JOURNAL OF VIROLOGY, Nov. 1991, p. 5910–5920
0022-538X/91/115910-11$02.00/0

Vol. 65, No. 11

Extracellular Vaccinia Virus Formation and Cell-to-Cell Virus Transmission Are Prevented by Deletion of the Gene Encoding the 37,000-Dalton Outer Envelope Protein

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Received 21 May 1991/Accepted 29 July 1991

There are two types of infectious vaccinia virus particles: intracellular naked virions and extracellular enveloped virions (EEV). To determine the biological role of the enveloped form of vaccinia virus, we produced and characterized a mutant that is defective in EEV formation. The strategy involved replacement by homologous recombination of the gene F13L, encoding a 37,000-Da protein (VP37) that is specific for the outer envelope of EEV, with a selectable antibiotic resistance marker, the *Escherichia coli gpt* gene. Initial experiments, however, suggested that such a mutation was lethal or prevented plaque formation. By employing a protocol consisting of high-multiplicity passages of intracellular virus from the transfected cells and then limiting dilution cloning, we succeeded in isolating the desired mutant, which was defective in production of plaques and extracellular virus but made normal amounts of intracellular naked virions. Electron microscopic examination indicated that the mutant virus particles, unlike wild type, were neither wrapped with Golgi-derived membranes nor associated with the cell surface. The absence of VP37 did not prevent the transport of the viral hemagglutinin to the plasma membrane but nevertheless abrogated both low-pH- and antibody-mediated cell fusion. These results indicate that VP37 is required for EEV formation and also plays a critical role in the local cell-to-cell transmission of vaccinia virus, perhaps via enveloped virions attached to or released from the cell membrane. By contrast, a mutated virus with a deletion of the K4L open reading frame, which is a homolog of the VP37 gene, was not defective in formation of plaques or EEV.

Vaccinia virus, the representative member of the *Poxviridae*, has a complex replicative cycle that occurs in the cytoplasm of infected cells (reviewed in reference 20). Late stages of virus assembly have been visualized by electron microscopy (19), but the biochemical mechanisms and protein interactions involved in the process are not understood. Two related but morphologically and antigenically distinct forms of infectious vaccinia virions have been recognized (2): intracellular naked virions (INV) and extracellular enveloped virions (EEV). The INV, which contain a complex core structure surrounded by a lipoprotein membrane, are more abundant and have been more thoroughly studied than the EEV, which have an additional envelope. The process leading to virus release involves wrapping of mature INV particles by two layers of Golgi-derived membranes and the subsequent fusion of the outer layer with the plasma membrane (15, 19, 25). The major protein in the external envelope of EEV, VP37, has an apparent molecular mass of 37,000 Da (11, 22) and is encoded by vaccinia virus open reading frame F13L (13). This protein contains bound palmitic acid and is localized in Golgi-derived membranes (12).

EEV may be important in animal infections since antisera to inactivated INV do not neutralize EEV and fail to protect against a live virus challenge (1, 23, 33). Conversely, antisera that neutralize EEV can protect animals from experimental infections. Antibody specific to EEV also inhibits the formation of comets in plaque assays of virus with a liquid overlay (2, 4). The comets are tails composed of small secondary plaques derived from the larger primary plaques. The ability of anti-EEV antiserum to inhibit comet formation indicates that long-distance spread of infectious virus in vitro is mediated by EEV. However, since INV are highly infectious, it seemed that isolation of virus mutants unable to generate extracellular virus would be possible. In this report, we describe the construction and characteristics of such a mutant.

MATERIALS AND METHODS

Cells and viruses. The IHD-J and IHD-W strains of vaccinia virus were kindly provided by S. Dales, University of Western Ontario, London, Ontario, Canada, and were routinely propagated and titered in green monkey kidney BSC-1 cells grown in minimal essential medium supplemented with 10% fetal calf serum. The plaque titers were determined after 2 days by staining with crystal violet. vRB10 mutant virus was also grown and titered in BSC-1 monolayers that were incubated for 5 to 7 days before staining.

Antibodies. Monoclonal antibody MAbC3 (27), specific for the vaccinia virus 14,000-Da protein, and monoclonal antibody B2D10, specific for the virus hemagglutinin (HA) (16), were kindly provided by M. Esteban, State University of New York Health Science Center, Brooklyn, and Y. Ichihashi, Niigata University, Asahimachi, Japan, respectively. Fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G for indirect immunofluorescence was obtained from Calbiochem Corp.

Plasmids. HA#9, a plasmid containing a *Bam*HI fragment derived from the vaccinia virus *Hin*dIII A fragment, was provided by N. Cole of this laboratory. HFR7 was obtained by cloning of a 7-kbp *Eco*RI subfragment of the vaccinia virus *Hin*dIII F fragment.

A plasmid containing the *Escherichia coli* gene encoding

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xanthine guanine phosphoribosyl transferase (*gpt*) under the control of the vaccinia virus 7.5 promoter and with suitable restriction sites for the cloning of vaccinia virus sequences to direct insertion of the *gpt* cassette into the vaccinia genome is described elsewhere (3). Sequences flanking genes F13L and K4L were amplified by polymerase chain reaction. The primers for the left flank of F13L were 5′-GCATATGCATGCTTTGTTAAAATAGATA-3′ (*Sph*I site underlined) and 5′-CATTTTGCTCGAGCAGGTACCGATGCAA-3′ (*Xho*I site underlined). The primers for the right flank of F13L were 5′-GAGAGAGCTCGGGTATCTAGCCACAGTA-3′ (*Sac*I site underlined) and 5′-GCGCCCACAATGCATCTCTAGATATGTA-3′ (*Nsi*I site underlined). The primers for the left flank of K4L were 5′-TGAGTGCATGCTGTATGTTCAATCTGGA-3′ (*Sph*I site underlined) and 5′-GCATACCTCGAGGAATAGTCTCTGTAATCA-3′ (*Xho*I site underlined). The primers for the right flank of K4L were 5′-CTGCGATGAGCTCCAACCGGAAAAAGAAA-3′ (*Sac*I site underlined) and 5′-TTGTCGAGCTCATTAGAATCTACTACATTA-3′ (*Sac*I site underlined). After polymerase chain reaction amplification, the flanking gene sequences were inserted into unique restriction sites in the plasmid pGEM-gpt.

Isolation of recombinant viruses. Monolayers of CV-1 cells were infected with vaccinia virus IHD-J at a multiplicity of infection (MOI) of 0.05 and transfected with plasmids by the calcium phosphate technique, following standard protocols. Recombinant viruses were selected by three consecutive rounds of plaque purification in the presence of mycophenolic acid (9). Subsequently, viruses were amplified by infection of cell monolayers, and the DNA was analyzed by Southern blot hybridization.

In the isolation of the deletion mutant vRB10, high-MOI passages were achieved by infecting BSC-1 cells in a 9-cm^2 well with 200 μl of a crude IHD-J virus stock. At 48 h after infection, the cells were harvested, freeze-thawed three times to release the virus, and sonicated. A limit dilution cloning procedure was performed from this virus stock as follows. Serial dilutions (10-fold) were used to infect BSC-1 cells in 96-well trays. Twelve wells were infected with each dilution and were observed at 48 h after infection for the presence or absence of virus plaques. Wells with no virus plaques were selected, and the cells were scraped, freeze-thawed three times, and sonicated. Two-thirds of the material obtained from each well was used to infect BSC-1 cells in 24-well plates. At 48 h, the monolayers showing cytopathic effect were selected, and virus stocks were grown by standard procedures.

Virus labeling. Monolayers of RK13 cells in six-well plates were infected at an MOI of 10. After 7 h, medium was replaced with 1 ml of methionine-free minimal essential medium supplemented with 25 μCi of [^{35}S]methionine. At 24 h after infection, 0.5 ml of complete medium with 5% fetal calf serum was added, and the incubation continued for another 24 h. Finally, the extracellular and cell-associated virus fractions were separated and the virus was pelleted through a 25% sucrose cushion. Buoyant density centrifugation was performed as described by Payne and Norrby (26).

Virus neutralization assays. Approximately 10^4 PFU of intra- or extracellular virus in 0.1 ml of minimal essential medium were incubated for 90 min at 37°C in the presence or absence of monoclonal antibody MAbC3 at a 1:10,000 dilution. Subsequently, the virus was diluted, and the titer was determined by plaquing in BSC-1 cell monolayers.

Electron microscopy. Monolayers of RK13 cells in 150-cm^2 flasks were infected with virus at an MOI of 10. At 16 h postinfection, the culture medium was removed and the cells were fixed by adding a solution of 2.5% glutaraldehyde in 0.13 M sodium phosphate (pH 7.4) directly to the monolayers. After 2 h of fixation, the cells were scraped and pelleted by centrifugation. Samples were postfixed in 1% osmium tetroxide in phosphate buffer and in-block stained with uranyl acetate. After dehydration, samples were embedded in Spurr's resin, cut to thin sections (about 70 nm thick), stained with lead citrate, and examined under the electron microscope.

Cell-cell fusion. Cell fusion experiments were done essentially as described previously (8). Briefly, confluent monolayers of BSC-1 cells in six-well plates were infected with vaccinia virus at an MOI of 10. After a 1-h adsorption, the virus inoculum was removed and fresh medium with or without monoclonal antibody B2D10 was added to the cell monolayers. At 12 h postinfection, the monolayers were washed with phosphate-buffered saline (PBS) and treated for 2 min at 37°C with fusion buffer [PBS with 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.4 or 5.5]. Subsequently, the fusion buffer was replaced with normal fresh medium, and the cells were then incubated at 37°C for 3 h before observation under the phase-contrast microscope. Cells treated with the antibody B2D10 were photographed at 20 h postinfection.

Immunofluorescence. BSC-1 cells grown on coverslips were infected at an MOI of 10 PFU per cell. At 18 h after infection, cells were washed twice with PBS and fixed by treatment with 3.7% paraformaldehyde for 10 min at room temperature. When permeabilization was desired, the fixed cells were incubated in PBS containing 0.1% Triton X-100 for 5 min at room temperature. The cells were washed three times in PBS and incubated for 1 h in monoclonal antibody B2D10. After being washed twice with PBS, cells were incubated with rabbit anti-mouse immunoglobulin G for 1 h, washed three times, dried, mounted, and observed in the fluorescence microscope.

RESULTS

Isolation of F13L and K4L deletion mutants. Since INV, the intracellular form of vaccinia virions, are infectious, we considered that it might be possible to isolate replication-competent virus mutants unable to produce EEV, the extracellular form. To test this hypothesis, we chose to delete the gene F13L encoding the outer envelope major protein VP37. The K4L gene also was selected for deletion because of its considerable sequence homology with F13L (5). The strategy followed was to replace most of the coding sequences of those genes with a selectable antibiotic marker, namely, the *E. coli gpt* gene. Plasmids suited to drive that process through homologous recombination were constructed by flanking the *gpt* gene of pGEM-gpt with sequences from the two ends of either F13L of K4L. Cells infected with the IHD-J strain of vaccinia virus, chosen because it produces large amounts of EEV, were transfected with the recombinant plasmids. The expected single- and double-crossover homologous recombination events are depicted in Fig. 1. Since single-crossover recombination intermediates contain tandemly repeated sequences, they are very unstable, giving rise via a second crossover event to antibiotic-sensitive wild-type and antibiotic-resistant stable mutant viruses (32) (Fig. 1). Therefore, plaques containing exclusively double-crossover recombinant viruses usually predominate after one or two successive plaque purifications under selection

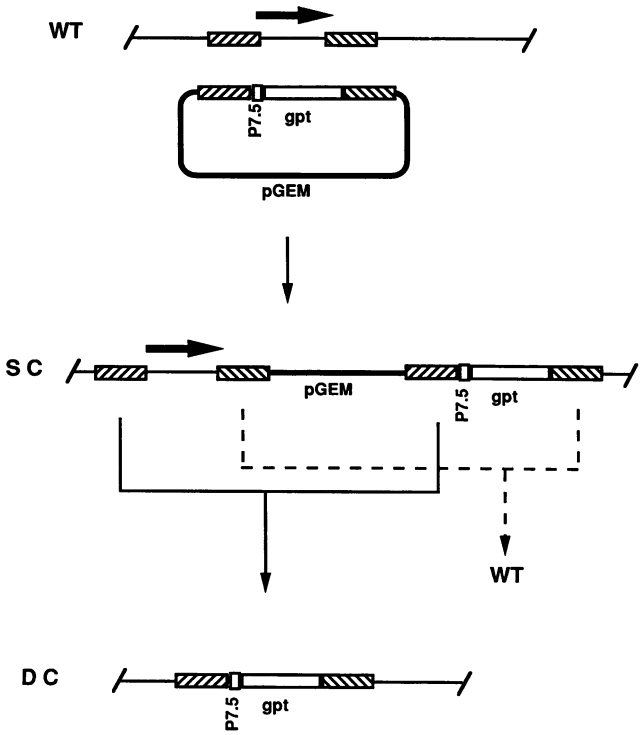

FIG. 1. Recombination processes leading to site-directed deletions in the vaccinia genome. WT, wild-type vaccinia virus. SC and DC, single and double recombinational crossover events, respectively. The thick black arrows represent the gene to be deleted in the vaccinia genome. P7.5 is an early-late vaccinia virus promoter. gpt designates the *E. coli* xanthine guanine phosphoribosyl transferase gene, and pGEM refers to the parental plasmid.

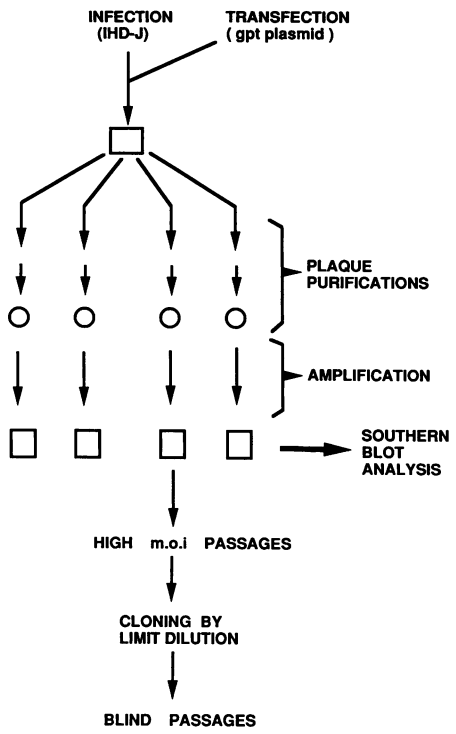

FIG. 2. Procedure to isolate a VP37$^-$ deletion mutant unable to form virus plaques. All steps from plaque purification onward were done in mycophenolic acid-containing selective medium.

conditions. To test whether F13L and K4L deletion mutants were obtained, the progeny virus was plaque purified three times in selection medium and then the DNA samples from several amplified plaques (Fig. 2, upper part) were analyzed by restriction enzyme digestion and Southern blotting. The results indicated that the K4L gene had been successfully deleted from the viral genome of several clones (data not shown). However, there were no plaque isolates that lacked the F13L gene. Instead, the Southern blot analysis revealed the presence of a 3.7-kbp band, which is diagnostic of the single-crossover virus, as well as 1.9- and 2.6-kbp bands (Fig. 3, SC).

The failure to isolate plaques containing only double-crossover recombinant virus usually signifies that the target gene is essential for virus replication. Strictly speaking, however, such a result only indicates that the desired deletion mutant cannot form plaques. Since the VP37 protein is present in the EEV but not in the INV, we originally thought that the F13L deletion mutant might be impaired in EEV formation and therefore might form plaques without comets. However, the lack of success in isolating the mutant by standard means raised the possibility of a nonplaquing phenotype. Therefore, the mutant isolation protocol was altered as shown in the lower part of Fig. 2. The cell-associated virus was freeze-thawed, sonicated, and passaged repeatedly at high MOI under selection conditions to allow the putative double-crossover mutant virus to become more abundant in the progeny. The purpose of using a high MOI was to diminish if not eliminate any advantage resulting from spontaneous virus spread that the single-crossover virus (which still has an intact F13L gene) would have over the double-crossover virus. The next steps consisted of cloning the mutant virus by a limiting dilution technique. All cultures containing virus plaques were discarded, and the remaining wells were harvested and freeze-thawed to release any intracellular virus, which was used to infect a fresh monolayer of cells. The harvesting and blind passage were repeated, and after the second passage, cytopathic effects were discernible in some wells. Further passages were done to generate large virus stocks. Analysis of the viral DNA by restriction endonuclease analysis and Southern blotting showed that the virus clones were double recombinants and lacked a complete F13L gene (Fig. 3, lane C). The IHD-J strain deletion mutant was code-named vRB10 but will be referred to here as VP37$^-$ to signify the protein that is no longer made. The K4L deletion mutant was named vRB11 and is referred to as I-K4L since the gene product has not yet been characterized.

Plaque formation by mutant viruses. The VP37$^-$ deletion mutant is unable to form plaques under standard conditions, which is 2 days at 37°C. Nevertheless, small distinct plaques could be detected with prolonged incubation. After 1 week, the mutant plaques were comparable in size to those made in 1 day by wild-type virus and considerably smaller than the standard 2-day plaques (Fig. 4). The presence of a VP37$^-$ mutant-infected cell could be discerned in 48 h, however, as an area of high cell density. This proliferation probably resulted from the secretion of the vaccinia virus growth factor, which stimulates the growth of the surrounding uninfected cells (6). This idea was corroborated by the formation of even more distinct foci of cell growth in A431 cells, which are highly responsive to both epidermal growth factor and vaccinia virus growth factor (6).

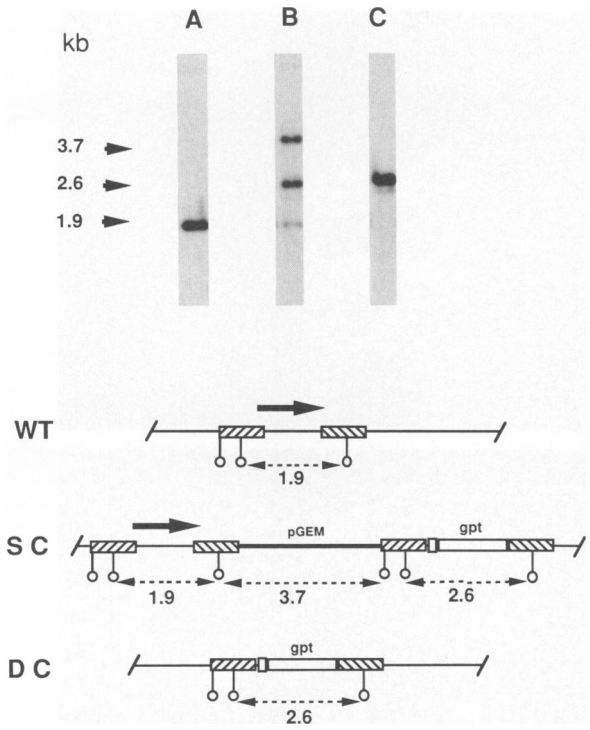

FIG. 3. Analysis of F13L gene recombinant virus. Upper part, Southern blot analysis of *Cla*I fragments derived from DNA of wild-type IHD-J virus (lane A), a virus stock derived from a plaque-purified mycophenolic acid-resistant virus (lane B), and the F13L deletion mutant (lane C). DNA fragment sizes, determined by electrophoresis of standards on the same gel, are indicated on the left. Lower part, schematics showing fragments in kilobase pairs generated by *Cla*I digestion (⏚) of DNA from wild-type (WT) and single (SC)- or double (DC)-crossover recombinant viral genomes. Other abbreviations are as defined in the legend to Fig. 1.

Marker rescue was performed to confirm that the defect in plaque formation by the VP37$^-$ mutant resulted exclusively from deletion of the F13L gene. Thus, plaque-forming virus was derived by transfecting plasmid HFR7 containing the F13L gene into cells infected with VP37$^-$. Controls showed that no virus plaques were detected if the cells were mock transfected or if plasmid HA#9 containing a different DNA fragment was used (Table 1). Marker rescue was then repeated with similar positive results by using smaller DNA fragments, derived by polymerase chain reaction, which contained only the F13L gene (data not shown).

In contrast to the VP37$^-$ mutant, the K4L gene deletion mutant produced plaques that were indistinguishable from those of the parental wild-type IHD-J virus (data not shown). These results indicate that VP37, but not the homologous product of gene K4L, is required for efficient cell-to-cell transmission of vaccinia virus IHD-J.

Formation of extracellular virus. Since VP37 is part of the EEV outer envelope, we considered that there might be a specific defect in formation of extracellular virus. We therefore compared the amounts of infectious intra- and extracellular virus produced by mutant and wild-type viruses in RK13 cell monolayers. Both mutants produced amounts of cell-associated virus which were comparable in infectivity to those produced by the parent IHD-J virus. In contrast, there was a 20- to 60-fold difference in the amount of infectious virus recovered from the culture medium of cells infected with wild-type and the VP37$^-$ mutant virus (Fig. 5). On the other hand, there was no significant difference in the amounts of extracellular virus produced by the K4L mutant and the parent virus (Fig. 5).

The marked decrease in infectious virus in the medium of cells infected with the VP37$^-$ mutant could reflect either a block in formation of EEV or the release of defective particles. To discriminate between these possibilities, the infected cells were labeled with [^{35}S]methionine and the medium was subjected to cesium chloride gradient centrifugation. The profiles obtained here (Fig. 6) showed a drastic decrease in EEV particles in the medium of mutant-infected cells that was comparable to that obtained by measuring infectivity. A small number of labeled virus particles were always found in the medium of VP37$^-$ mutant-infected cells, however, and some of those banded at the density of EEV. This result suggests that at least a portion of the mutant extracellular virus contains an additional wrapping membrane as does wild-type IHD-J virus. The quantity of naked virions in the medium did not increase significantly even after 72 h postinfection, suggesting that spontaneous cell disruption was not a major factor in release of VP37$^-$ virus. In contrast to the differences in extracellular virus, similar amounts of labeled virus particles were recovered from the mutant and wild-type virus-infected cells (data not shown).

Neutralization of intra- and extracellular VP37$^-$ virus. To further characterize the intra- and extracellular virus particles made by the VP37$^-$ mutant, we determined their interaction with monoclonal antibody MAbC3, which is directed to the 14-kDa INV membrane protein (27). As expected, extracellular IHD-J virus was much more resistant than intracellular IHD-J virus to neutralization. Comparable results were obtained with the small amounts of extracellular VP37$^-$ mutant virus (Fig. 7), consistent with its sedimentation density in CsCl and suggesting that it is membrane wrapped as is normal EEV and consequently not derived by cell lysis.

Electron microscopy of VP37$^-$-infected cells. To further investigate the defect in VP37$^-$ virus formation, we prepared thin sections of infected RK13 cells and examined them under the electron microscope. The cells were fixed at 16 h postinfection, when mature and immature virus particles are numerous. Immature virus particles, as well as mature INV particles, were present in similar amounts and were morphologically indistinguishable in IHD-J- and VP37$^-$-infected cells. Mature VP37$^-$ intracellular virions were dispersed in the cytoplasm, indicating that mutant progeny virus was still able to migrate from the viral factories toward the cell periphery (Fig. 8). The most striking difference was the presence of numerous IHD-J particles in association with the plasma membrane and the virtual absence of such particles in cells infected with the VP37$^-$ mutant (Fig. 8). Higher magnifications also showed a difference in the association of virions with intracellular Golgi membranes; we could not detect wrapped virions in VP37$^-$-infected cells (Fig. 9A), whereas they were easily detected in IHD-J-infected cells (Fig. 9B to E).

Fusion from within. We tested the ability of the VP37$^-$ mutant to induce cell fusion activity in the membrane of infected cells. Polykaryons form when the pH of vaccinia virus-infected cells is lowered (8, 10, 18). Cell fusion was induced in IHD-J-infected cells by treatment with pH 5.5 buffer (Fig. 10C), whereas no fusion was induced by incubation at pH 7.4 (Fig. 10A). When VP37$^-$ mutant-infected cells were subjected to either treatment, no fusion could be detected (Fig. 10B and D). Cell fusion can also be induced

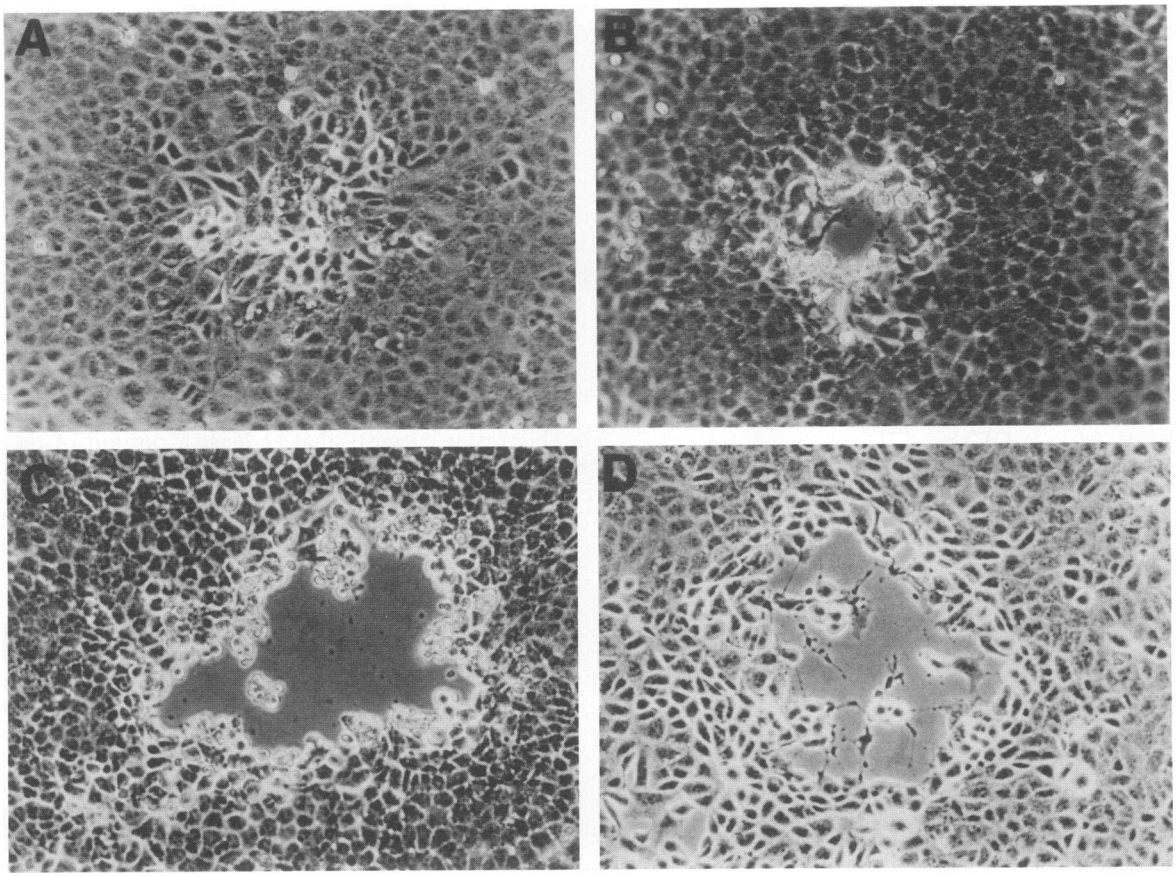

FIG. 4. Plaque formation by VP37⁻ virus. Monolayers of BSC-1 cells infected with VP37⁻ virus were infected and photographed at 2 days (A), 4 days (B), and 7 days (C) postinfection. A plaque of wild-type virus at 1 day postinfection (D) is shown for comparison.

with monoclonal antibody B2D10, specific for the virus HA (30). We found this fusion to occur more slowly than low-pH-induced fusion, but it could still be easily detected in IHD-J-infected cells (Fig. 10E). When VP37⁻ mutant-infected cells were incubated in the presence of the antibody, no fusion was induced (Fig. 10F). This difference was not a matter of timing, since no cell fusion was detected after prolonged incubation (up to 48 h) with the antibody, at which time IHD-J-infected cells had formed large syncytia (data not shown).

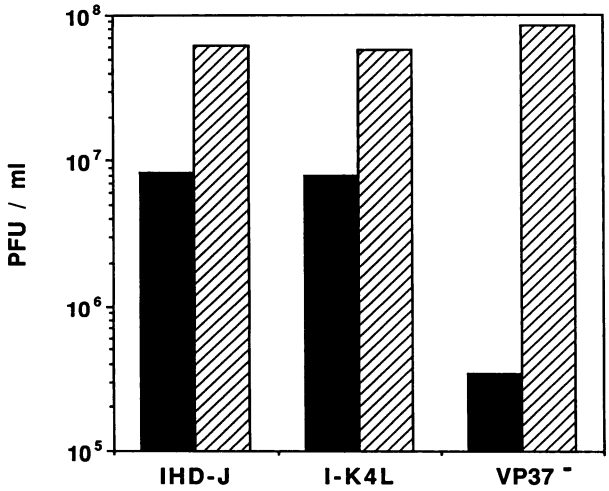

FIG. 5. Extracellular virus production by K4L and F13L mutant viruses. Plaque titers are shown for virus free in the extracellular culture medium (black bars) and for cell-associated virus (hatched bars).

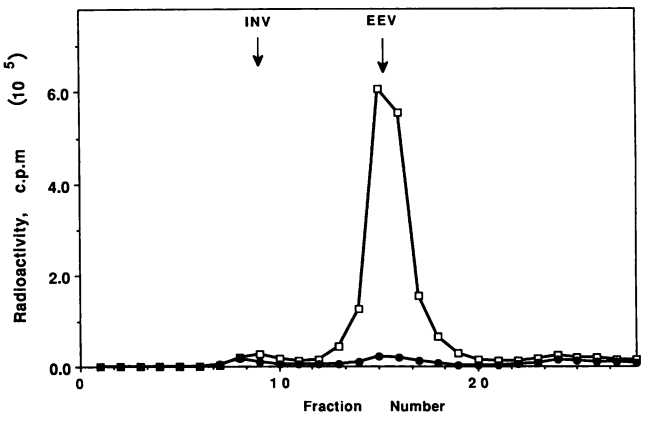

FIG. 6. CsCl centrifugation of extracellular virus. Virus in the medium of infected RK13 cells was subjected to centrifugation in CsCl gradients as described in Materials and Methods. After centrifugation, fractions were collected from the bottom of the tube. □, IHD-J virus; ●, VP37⁻ virus.

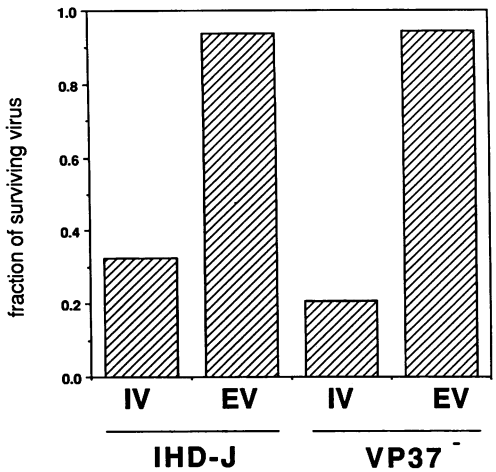

FIG. 7. Neutralization of VP37⁻ virus. Plaque titers were determined after incubation of intracellular (IV) or extracellular (EV) parental IHD-J or mutant VP37⁻ virus with or without monoclonal antibody MAbC3. The ratios of plaque titers (with MAbC3/without MAbC3) are presented as the fractions of surviving virus.

Localization of viral HA in plasma membrane. The lack of cell fusion activity in the membranes of VP37⁻ virus-infected cells can be explained if the proteins involved in the fusion activity were not transported properly to the plasma membrane. Accordingly, the failure of monoclonal antibody B2D10 to induce cell fusion could correlate with the absence of HA molecules in the surface of VP37⁻-infected cells. This was not the case, however, since the presence of cell surface HA in unpermeabilized mutant-infected cells was clearly seen by immunofluorescence with monoclonal antibody B2D10 (Fig. 11). As controls, the antibody stained the surface of unpermeabilized IHD-J-infected cells but not the surface of unpermeabilized IHD-W-infected cells, which have a mutated version of the HA that is not transported to the cell membrane (31) (Fig. 11C and D).

TABLE 1. Marker rescue of the VP37⁻ mutation

Transfected DNA	Wild-type titer (PFU/ml) (% of total virus)[a]	
	Cells	Medium
None	$<10^4$ (<0.01)	<10 (<0.01)
HA#9	$<10^4$ (<0.01)	<10 (<0.01)
HFR7	8×10^5 (1.5)	2.3×10^4 (7.7)

[a] Mutant virus titer was determined by counting microplaques that formed in 7 days. Wild-type virus titer was determined by counting standard-size plaques that formed in 2 days.

DISCUSSION

We isolated a novel deletion mutant of vaccinia virus that makes normal amounts of infectious INV but is defective in production of EEV. The mutant was produced by replacement of the gene encoding VP37, the major protein component of the viral outer envelope, with a selectable marker. The mutant also was defective in plaque formation, which necessitated an isolation protocol involving high-multiplicity passages of intracellular virus and limiting dilution cloning. Marker rescue confirmed that the defective plaque phenotype was due solely to deletion of the one gene.

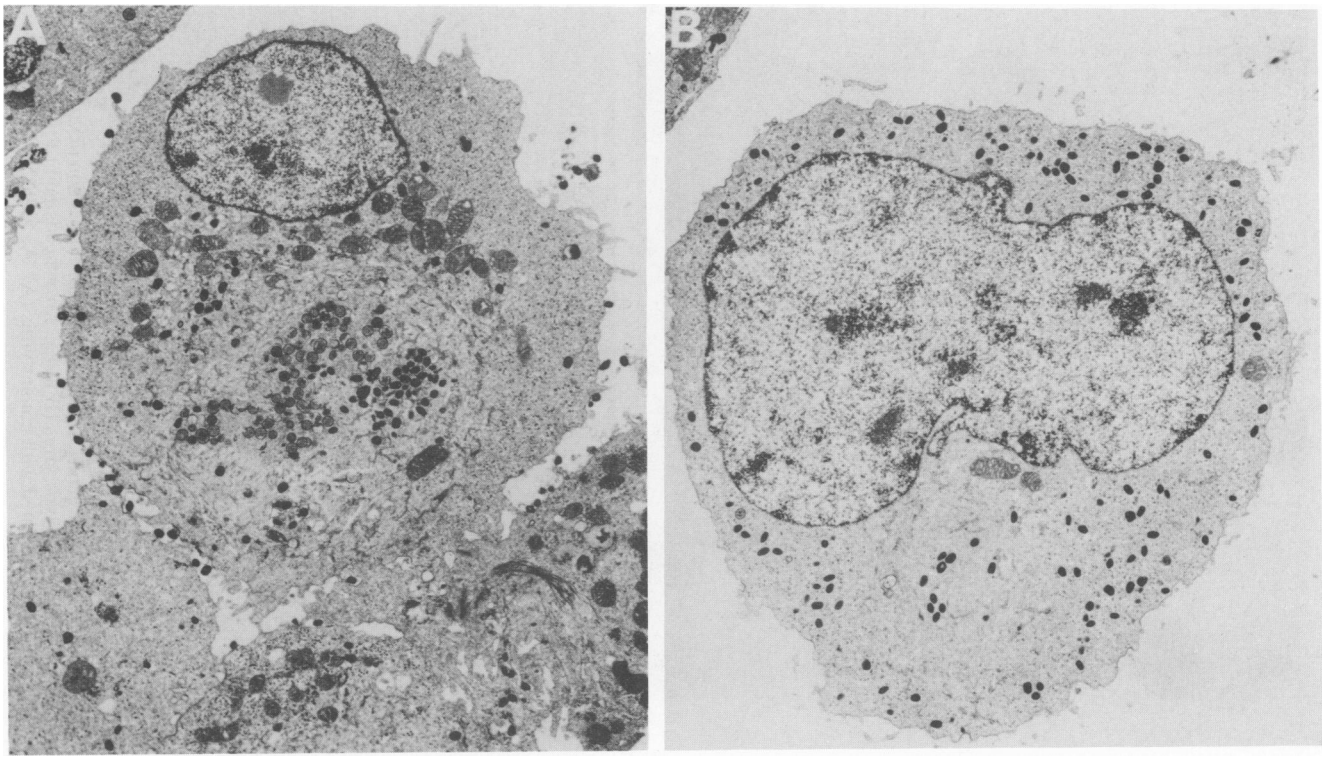

FIG. 8. Electron microscopy. Thin sections of RK13 cells infected with IHD-J (A) or the VP37⁻ mutant virus (B) are shown. Note the absence of virions at the cell surface in cells infected with the mutant. Magnification, ×5,280.

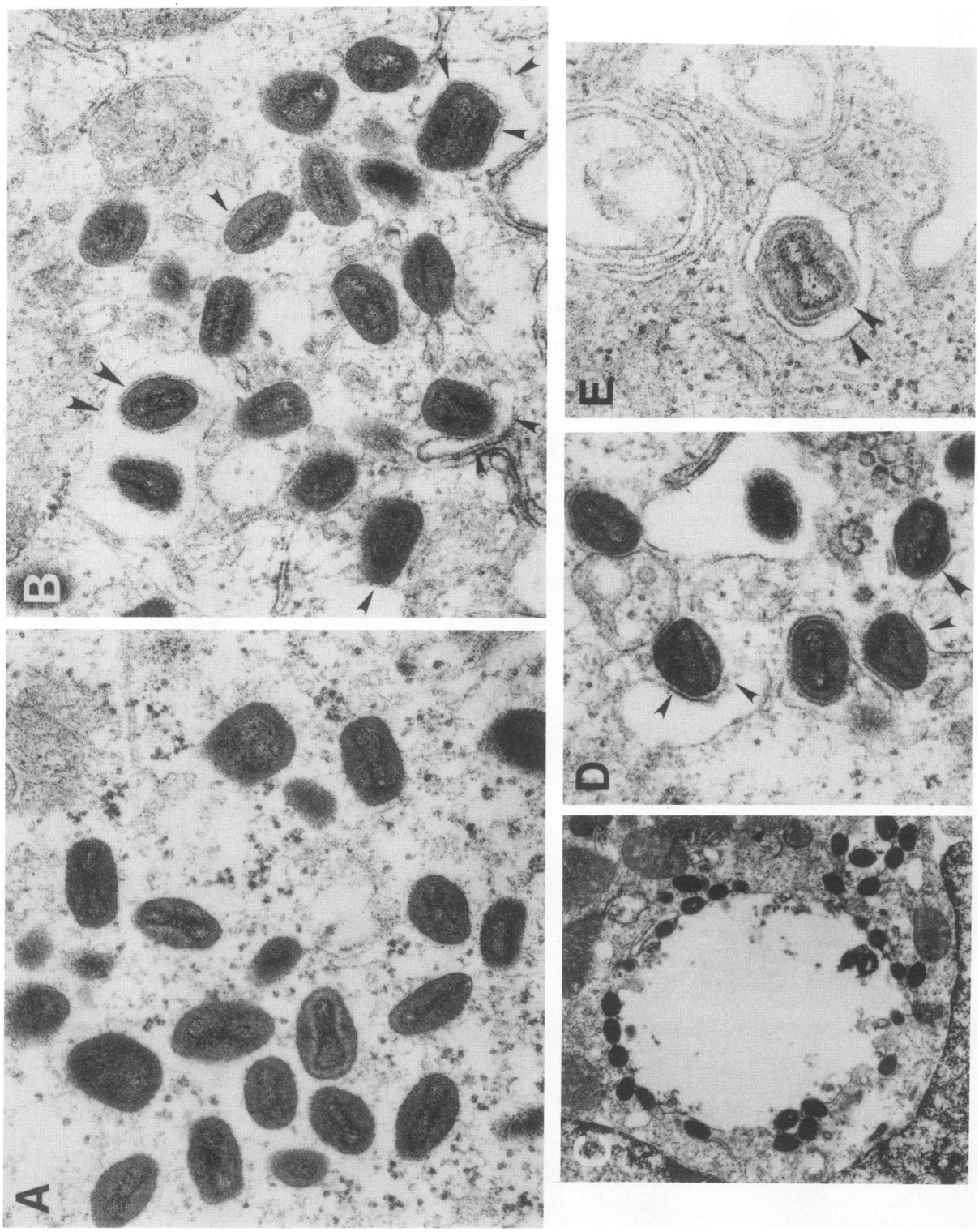

FIG. 9. Effect of the F13L mutation on the interaction of virus with cytoplasmic membranes. The electron micrographs show cells infected with VP37$^-$ mutant (A) or IHD-J wild-type virus (B to E). Arrowheads point to membranes associated with or surrounding cytoplasmic vaccinia virions. Note the absence of such membranes associated with mutant virus (A). A, ×69,150; B, ×62,000; C, ×18,150; D, ×62,000; E, ×69,150.

Our initial belief that EEV might not be required for plaque formation was based on (i) the known infectivity of INV, which has a lipoprotein envelope that can fuse with the cell membrane, (ii) the large variability in EEV production by different virus strains and the lack of correlation between plaque size and EEV yield (23), and (iii) the ability of EEV-specific antiserum to block the cometlike spread of vaccinia virus but not plaque formation when a liquid overlay is used (2, 4). One interpretation of those observations was that INV are primarily responsible for plaque formation by cell-to-cell spread of virus and that EEV provide the means for long-range spread. The present experiments, however, demonstrated that production of INV is not sufficient for plaque formation. The previous lack of correlation between EEV production and plaque formation could be explained if the latter is mediated by virions that are wrapped with VP37-containing Golgi-derived membranes but have not been released from the cells into the medium and therefore would not be counted as EEV. Cell-associated virions are commonly seen in electron micrographs of in-

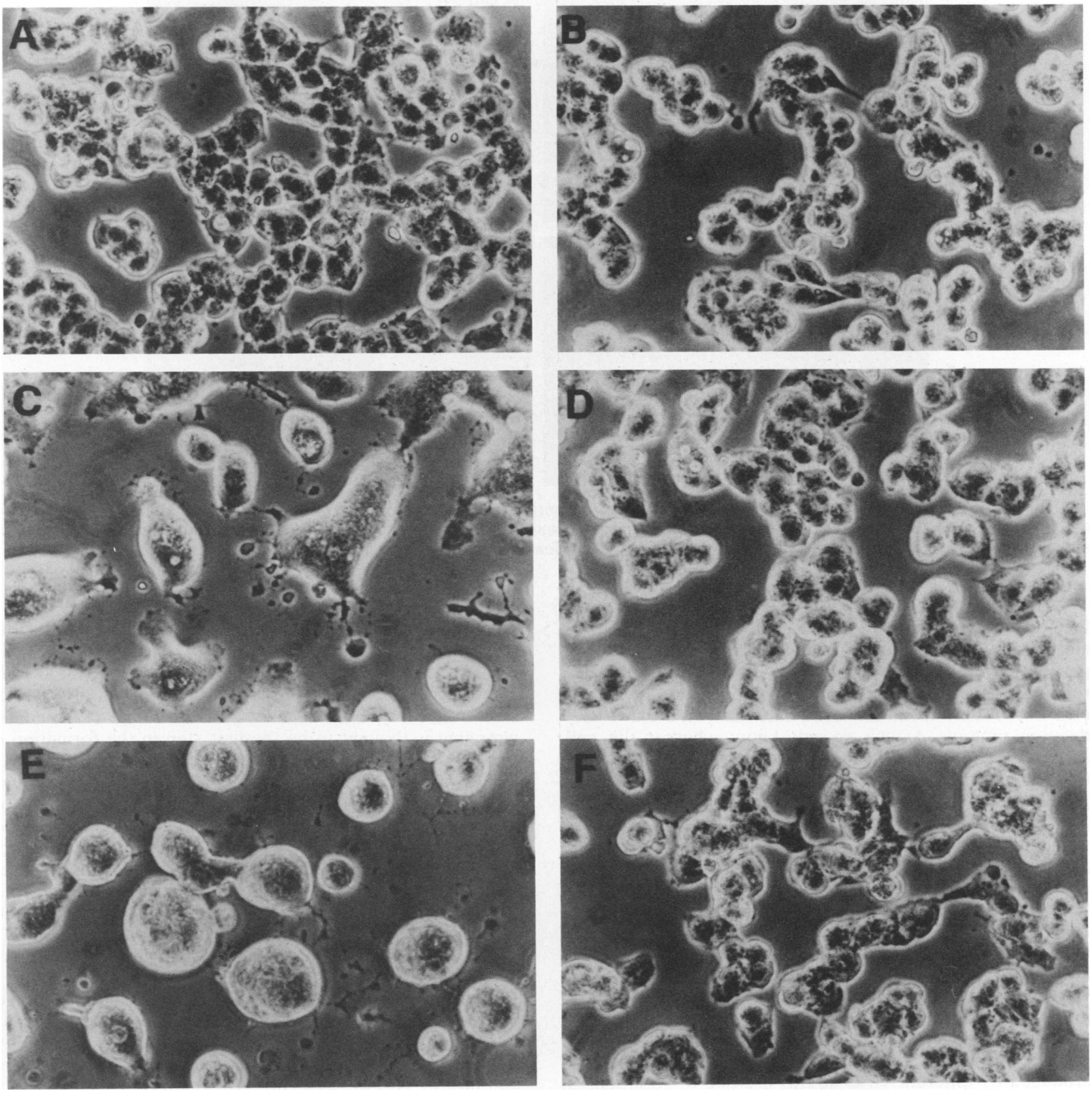

FIG. 10. Fusion from within. BSC-1 cells infected with IHD-J virus (A, C, and E) or with VP37$^-$ mutant virus (B, D, and F) were treated with either pH 7.4 (A and B) or pH 5.5 (C and D) fusion buffer as described in Materials and Methods. In panels E and F, the cells were incubated in the presence of monoclonal antibody B2D10.

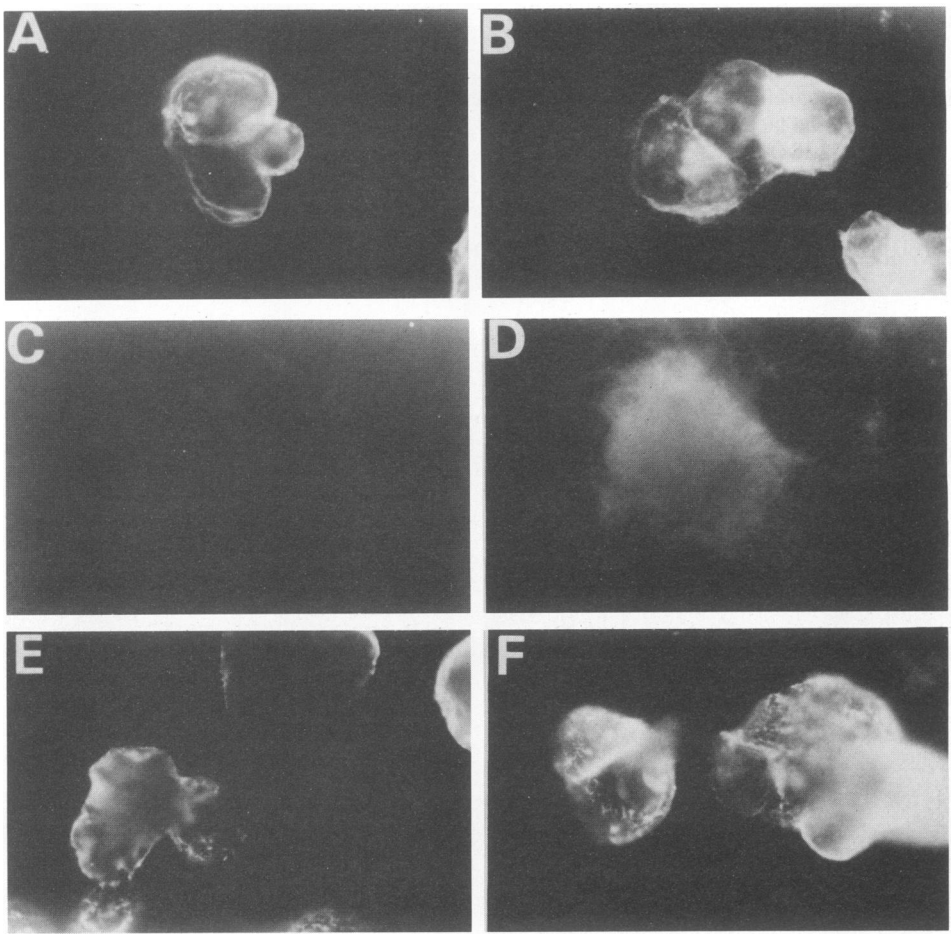

FIG. 11. Localization of HA. BSC-1 cells infected with vaccinia virus IHD-J (A and B), IHD-W (C and D), or VP37$^-$ (E and F) were fixed (A, C, and E) or fixed and permeabilized (B, D, and F) and subjected to immunofluorescence with monoclonal antibody B2D10. Note the surface fluorescence of VP37$^-$-infected cells without permeabilization (E).

fected cells. For example, the electron micrographs in Fig. 8 clearly show the presence of numerous progeny IHD-J virus particles contiguous with the outside surface of cells after removal of the medium. Significantly, however, such particles are absent from the cells infected with the VP37$^-$ deletion mutant. As we do not know whether the cell-associated enveloped virions are identical to EEV, we refer to them descriptively as CEV. The CEV appear to be in position to mediate the spread of infection to neighboring cells. Moreover, some CEV are located in intercellular spaces that would seem difficult for antibody to penetrate, perhaps accounting for the inability of EEV antisera to block plaque formation. Procedures to gently strip the CEV from the plasma membranes of infected cells are needed to compare their biochemical and physical properties with those of INV and EEV. In addition, it would be useful to determine whether the size of plaques produced by different virus strains correlates better with the amount of CEV than the amount of EEV. The direct involvement of the VP37 protein in the generation of EEV raises the question of whether the genetic differences affecting the yields of EEV produced by different virus strains are due to variations in the sequence of the VP37 proteins coded by those strains. Experiments are under way to test this possibility.

In cells infected with vaccinia virus IHD-J, it is possible to see numerous particles apparently being wrapped with Golgi membrane. No such association of particles with Golgi membrane was detected in cells infected with the VP37$^-$ deletion mutant. This is a particularly important observation since VP37 has been localized to the Golgi (12) and the wrapping is thought to be the first step in the formation of EEV (15, 19). It seems likely, therefore, that the VP37 protein is the component in the modified Golgi membranes that interacts with the INV surface. Small amounts of infectious virus particles were released into the medium of cells infected with the VP37$^-$ deletion mutant, and tiny plaques did form after prolonged incubation. This extracellular virus was similar to EEV with regard to its sedimentation in CsCl density gradients and relative resistance to neutralization by a monoclonal antibody to the 14-kDa INV protein, suggesting that inefficient EEV production can occur without VP37 protein.

Effects that appear similar to those obtained by deletion of the gene encoding VP37 have been obtained by other means. The drug *N*1-isonicotinoyl-*N*2-3-methyl-4-chlorobenzoylhydrazine inhibits virus release but does not affect the production of INV (11, 17, 24). Although the mechanism of action of the drug is not known, it has been shown to affect EEV production, possibly at the stage of INV wrapping. The drug also affects virus spread and plaque formation in vitro,

suggesting that EEV are involved in this process. When the synthesis of a 14-kDa protein that forms covalent trimers on the surface of INV (28) was inhibited by the *E. coli lac* repressor, Golgi wrapping of virions and plaque formation also was blocked (29). It seems possible that interaction of Golgi-associated VP37 protein with the INV-associated 14-kDa protein leads to wrapping, as has been suggested previously (29), and that this interaction may be affected by *N*1-isonicotinoyl-*N*2-3-methyl-4-chlorobenzoylhydrazine.

The viral HA is present in both EEV and plasma membranes (14, 26). As one possible mechanism to account for the dual localization, we considered that after transit through the endoplasmic reticulum, the HA might remain in the Golgi membrane until carried to the plasma membrane in association with wrapped virions. Following fusion of the doubly wrapped virus with the plasma membrane, the HA in the outer wrapping membrane would be deposited there and the HA in the inner wrapping membrane would remain associated with EEV. If this were the only mechanism of transport, then the HA should not be associated with the plasma membrane in cells infected with the VP37$^-$ deletion mutant. Immunofluorescence microscopy revealed, however, that HA was at the cell surface. As an alternative model, we suggest that the HA can associate with VP37 in the Golgi membrane but that independent transport occurs in the absence of VP37. This hypothesis may be tested by comparing the kinetics of HA movement to the surface in IHD-J- and VP37$^-$-infected cells or by coexpression of the two proteins in uninfected cells. In vitro evidence for binding of HA to a virion protein has been reported (21).

Fusion of vaccinia virus-infected cells has been described under circumstances in which (i) the HA is mutated (30); (ii) monoclonal antibody to HA was added (30); and (iii) the pH of the medium was lowered (8, 10, 18). A role for the 14-kDa INV envelope protein has been proposed based on the ability of antibody to that protein to block acid-mediated fusion and the inability of mutants with N-terminal deletions of the 14-kDa protein to mediate fusion (10). In view of the proposed association of VP37 with the HA and the 14-kDa protein, it was of interest to learn the consequences of deletion of the VP37 gene on fusion phenomena. We found that the mutant-infected cells did not fuse in response to the anti-HA monoclonal antibody or low pH even though the HA was still associated with the cell surface. Therefore, transport of mature virus particles to the cell surface (which does not occur in VP37$^-$ mutant-infected cells) may be required for fusion activity. The fact that rifampin blocks low-pH-mediated fusion (7) also provides evidence that assembled virus particles are needed for fusion from within. This can be explained in two ways: (i) pH-dependent fusion is mediated by CEV or (ii) the fusion activity is localized to the outer membrane of the doubly wrapped virions and gets to the cell surface by fusion of this membrane with the plasma membrane. The former possibility would point to a difference between CEV and EEV, since EEV as well as INV can fuse with cells at neutral pH (8). However, cell-to-cell fusion from without, mediated by INV, also occurs at low pH (10), so there may be a difference in the requirements for virus-cell and cell-cell fusion. More detailed studies will be needed to test those possibilities.

The similarity in the predicted protein sequences of VP37 and the product of the K4L gene suggested that the two have related functions. To test this possibility, we also made a K4L deletion mutant. However, this deletion produced no discernible phenotypic changes and normal amounts of extracellular virus were produced. Although we cannot suggest a role for the K4L gene product at this time, the deletion mutant should be valuable in future studies.

Finally, we wish to point out that the VP37 gene deletion or similar mutant viruses may have practical use for a new and highly efficient recombinant virus selection system under development. Experiments to test the degree of attenuation of vaccinia virus pathogenicity achieved by deletion of the VP37 gene also are in progress. Construction of safer vectors is essential for the use of recombinant vaccinia viruses as live vaccines.

ADDENDUM IN PROOF

Schmutz et al. (C. Schmutz, L. G. Payne, J. Gubser, and R. Wittek, J. Virol. **65**:3435–3442, 1991) have reported that a mutation in the gene encoding VP37 confers resistance to the inhibition of virus envelopment and release caused by N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine.

REFERENCES

1. **Appleyard, G., and C. Andrews.** 1974. Neutralizing activities of antisera to poxvirus soluble antigens. J. Gen. Virol. **23**:197–200.
2. **Appleyard, G., A. J. Hapel, and E. A. Boulter.** 1971. An antigenic difference between intracellular and extracellular rabbitpox virus. J. Gen. Virol. **13**:9–17.
3. **Blasco, R., N. B. Cole, and B. Moss.** 1991. Sequence analysis, expression, and deletion of a vaccinia virus gene encoding a homolog of profilin, a eukaryotic actin-binding protein. J. Virol. **65**:4598–4608.
4. **Boulter, E. A., and G. Appleyard.** 1973. Differences between extracellular and intracellular forms of poxvirus and their implications. Prog. Med. Virol. **16**:86–108.
5. **Boursnell, M. E., I. J. Foulds, J. I. Campbell, and M. M. Binns.** 1988. Non-essential genes in the vaccinia virus HindIII K fragment: a gene related to serine protease inhibitors and a gene related to the 37K vaccinia virus major envelope antigen. J. Gen. Virol. **69**:2995–3003.
6. **Buller, R. M., S. Chakrabarti, B. Moss, and T. Fredrickson.** 1988. Cell proliferative response to vaccinia virus is mediated by VGF. Virology **164**:182–192.
7. **Doms, R. W.** Personal communication.
8. **Doms, R. W., R. Blumenthal, and B. Moss.** 1990. Fusion of intra- and extracellular forms of vaccinia virus with the cell membrane. J. Virol. **64**:4884–4892.
9. **Falkner, F. G., and B. Moss.** 1988. *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol. **62**:1849–1854.
10. **Gong, S. C., C. F. Lai, and M. Esteban.** 1990. Vaccinia virus induces cell fusion at acid pH and this activity is mediated by the N-terminus of the 14-kDa virus envelope protein. Virology **178**:81–91.
11. **Hiller, G., H. Eibl, and K. Weber.** 1981. Characterization of intracellular and extracellular vaccinia virus variants: *N*1-isonicotinoyl-*N*2-3-methyl-4-chlorobenzoylhydrazine interferes with cytoplasmic virus dissemination and release. J. Virol. **39**:903–913.
12. **Hiller, G., and K. Weber.** 1985. Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. J. Virol. **55**:651–659.
13. **Hirt, P., G. Hiller, and R. Wittek.** 1986. Localization and fine structure of a vaccinia virus gene encoding an envelope antigen. J. Virol. **58**:757–764.
14. **Ichihashi, Y.** 1977. Vaccinia-specific hemagglutinin. Virology **76**:527–538.
15. **Ichihashi, Y., S. Matsumoto, and S. Dales.** 1971. Biogenesis of poxviruses: role of A-type inclusions and host cell membranes in virus dissemination. Virology **46**:507–532.
16. **Ichihashi, Y., and M. Oie.** 1988. Epitope mosaic on the surface proteins of orthopoxviruses. Virology **163**:133–144.
17. **Kato, N., H. J. Eggers, and H. Rolly.** 1969. Inhibition of release of vaccinia virus by *N*1-isonicotinoyl-*N*2-3-methyl-4-chloroben-

zoylhydrazine. J. Exp. Med. **129**:795–808.
18. **Kohno, K., J. Sambrook, and M. J. Gething.** 1988. Effect of lysomotropic agents on the entry of vaccinia virus into CV-1 cells. J. Cell. Biochem. Suppl. **12**:29.
19. **Morgan, C.** 1976. Vaccinia virus reexamined: development and release. Virology **73**:43–58.
20. **Moss, B.** 1990. Poxviridae and their replication, p. 2079–2112. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. Melnick, T. P. Monath, and B. Roizman (ed.), Virology. Raven Press, New York.
21. **Oie, M., H. Shida, and Y. Ichihashi.** 1990. The function of the vaccinia hemagglutinin in the proteolytic activation of infectivity. Virology **176**:494–504.
22. **Payne, L.** 1978. Polypeptide composition of extracellular enveloped vaccinia virus. J. Virol. **27**:28–37.
23. **Payne, L. G.** 1980. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J. Gen. Virol. **50**:89–100.
24. **Payne, L. G., and K. Kristenson.** 1979. Mechanism of vaccinia virus release and its specific inhibition by N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine. J. Virol. **32**:614–622.
25. **Payne, L. G., and K. Kristenson.** 1985. Extracellular release of enveloped vaccinia virus from mouse nasal epithelial cells in vivo. J. Gen. Virol. **66**:643–646.
26. **Payne, L. G., and E. Norrby.** 1976. Presence of haemagglutinin in the envelope of extracellular vaccinia virus particles. J. Gen. Virol. **32**:63–72.
27. **Rodriguez, J. F., R. Janeczko, and M. Esteban.** 1985. Isolation and characterization of neutralizing monoclonal antibodies to vaccinia virus. J. Virol. **56**:482–488.
28. **Rodriguez, J. F., E. Paez, and M. Esteban.** 1987. A 14,000-M_r envelope protein of vaccinia virus is involved in cell fusion and forms covalently linked trimers. J. Virol. **61**:395–404.
29. **Rodriguez, J. F., and G. L. Smith.** 1990. IPTG-dependent vaccinia virus: identification of a virus protein enabling virion envelopment by Golgi membrane and egress. Nucleic Acids Res. **18**:5347–5351.
30. **Seki, M., M. Oie, Y. Ichihashi, and H. Shida.** 1990. Hemadsorption and fusion inhibition activities of hemagglutinin analyzed by vaccinia virus mutants. Virology **175**:372–384.
31. **Shida, H., and S. Dales.** 1982. Biogenesis of vaccinia: molecular basis for the hemagglutination-negative phenotype of the IHD-W strain. Virology **117**:219–237.
32. **Spyropoulos, D. D., B. E. Roberts, D. L. Panicalli, and L. K. Cohen.** 1988. Delineation of the viral products of recombination in vaccinia virus-infected cells. J. Virol. **62**:1046–1054.
33. **Turner, G. S., and E. J. Squires.** 1971. Inactivated smallpox vaccine: immunogenicity of inactivated intracellular and extracellular vaccinia virus. J. Gen. Virol. **13**:19–25.