during VV infection (1, 6, 15). Thus, the function of IMV may be associated with the initial infection event in a susceptible host. An alternative, but not exclusive, function may be facilitation of infection of immune hosts previously exposed to the virus.

From previously published data (22) and that shown in Fig. 5, it seems that EV production reaches a plateau before IMV production and that the bulk of IMV production is delayed until after that point. This suggests that EV production is limited by an as-yet-unknown mechanism, possibly involving depletion of membranes required for envelopment (22) and that IMV is simply what is left. However, the demonstration that IMV particles possess unique viral proteins not found on EV suggests that, in fact, IMV is a differentiated form rather than an unused precursor. The first detectable signal for 4c production at 6 to 9 h p.i., later than the first such signal for P37 at 4 to 6 h p.i. (Fig. 4), coupled with the apparent precedence of EV production in the earlier stages of differentiation and the observation that IMV production reaches a plateau concomitantly with high level synthesis of 4c raises the possibility that the onset of 4c production may act as a differentiation switch that facilitates maturation of nascent virions as IMV, especially as the production of 4c is not maximal until after 18 h p.i. (Fig. 4). Further experimentation will be required to determine if this is the case. The continued production of both 4c and P37 until very late times p.i. suggests that at these times production of IMV and EV may occur simultaneously. Thus it appears that VV ensures that both IMV and EV forms are produced during an infection but that the first virions to fully mature do so as EV. It is not clear what advantage is gained from the precedence of EV production in the early stages of maturation; however, this may be a mechanism to facilitate rapid dissemination of the virus after the initial infection event.

The data presented in Fig. 4 and 5 do not demonstrate that the onset of production of 4c is a trigger for virions to mature as IMV. However, the hypothesis is supported by the observation that at 24 h p.i., the ratio of IMV/EV in IHD-J-infected cultures is 2.0, while in cultures infected with the Copenhagen strain, which does not produce 4c, the ratio is 0.63, suggesting that the Copenhagen strain has reduced ability to produce IMV progeny (30a). Reduced production of IMV would not be expected to affect the ability of the Copenhagen strain to replicate in tissue culture cells or to infect naive animals, as the EV form of the virus should be capable of accomplishing this. The effect of 4c deletion on IMV production and infectivity in naive and immune animals would be best studied in a 4c-knockout virus, in which the absence of 4c is the only difference between parental and recombinant viruses. This issue is under further investigation.

In summary, we present data identifying two VV proteins, 4c and the ATI protein, as being unique to the IMV form of the virus. The proteins are not found in association with EEV particles, indicating that IMV particles are not infectious precursors of EV but are, in fact, mature, differentiated particles destined not to acquire a Golgi-derived envelope. The identification of the ATI protein of VV as IMV restricted suggests that the function of IMV is intimately associated with that protein in orthopoxviruses which do not produce intact ATIs as well as in those that do, and we speculate that the role(s) of IMV relates to establishment of an initial infection at the portal of entry and/or establishment of infection in an immune host. Furthermore, we present data suggesting the 4c protein as a candidate for a differentiation switch, facilitating maturation of virions as IMV rather than EV particles.

## ACKNOWLEDGMENTS

We thank D. Pickup for the gift of CPV strains BR and BR<sup>v+</sup> and for permission to quote his unpublished data concerning the occlusion-positive phenotype of BR<sup>v+</sup>. We also thank B. Moss for the gift of the P37 knockout virus vRB10.

This work was supported by grant AI 20563 from the National Institutes of Health.

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