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Deletion of the Vaccinia Virus B5R Gene Encoding a 42-Kilodalton Membrane Glycoprotein Inhibits Extracellular Virus Envelope Formation and Dissemination

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The structure, formation, and function of the virion membranes are among the least well understood aspects of vaccinia virus replication. In this study, we investigated the role of gp42, a glycoprotein component of the extracellular enveloped form of vaccinia virus (EEV) encoded by the B5R gene. The B5R gene was deleted by homologous recombination from vaccinia virus strains IHD-J and WR, which produce high and low levels of EEV, respectively. Isolation of recombinant viruses was facilitated by the insertion into the genome of a cassette containing the *Escherichia coli gpt* and *lacZ* genes flanked by the ends of the B5R gene to provide simultaneous antibiotic selection and color screening. Deletion mutant viruses of both strains formed tiny plaques, and those of the IHD-J mutant lacked the characteristic comet shape caused by release of EEV. Nevertheless, similar yields of intracellular infectious virus were obtained whether cells were infected with the B5R deletion mutants or their parental strains. In the case of IHD-J, however, this deletion severely reduced the amount of infectious extracellular virus. Metabolic labeling studies demonstrated that the low extracellular infectivity corresponded with a decrease in EEV particles in the medium. Electron microscopic examination revealed that mature intracellular naked virions (INV) were present in cells infected with mutant virus, but neither membrane-wrapped INV nor significant amounts of plasma membrane-associated virus were observed. Syncytium formation, which occurs in cells infected with wild-type WR and IHD-J virus after brief low-pH treatment, did not occur in cells infected with the B5R deletion mutants. By contrast, syncytium formation induced by antibody to the viral hemagglutinin occurred, suggesting that different mechanisms are involved. When assayed by intracranial injection into weanling mice, both IHD-J and WR mutant viruses were found to be significantly attenuated. These findings demonstrate that the 42-kDa glycoprotein of the EEV is required for efficient membrane enwrapment of INV, externalization of the virus, and transmission and that gp42 contributes to viral virulence in strains producing both low and high levels of EEV.

Vaccinia virus, the representative member of the *Poxviridae*, is a large DNA virus that replicates entirely within the cytoplasm of the infected cell (for a review, see reference 25). The assembly of the complex virion structure, and in particular the formation of the multiple viral membranes, is the least well understood aspect of the replication cycle. Two related but antigenically and morphologically distinct infectious forms of the virus have been characterized, intracellular naked virions (INV) and extracellular enveloped virions (EEV). INV, the principal mature form of vaccinia virus in infected cells, has a complex core structure surrounded by a lipoprotein membrane, the origin of which is under investigation (38). During the course of infection, some of the INV become enwrapped by a double-layer cisternal membrane and move to the cell periphery (19, 24, 30). The outermost viral membrane fuses with the plasma membrane of the cell, resulting in the externalization of virions that after exit still retain an extra membrane relative to INV. With some virus strains, such as WR, most of the extracellular virus remains associated with the cell membrane and has been called cell-associated enveloped virions (CEV) (3, 4), whereas in other strains, such as IHD-J and IHD-W, considerable amounts of the extracellular virus are released into the medium as EEV (27, 28). The surrounding membrane of EEV contains protein components that distinguish it antigenically from INV (2, 6, 26, 29). The EEV-specific proteins that have been identified include at least three glycoproteins: the hemagglutinin (HA) (27, 31), a product of the A56R gene (36); gp42 (29) encoded by the B5R gene (12, 21); and a group of proteins with molecular masses of 22 to 24 kDa that share a protein backbone encoded by the gene designated SalL4R or A34R (10). In addition, the 37-kDa nonglycosylated product of the F13L gene is also specifically associated with EEV (16, 26). While INV are infectious, CEV and EEV are thought to be important for cell-to-cell spread (4) and long-range transmission (28), respectively. The latter property of EEV has been considered an important contributor to virulence (2, 6).

The roles of the individual membrane proteins of extracellular virus are largely unknown. The 37-kDa protein is required for membrane wrapping of the INV (3, 34), and the 22- to 24-kDa proteins regulate the formation (10) and release (5) of EEV. Although the HA is not required for EEV production (27), mutations can result in a syncytial phenotype (18). The small plaque size and host range properties of a highly passaged and greatly attenuated Lister strain of vaccinia virus have been explained in part by a frameshift mutation in the gene encoding gp42 (39). The latter gene is of additional interest as it has homology with members of the family of complement control proteins (14, 37). To investigate the function of gp42 in virus envelope assembly, EEV formation,

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and virulence, we made recombinant viruses in which the B5R open reading frame (ORF) was almost completely deleted. The effects of this deletion in strains yielding low (WR) and high (IHD-J) levels of EEV are described.

MATERIALS AND METHODS

Cells and viruses. HeLa cell monolayers were maintained in Dulbecco's medium (Quality Biological, Inc., Gaithersburg, Md.) containing 10% fetal bovine serum (FBS). Both BS-C-1 (ATCC CCL 26) and RK_{13} (ATCC CCL 37) cells were maintained in minimal essential medium containing 10% FBS. Vero cells (ATCC CCL 81) were also grown in minimal essential medium containing 10% FBS. Vaccinia viruses, strain WR (ATCC VR1354) or IHD-J (obtained from S. Dales, University of Western Ontario, London, Canada), were routinely propagated in HeLa cells, and titers were determined in BS-C-1 cells which were stained with crystal violet at 2 days postinfection. Purified virus stocks were prepared as previously described (11). Unless otherwise specified, cells were infected in medium containing 2.5% FBS with 5 to 10 PFU of a crude vaccinia virus stock per cell and incubated at 37°C in a 5% CO_2 atmosphere.

Antibodies. Monoclonal antibody 20, which is specific for gp42 of EEV, has been described previously (29). Monoclonal antibody B2D10, which is specific for the vaccinia virus HA (20), was kindly provided by Y. Ichihashi, Niigata University, Asahimachi, Japan.

Plasmids. A portion of the vaccinia virus WR *Hin*dIII B fragment, from the left *Hin*dIII site to the first *Sal*I site contained in pBHS (from M. Merchlinsky, National Institutes of Health, Bethesda, Md.), was digested with *Sca*I and *Nsi*I to yield a 2.1-kbp fragment containing the B5R ORF. This DNA fragment was ligated to pUC19, which had been digested with *Sma*I and *Pst*I, to yield plasmid pSI-80. A copy of the *Escherichia coli* gene encoding xanthine guanine phosphoribosyl transferase (*gpt*) under the control of the vaccinia virus P7.5 promoter from pTK61gpt (13) and a copy of the *E. coli lacZ* gene under the control of the vaccinia virus P11 promoter were sequentially inserted into pSI-80. First, pSI-80 was cut with *Hin*cII and the *gpt* cassette was inserted by blunt-end ligation. The resulting plasmid, pSI-81, was checked for the specific orientation of the *gpt* cassette relative to the 5′- and 3′-flanking sequences of B5R. The *lacZ* cassette was ligated into *Bcl*I-cut pSI-81, yielding pSI-82.2, and its orientation was confirmed by restriction endonuclease digestion. In this manner, approximately 710 bp of the B5R ORF were deleted. Plasmid pSI-82.2, which was used for homologous recombination, contained the *E. coli gpt* and *lacZ* cassettes flanked on one side by 750 bp of viral DNA of which 142 bp are the B5R gene and on the other by 680 bp of which 99 bp are the B5R gene (see Fig. 1).

Construction of recombinant vaccinia viruses. RK_{13} cells were infected with 0.05 PFU of either vaccinia virus strain WR or IHDJ per cell and then transfected with pSI-82.2 by the calcium phosphate technique by standard protocols (11). The infected cells were harvested, and recombinant viruses from which the B5R gene had been deleted were isolated on RK_{13} cells by selecting virus plaques that were resistant to mycophenolic acid (13) and stained blue in the presence of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal). The recombinant virus derived from WR, with a deletion of the B5R ORF, is code named vSI-14 and referred to as W-B5R$^-$. The corresponding recombinant virus derived from IHD-J, vSI-15, is referred to as I-B5R$^-$.

Southern blot analysis. Southern blot analysis of DNA isolated from putative recombinant viruses was carried out as previously described (11). DNA was digested with restriction enzyme *Spe*I or *Sna*BI (GIBCO-BRL Life Technologies) by the manufacturer's instructions. The digested DNA was separated on a 0.6% agarose gel and transferred to Nytran membranes (Schleicher and Schuell, Inc.). Membranes were then hybridized to an α-^{32}P-labeled probe, either the *lacZ* gene in pUC19 (no vaccinia virus sequences present) or a 580-bp gel-purified polymerase chain reaction product derived from the 5′ end of the B5R gene.

Virus labeling and purification. Monolayers of RK_{13} cells, grown in 150-cm^2 flasks, were infected with purified virus at multiplicities of 10. After 4 h, medium was replaced with 9 ml of methionine-free minimal essential medium supplemented with 1 ml of normal medium, 1 mCi of [^{35}S]methionine [ICN] per ml, and 2.5% dialyzed FBS. At 24 h after infection, 5 ml of complete medium with 2.5% FBS was added and the incubation was continued for a further 24 h. The medium was removed from the cells and cleared of cellular debris by low-speed centrifugation, and the virus was collected by sedimentation through a 36% sucrose cushion. Pelleted extracellular virus was banded on a cesium chloride gradient as previously described (31). Gradients were fractionated from the bottom of the tube, and an aliquot of each fraction was mixed with Beckman Readysolve and the mixtures were counted with a Beckman LS 3801 scintillation spectrometer.

Western blot (immunoblot) analysis. Proteins were separated on a 0.1% sodium dodecyl sulfate (SDS) gel with stacking and separating portions containing 5 and 10% polyacrylamide, respectively. The resolved protein bands were electrophoretically transferred onto nitrocellulose. The blots were then incubated in 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)–0.2% Tween 20 and then with a 1:500 dilution of monoclonal antibody 20 in PBS-Tween 20. After washing with PBS-Tween, the nitrocellulose was incubated with 0.1 μCi of ^{125}I-protein A (Amersham) in PBS-Tween 20; incubation was followed by additional washing and autoradiographic analysis.

Electron microscopy. For electron microscopy, monolayers of RK_{13} cells grown in 75-cm^2 flasks were infected with virus at multiplicities of 10. At 16 h after infection, the culture medium was removed and the monolayer was fixed in 2.5% glutaraldehyde in 0.13 M sodium phosphate, pH 7.4. The cells were gently scraped and collected by centrifugation. Cell pellets were post fixed in osmium tetroxide, stained with uranyl acetate, and dehydrated in increasing concentrations of ethanol before infiltration and embedding in Spurr's resin. The embedded samples were ultra-thin sectioned, collected on grids, and stained with lead citrate.

Syncytium formation. Cell fusion experiments were carried out as described previously (3). Confluent monolayers of BS-C-1 cells in six-well plates were infected with virus at a multiplicity of 10. Virus was allowed to adsorb to the cells for 1 h, and the inoculum was removed and replaced with either fresh medium alone or medium containing anti-HA antibody B2D10 or an irrelevant antibody. For acid-dependent fusion, the monolayers were washed at 12 h after infection with warm 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 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 5.5 or 7.4)]. Fusion buffer was replaced with fresh medium, and the cells were incubated at 37°C before observation under the phase-contrast microscope. Cells incubated with antibody were photographed at 20 to 24 h after infection.

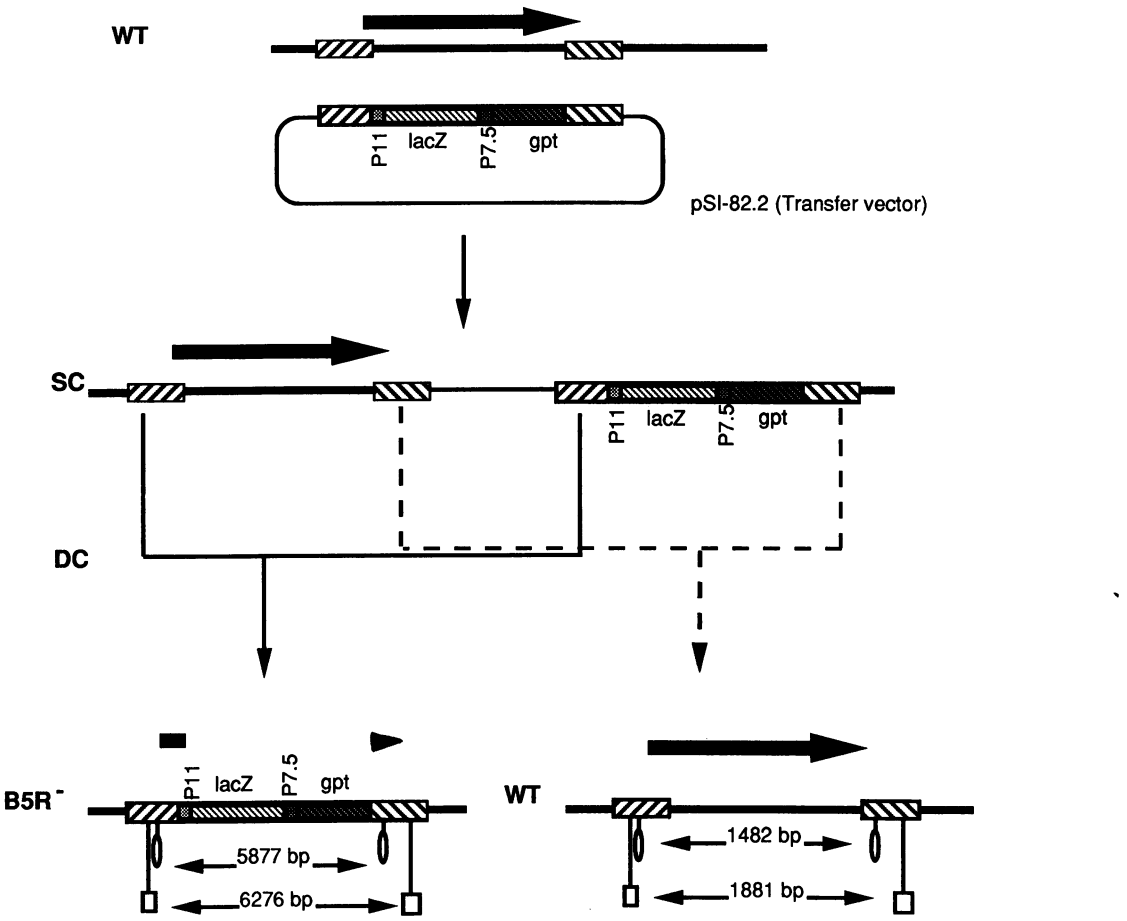

FIG. 1. Insertion/deletion mutagenesis of the B5R gene. WT, segment of genome of wild-type virus containing the B5R ORF, indicated by an arrow. The hatched bars represent the ends of the B5R ORF and neighboring sequence. Transfer vector, pSI-82.2 containing B5R flanking sequences indicated by hatched bars, the *E. coli gpt* gene under the control of the vaccinia P7.5 promoter and the *lacZ* gene under the control of the P11 promoter. SC and DC refer to recombinant genomes that have undergone single and double crossover events, respectively. Bottom, schematic diagrams showing expected restriction fragment sizes of products generated by digestion of DNA from the wild-type (WT) and the B5R$^-$ mutant with *Sna*BI (ovals) and *Spe*I (open squares).

Assay of 50% lethal dose (LD_{50}) in mice. Test groups of six female, weanling BALB/c ByJ mice were injected intracranially with 20 μl of purified virus diluted in sterile PBS. Mice were observed daily over a 3-week period, and survivors were sacrificed by cervical dislocation at the end of the experiment.

RESULTS

Isolation of B5R deletion mutants. Having established that the product of the B5R ORF, gp42, is a type I integral membrane glycoprotein component of the EEV (21), we proceeded to investigate its functions by constructing recombinant vaccinia viruses in which the B5R gene had been disrupted and largely deleted. As there was reason to believe that the recombinant virus might form small plaques which could be difficult to discern, we used the *E. coli gpt* gene to provide mycophenolic acid antibiotic selection (13) and the *E. coli lacZ* gene to provide a β-galactosidase color screening (7). Thus, a plasmid, pSI-82.2 (Fig. 1), was constructed in which a copy of the *gpt* gene and a copy of the *lacZ* gene under the control of vaccinia virus promoters were flanked by sequences from the two ends of the B5R ORF to direct homologous recombination. Although BS-C-1 cells are commonly used for plaque isolation, we chose RK_{13} rabbit cells because a mutation in the B5R gene of the Lister strain of vaccinia virus prevented plaque formation in Vero cells, which, like BS-C-1 cells, are derived from African green monkey kidneys (39). Cells were infected with either the WR or IHD-J strain of vaccinia virus and then transfected with pSI-82.2. After 48 h, cells were harvested and diluted lysates were used to infect RK_{13} cell monolayers in the presence of mycophenolic acid. The infected cells were covered with agar and subsequently stained with X-Gal. The antibiotic-resistant plaques and surrounding cells which expressed β-galactosidase and stained blue were picked and used to reinfect new monolayers of RK_{13} cells. In this way, the recombinant virus was purified by three successive rounds of plaque isolation under mycophenolic acid selection and X-Gal screening.

Single and double crossover homologous recombination events are depicted schematically in Fig. 1. DNA samples from several amplified plaques were analyzed by digestion with restriction enzymes and Southern blotting. The predicted fragment sizes for B5R deletion mutant and wild-type viruses are shown (Fig. 1). Restriction fragments of the expected sizes were seen when the Southern blots contain-

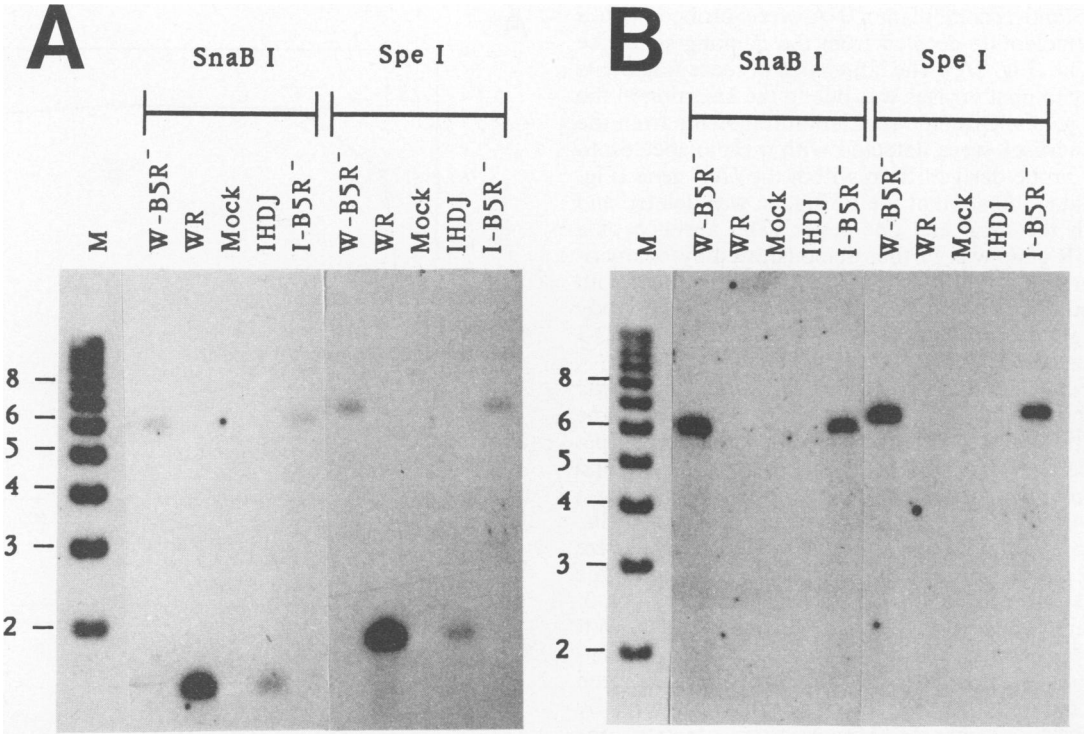

FIG. 2. Southern blot analysis showing deletions of the B5R gene in WR and IHD-J strains of vaccinia virus. Southern blot analysis of *Sna*BI and *Spe*I restriction digest fragments derived from DNA of mock-infected cells (Mock), cells infected with wild-type strains of vaccinia virus (WR and IHD-J), and cells infected with the respective B5R deletion mutants (W-B5R$^-$ and I-B5R$^-$). Blots were probed with either a radiolabeled oligonucleotide sequence from within the B5R coding/flanking region (A) or an oligonucleotide sequence from within the inserted *E. coli lacZ* gene (B). DNA molecular size markers are designated M and show sizes in kilobase pairs.

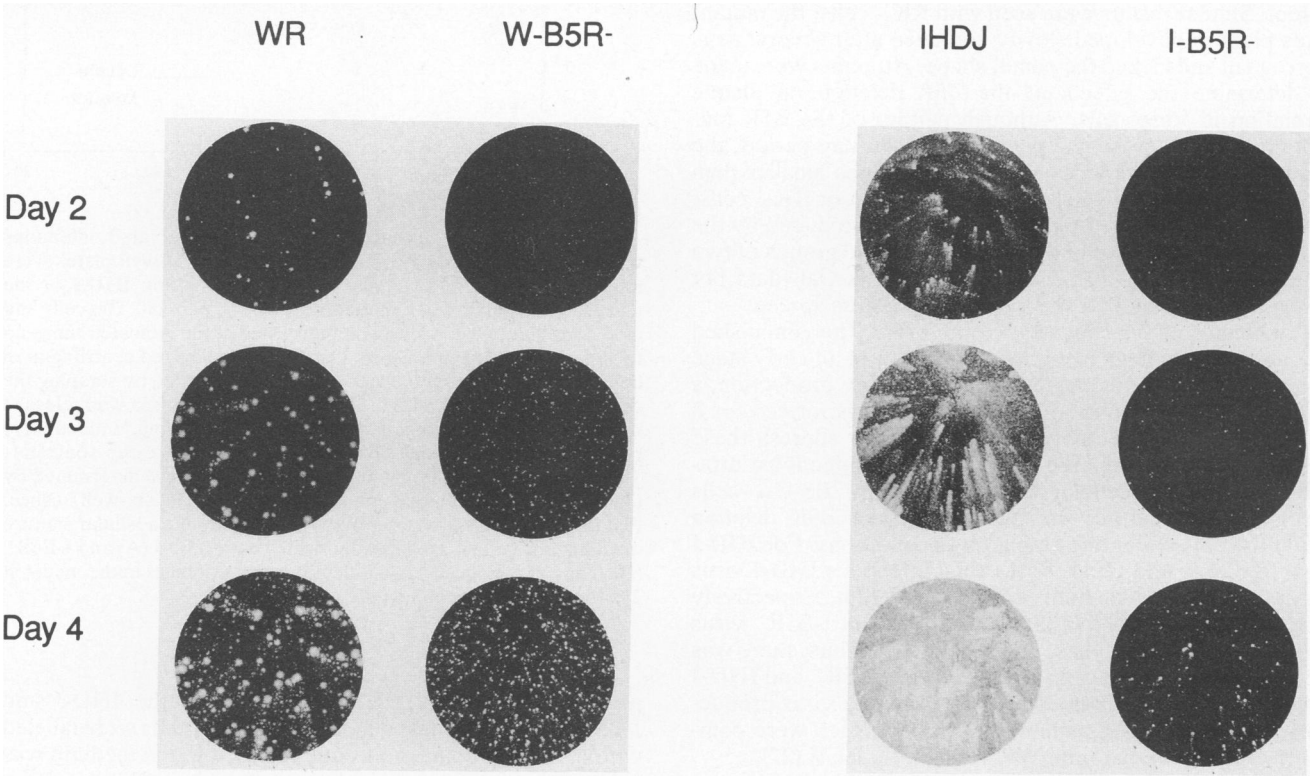

FIG. 3. Comparison of plaques produced by the WR and IHD-J strains of vaccinia virus and their corresponding B5R deletion mutants. Viruses WR, W-B5R$^-$, IHD-J, and I-B5R$^-$ were used to infect monolayers of BS-C-1 cells which were covered with a liquid overlay. The monolayers were fixed and stained at 2, 3, and 4 days with 0.1% crystal violet in 20% ethanol to visualize plaques.

ing wild-type and recombinant DNA were probed with a labeled oligonucleotide derived from the flanking sequence of the B5R gene (Fig. 2A). The larger size of these fragments from the recombinant viruses was due to the insertion of the *gpt* and *lacZ* genes. Only the restriction fragments from the recombinant viruses were detected with a radiolabeled oligonucleotide probe derived from within the *lacZ* gene (Fig. 2B). These data indicate that the B5R gene was deleted and replaced with the *lacZ* and *gpt* genes. The absence of a functional B5R gene was further demonstrated by comparison of Western blots containing lysates of cells infected with wild-type and recombinant viruses by using an antibody specific for gp42 (data not shown). The WR and IHD-J mutants were called W-B5R$^-$ and I-B5R$^-$, respectively.

Plaque formation by B5R deletion mutants. During the isolation of recombinant viruses on RK_{13} cells, it became obvious that the viruses displayed a small-plaque phenotype. We compared the plaque formation of both W-B5R$^-$ and I-B5R$^-$ recombinants to that of the parental viruses over a period of several days in RK_{13} cells as well as BS-C-1 cells, which are most commonly used for plaque assays. The size and shape of plaques formed by recombinant and wild-type WR and IHD-J viruses on BS-C-1 cells are shown in Fig. 3. Under standard conditions of 2 days of infection at 37°C, WR and IHD-J recombinant viruses formed round and comet-shaped plaques, respectively. Comet formation has been shown to be due to released EEV, as it can be inhibited by specific antibody (2, 6). At 2 days after infection, the W-B5R$^-$ and I-B5R$^-$ plaques were barely discernible. By day 4, the mutant plaques were large enough to count, although they were still smaller than the wild-type virus plaques at 2 days. In addition, the I-B5R$^-$ plaques never exhibited the extensive comet formation characteristic of its parent. Similar results were seen with RK_{13} cells: the mutant virus plaques developed slowly and even after several days were small and lacked the comet shape. Attempts were made to determine the effects of the B5R deletion on plaque formation in Vero cells. Although neither of the B5R mutants formed macroscopic plaques over a 3-day period, the sizes of the parental virus plaques were much smaller than those of the plaques which formed on BS-C-1 or RK_{13} cells. By taking advantage of the β-galactosidase produced by the mutants, we could demonstrate individual and groups of two to three Vero cells that stained blue with X-Gal (data not shown), suggesting that the block was in virus spread.

Production of infectious INV and EEV. The diminished plaque size may have been due to a defect in an early stage of virus assembly affecting both INV and EEV production, a reduction in the amount of extracellular virus formed, or a reduction in the infectivity of the latter. To address these possibilities, we compared the amounts of infectious intracellular and extracellular virus formed by BS-C-1 cells inoculated with either the parental or the B5R deletion mutant strains under one-step growth conditions. For IHD-J virus, 0.8, 6.9, and 12.5% of the total infectious IHD-J virus was present in the medium at 21, 48, and 72 h, respectively (Fig. 4A). By contrast, 0.08, 0.6, and 1.9% of I-B5R$^-$ virus was in the medium at these times (Fig. 4B). Thus, there was a 6- to 12-fold difference in the release of I-B5R$^-$ and IHD-J viruses. Differences in infectious extracellular virus production were less obvious when WR and W-B5R$^-$ were compared, since even parental WR yields little EEV (27).

It appeared that the deletion of the B5R gene did not significantly affect INV production but severely diminished the amount or infectivity of EEV released into the medium. To distinguish between these possibilities, we compared the

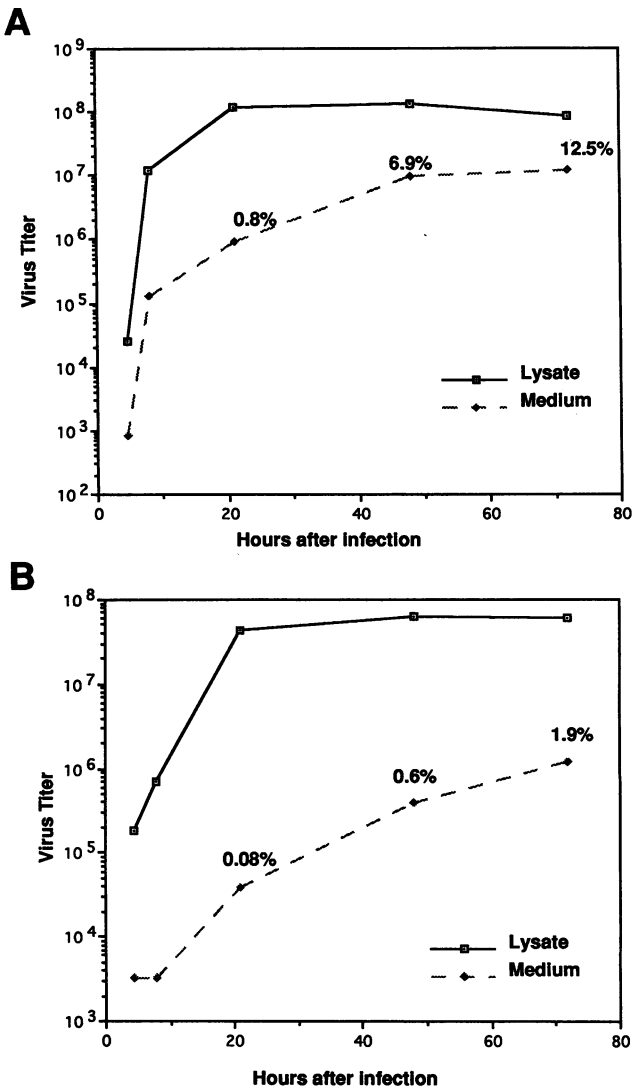

FIG. 4. Yields of extracellular and cell-associated infectious virus. Confluent monolayers of BS-C-1 cells in 24-well plates were infected with 5 PFU of either vaccinia virus strain IHD-J or the corresponding B5R deletion mutant, I-B5R$^-$, per cell. The cells and media from duplicate wells were harvested at the indicated times up to 48 h. Medium samples were clarified by low-speed centrifugation before titration. Cell-associated virus was harvested by scraping the cell monolayer into 0.5 ml of fresh medium, and virus was released by three successive rounds of freezing and thawing. Immediately prior to titration, the cell lysate was dispersed in a cup sonicator. The yields of virus from media and cell lysates were determined by plaque assay on monolayers of BS-C-1 cells in six-well dishes. Plaque titers are shown for free virus in the extracellular culture medium and for cell-associated virus for both IHD-J (A) and I-B5R$^-$ (B). The percentages of total infectious virus present in the media at 21, 48, and 72 h are indicated.

physical amounts of EEV released from the IHD-J and I-B5R$^-$ virus-infected cells. Infected RK_{13} cells were labeled with [^{35}S]methionine, and virus released in the medium was subjected to cesium chloride centrifugation (31). A prominent peak of radioactivity was found at the density expected for EEV when the medium from IHD-J-infected cells was analyzed (Fig. 5A). By contrast, there was much less radio-

active material sedimenting with the density of EEV when the medium was from I-B5R$^-$-infected cells (Fig. 5B). The approximately fivefold difference in metabolically labeled EEV could account in large measure for the difference in infectivity (Fig. 4).

Electron microscopy of W-B5R$^-$-infected cells. Although the WR strain of vaccinia virus produces low levels of EEV, this is due not to a block in wrapping and externalization of virions but to the failure of enveloped virions to be released into the medium (4). To further investigate the defect in B5R deletion mutant viruses, we examined thin sections of cells at 16 h after infection by electron microscopy (Fig. 6). At this time after infection, large numbers of mature and immature INV (Fig. 6A) as well as membrane-wrapped forms of the virus are present in WR-infected cells (Fig. 6C). Immature and mature forms of INV also were observed in W-B5R$^-$-infected cells (Fig. 6B), and these appeared indistinguishable in appearance or amount from those in cells infected with the wild-type virus. However, no examples of fully wrapped virus were seen and mature INV were sparse near the periphery of the cells. Occasionally, INV were found around large intracellular cisternae (Fig. 6D), suggesting that the viruses had some capacity to interact with membranes without being enveloped. Rarely, virus which appeared to be in the initial process of being wrapped was seen in sections from the W-B5R$^-$-infected cells (data not shown). These data indicate that the primary defect in the WR strain B5R deletion mutant virus is in the wrapping and release of INV and are consistent with the diminished EEV production for the IHD-J mutant.

Cell-cell fusion from within. Fusion of cells infected with either WR or IHD-J virus has been observed when cells were briefly treated with an acidic (pH 5.5) buffer (9, 15). We looked at the ability of the B5R deletion mutants to mediate acid-induced syncytium formation. Data are shown for cells infected with WR and W-B5R$^-$, although similar results were seen when cells were infected with IHD-J and I-B5R$^-$. No significant polykaryon formation was seen 3 h after cells infected for 12 h with the deletion mutants were briefly treated (2 min) with acid buffer (pH 5.4) (Fig. 7D), whereas polykaryon formation was well advanced in the wild-type virus-infected cells at this time (Fig. 7C). Even after prolonged periods of incubation at 37°C (8 to 10 h), there was no increase in the formation of syncytia in cells infected with the mutant viruses, suggesting that this was not simply a kinetic difference between the two systems. Control experiments indicated that polykaryon formation did not occur at pH 7.4 (Fig. 7A and B).

Syncytium formation also occurs when vaccinia virus-infected cells are incubated with certain antibodies against the virus-encoded HA (35). We found that cells infected with either the wild type or WB5R$^-$ mutant formed syncytia at similar rates over a period of 15 to 20 h when treated with the anti-HA antibody B2D10 (Fig. 7E and F). In fact, syncytium formation appeared to be more extensive in cells infected with the mutant viruses compared with the wild type. No syncytium formation was observed when infected cells were treated with an irrelevant antibody or when uninfected cells were treated with B2D10 at a similar dilution (data not shown).

Effect of deletion of the B5R gene on virulence in mice. The EEV form of vaccinia virus may be an important factor in animal infections, as antisera that neutralize EEV but not INV protect animals against live virus challenge (1, 28, 40). The virulence of the wild-type and B5R deletion mutant strains was assessed by intracranial injection of weanling mice with serial dilutions of each virus. LD_{50} measurements of the wild-type strains (Table 1) agreed closely with previously reported data (7, 8, 22). Significantly, we found that the deletion of the B5R gene resulted in a marked decrease in the virulence of both the IHD-J and WR strains of virus. This decrease was more dramatic for I-B5R$^-$, which showed a five- to six-log reduction in the intracranial LD_{50}, than for W-B5R$^-$, which gave a three- to four-log attenuation.

DISCUSSION

Recent work has identified the product of the B5R gene as a component of the EEV form of vaccinia virus (12, 21), corresponding to the 42-kDa acylated glycoprotein described by Payne (29). Biochemical studies further showed that gp42 is a

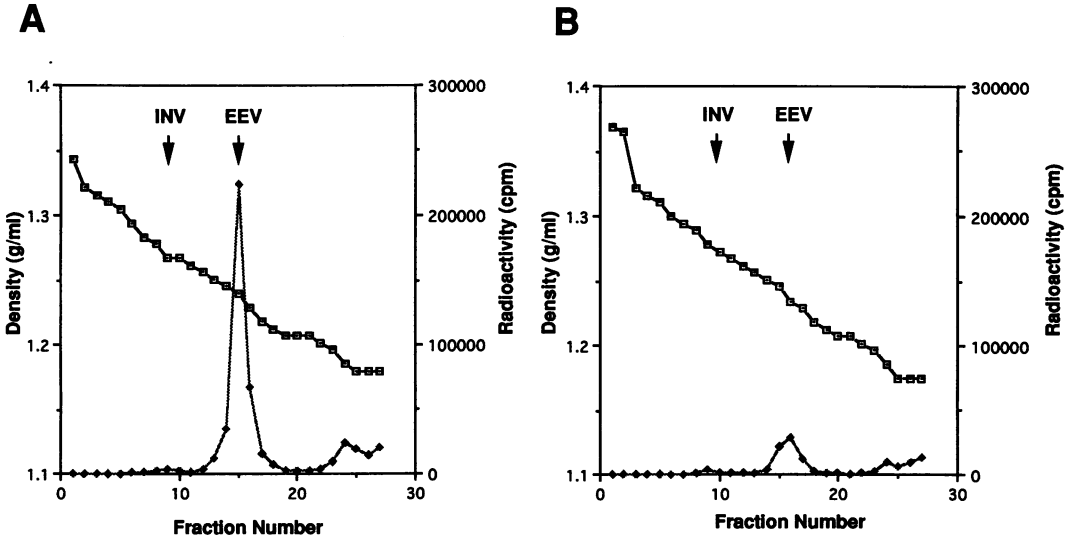

FIG. 5. CsCl equilibrium density centrifugation of extracellular virus. Virus in the medium of RK_{13} cells infected with IHD-J (A) or I-B5R$^-$ (B) in the presence of [^{35}S]methionine was subjected to centrifugation in CsCl gradients. After centrifugation, fractions were collected from the bottoms of the tubes, the refractive index of each was measured and converted to density (⊡), and aliquots were counted in a liquid scintillation spectrometer (◆). Densities at which INV and EEV characteristically band (1.27 g/ml and 1.23 g/ml, respectively) are indicated by arrows.

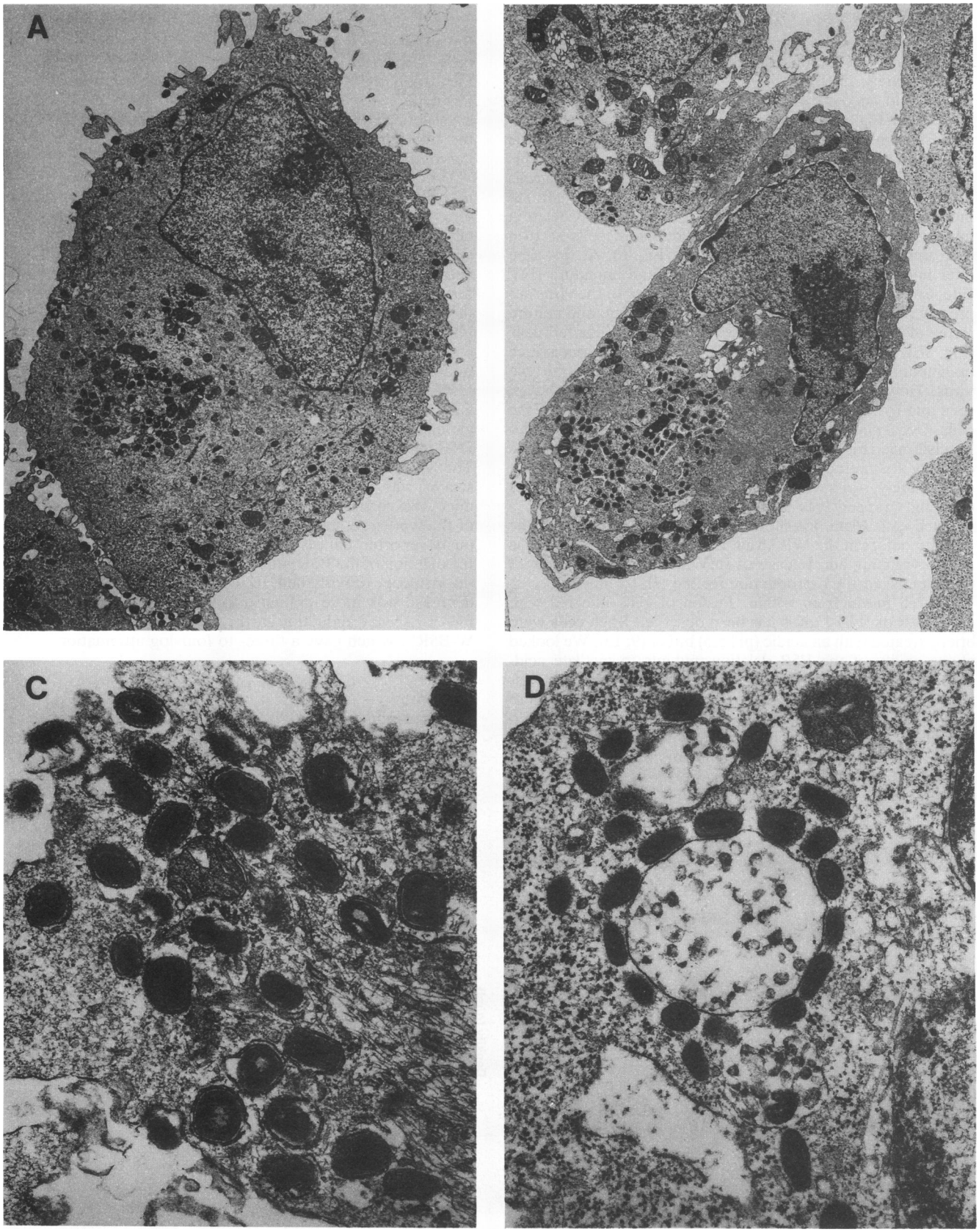

FIG. 6. Electron microscopy of cells infected with either wild-type vaccinia virus or the B5R deletion mutant. Thin sections were made of RK_{13} cells that were infected with either WR (A and C) or W-B5R$^-$ (B and D) virus and viewed at a magnification of ×5,714 (A and B) or ×35,714 (C and D). Note the wrapped forms of virus in wild-type virus-infected cells (C) and mature INV of the mutant virus aligned at an intracellular cisternal membrane (D).

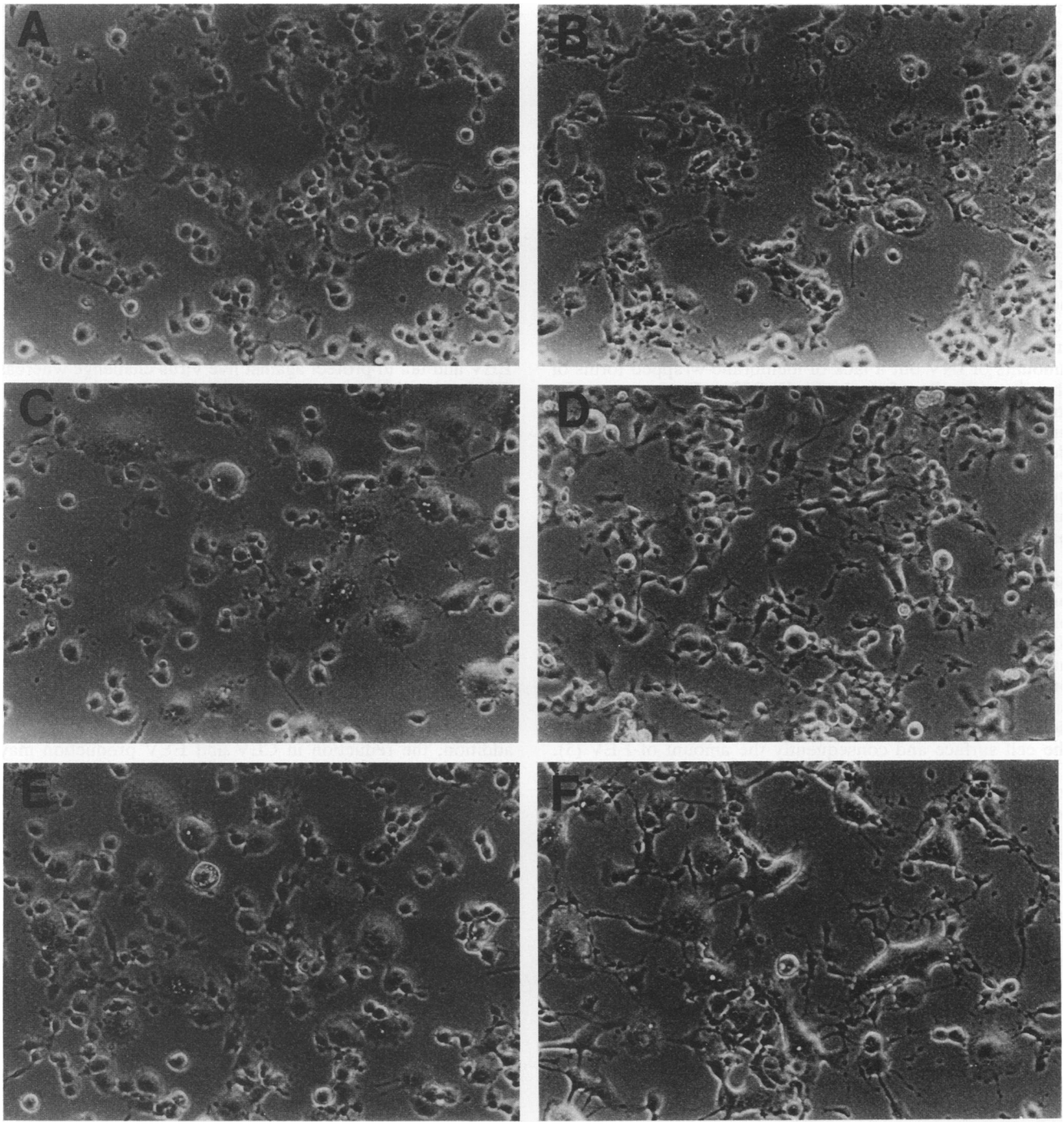

FIG. 7. Syncytium formation. BS-C-1 cells infected with WR virus (A, C, and E) or W-B5R$^-$ (B, D, and F) were treated with fusion buffer at pH 7.4 (A and B) or pH 5.5 (C and D) or incubated in the presence of monoclonal antibody B2D10 to HA (E and F).

type I membrane glycoprotein with an N-terminal signal peptide that is cleaved and a near C-terminal transmembrane domain (21). The presence of gp42 in the EEV membrane and the external orientation of the N-terminal segment were determined by immunogold labeling (21).

To investigate the putative role of this protein, we have deleted the B5R gene from vaccinia virus strains producing low (WR) and high (IHD-J) levels of EEV to yield mutant viruses W-B5R$^-$ and I-B5R$^-$, respectively. Compared with the parental viruses, the deletion mutants form much smaller plaques on BS-C-1 and RK_{13} cell monolayers. Under one-step growth conditions, the amounts of infectious cell-associated virus formed were nearly equivalent but the percentage of the total infectious I-B5R$^-$ virus released into the media was reduced by approximately 1/6 to 1/12 compared with IHD-J. Metabolic labeling of I-B5R$^-$ virus-

TABLE 1. Determination of attenuation of B5R deletion mutants in a mouse intracranial model

Virus	LD_{50}[a] (PFU) in:	
	Exp 1	Exp 2
IHD-J	3.9	1.0
I-B5R$^-$	2.0×10^5	2.6×10^5
WR	4.9	1.6
W-B5R$^-$	2.7×10^3	1.5×10^4

[a] The Spearman-Karber method was used to calculate the LD_{50}s (23).

infected cells revealed that the reduction in infectivity was accompanied by a decrease in the physical amount of released virus isolated by equilibrium density gradient centrifugation. Electron microscopic analysis revealed normal amounts of INV but a lack of membrane-wrapped forms of the virus in cells infected with W-B5R$^-$. Furthermore, the mutant virus was largely absent from the cell periphery and unassociated with the plasma membrane. Thus, a primary defect of the mutant viruses is in the inability of INV to become wrapped with cellular membranes. As a consequence, cells infected with the deletion mutants produce much smaller amounts of CEV and EEV than the respective parental virus.

Thus far, proteins encoded by four genes have been localized to the EEV membrane. Of these, three are glycoproteins. One glycoprotein is gp42; the other two are the HA product of the A56R gene and the 22- to 24-kDa products of the A34R gene. HA$^-$ mutants still make EEV (27) and may have a syncytium-forming phenotype (18). Repressed expression of the A34R gene gave a small-plaque phenotype and reportedly decreased EEV formation (10). Point mutations in the A34R gene also affect the release of CEV from the cell surface and consequently the amount of EEV (5). The 37-kDa nonglycosylated product of the F13L ORF is the major component of the EEV membrane (16), yet it does not resemble a transmembrane protein on the basis of sequence analysis (17). Nevertheless, deletion of F13L produces a phenotype that closely resembles that of the B5R$^-$ mutants (3). Actually, the F13L deletion mutants appear to be even more seriously affected in plaque formation. It is possible that the 37-kDa protein binds to gp42 or another integral membrane glycoprotein and mediates attachment to an INV protein. A candidate INV protein would be the 14-kDa product of the A27L gene (32). Like the 37-kDa protein, the 14-kDa protein lacks a signal sequence and an obvious transmembrane region. Nevertheless, the protein is located on the surface of INV apparently as disulfide-bonded trimers (33). The interactions between the membrane-associated proteins of INV and EEV need to be fully investigated.

Cells infected with gp42$^-$ mutants, unlike those infected with HA$^-$ mutants, do not spontaneously form syncytia at neutral pH, nor do they form syncytia when treated with low-pH buffer, as do cells infected with wild-type virus. In this respect, the gp42$^-$ mutant is like the F13L deletion mutant (3) and supports the hypothesis that acid-dependent fusion is mediated by enveloped virions at the cell surface. Fusion also occurs when vaccinia virus-infected cells are incubated with antibodies to HA, and deletion of the B5R gene did not abrogate this phenomenon although deletions of the gene encoding the 37-kDa protein did (3). Whether acid- and HA antibody-mediated fusions are caused by different mechanisms or whether the sensitivities of the triggering stimuli are different remains to be determined.

Formation of small plaques on RK_{13} cells by a multiply passaged variant of the Lister strain was attributed to a frameshift mutation in the B5R gene (39), which is consistent with our data for deletion of the B5R gene. The inability of the Lister mutant to form plaques in Vero cells, however, was considered to be a host range defect even though normal yields of infectious cell-associated mutant virus formed in Vero cells. We found that the W-B5R$^-$ and I-B5R$^-$ viruses both had a small-plaque rather than a host range phenotype in BS-C-1 cells, which, like Vero cells, are derived from African green monkey kidneys. Although we agree with Takahasi-Nishimaki et al. (39) on the more severe effect of the B5R mutations on plaquing in Vero cells, we attribute this to the generally less efficient spread of vaccinia virus in these cells rather than to an additional host range defect.

Previous reports suggested that EEV may be important in animal infections, as INV-specific antisera do not neutralize EEV and fail to protect against live virus challenge whereas EEV-neutralizing antiserum is protective (1, 6). In addition, a correlation was found between the virulence of vaccinia virus strains and their ability to form EEV in cultured cells (28). The WR strain, however, was considered to be an exception since it is virulent yet forms little EEV. To determine the effects of the B5R gene deletions on virulence, we used the mouse intracranial challenge system. This route of inoculation provides the ability to measure a larger range of attenuation than the intraperitoneal and intranasal routes (22). A relationship between increased intracranial LD_{50}s and decreased virus titers in the brain has been demonstrated (8, 22). It was of interest that we found that the virulence of both the IHD-J and WR strains decreased significantly when the B5R gene was deleted. The slightly lesser attenuation of the W-B5R$^-$ virus compared with the I-B5R$^-$ virus, however, might be partly explained by the reliance of WR on other mechanisms of spread in vivo. In addition, the reduction in CEV and EEV production may only partly contribute to the attenuation of B5R$^-$ mutants. The product of the B5R gene shares significant homology with members of the family of complement control proteins and is most closely related to factor H (14, 37). Although there is as yet no evidence that gp42 inhibits complement activation or helps vaccinia virus to resist cellular humoral defenses, these possibilities need to be considered. In conclusion, we also point out that the identification and characterization of the viral components necessary for the production of EEV may assist in the development of attenuated vaccinia virus strains for use as recombinant vaccines.

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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., and B. Moss.** 1991. 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. J. Virol. **65**:5910–5920.
4. **Blasco, R., and B. Moss.** 1992. Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. J. Virol. **66**:4170–4179.

5. **Blasco, R., J. R. Sisler, and B. Moss.** 1993. Dissociation of progeny vaccinia virus from the cell membrane is regulated by a viral envelope glycoprotein: effect of a point mutation in the lectin homology domain of the A34R gene. J. Virol. **67**:3319–3325.
6. **Boulter, E. A., and G. Appleyard.** 1973. Differences between extracellular and intracellular forms of poxvirus and their implications. Prog. Med. Virol. **16**:86–108.
7. **Buller, R. M. L., S. Chakrabarti, J. A. Cooper, D. R. Twardzik, and B. Moss.** 1988. Deletion of the vaccinia virus growth factor gene reduces virus virulence. J. Virol. **62**:866–874.
8. **Buller, R. M. L., G. L. Smith, K. Cremer, A. L. Notkins, and B. Moss.** 1985. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature (London) **317**:813–815.
9. **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.
10. **Duncan, S. A., and G. L. Smith.** 1992. Identification and characterization of an extracellular envelope glycoprotein affecting vaccinia virus egress. J. Virol. **66**:1610–1621.
11. **Earl, P. L., N. Cooper, and B. Moss.** 1991. Preparation of cell cultures and vaccinia virus stocks, p. 16.16.1–16.16.7. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. F. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. Greene Publishing Associates & Wiley Interscience, New York.
12. **Engelstad, M., S. T. Howard, and G. L. Smith.** 1992. A constitutively expressed vaccinia gene encodes a 42-kDa glycoprotein related to complement control factors that forms part of the extracellular virus envelope. Virology **188**:801–810.
13. **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.
14. **Goebel, S. J., G. P. Johnson, M. E. Perkus, S. W. Davis, J. P. Winslow, and E. Paoletti.** 1990. The complete DNA sequence of vaccinia virus. Virology **179**:247–266.
15. **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.
16. **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.
17. **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.
18. **Ichihashi, Y., and S. Dales.** 1971. Biogenesis of poxviruses: interrelationship between hemagglutinin production and polykaryocytosis. Virology **46**:533–543.
19. **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.
20. **Ichihashi, Y., and M. Oie.** 1988. Epitope mosaic on the surface proteins of Orthopoxviruses. Virology **163**:133–144.
21. **Isaacs, S. N., E. J. Wolffe, L. G. Payne, and B. Moss.** 1992. Characterization of a vaccinia virus-encoded 42-kilodalton class I membrane glycoprotein component of the extracellular virus envelope. J. Virol. **66**:7217–7224.
22. **Lee, M. S., J. M. Roos, L. C. McGuigan, K. A. Smith, N. Cormier, L. K. Cohen, B. E. Roberts, and L. G. Payne.** 1992. Molecular attenuation of vaccinia virus: mutant generation and animal characterization. J. Virol. **66**:2617–2630.
23. **Miller, R. G.** 1973. Nonparametric estimators of the mean tolerance in bioassay. Biometrika **60**:535–542.
24. **Morgan, C.** 1976. Vaccinia virus reexamined: development and release. Virology **73**:43–58.
25. **Moss, B.** 1991. Poxviridae and their replication, p. 2079–2111. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. C. Melnick, T. P. Monath, and B. Roizman (ed.), Virology. Raven Press, New York.
26. **Payne, L.** 1978. Polypeptide composition of extracellular enveloped vaccinia virus. J. Virol. **27**:28–37.
27. **Payne, L. G.** 1979. Identification of the vaccinia hemagglutinin polypeptide from a cell system yielding large amounts of extracellular enveloped virus. J. Virol. **31**:147–155.
28. **Payne, L. G.** 1980. Significance of extracellular virus in the in vitro and in vivo dissemination of vaccinia virus. J. Gen. Virol. **50**:89–100.
29. **Payne, L. G.** 1992. Characterization of vaccinia virus glycoproteins by monoclonal antibody preparations. Virology **187**:251–260.
30. **Payne, L. G., and K. Kristensson.** 1985. Extracellular release of enveloped vaccinia virus from mouse nasal epithelial cells *in vivo*. J. Gen. Virol. **66**:643–646.
31. **Payne, L. G., and E. Norrby.** 1976. Presence of haemagglutinin in the envelope of extracellular vaccinia virus particles. J. Gen. Virol. **32**:63–72.
32. **Rodriguez, J. F., and M. Esteban.** 1987. Mapping and nucleotide sequence of the vaccinia virus gene that encodes a 14-kilodalton fusion protein. J. Virol. **61**:3550–3554.
33. **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.
34. **Schmutz, C., L. G. Payne, J. Gubser, and R. Wittek.** 1991. A mutation in the gene encoding the vaccinia virus 37,000-M_r protein confers resistance to an inhibitor of virus envelopment and release. J. Virol. **65**:3435–3442.
35. **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.
36. **Shida, H.** 1986. Nucleotide sequence of the vaccinia virus hemagglutinin gene. Virology **150**:451–462.
37. **Smith, G. L., Y. S. Chan, and S. T. Howard.** 1991. Nucleotide sequence of 42 kbp of vaccinia virus strain WR from near the right inverted terminal repeat. J. Gen. Virol. **72**:1349–1376.
38. **Sodeik, B., R. W. Doms, M. Ericsson, G. Hiller, C. E. Machamer, W. van't Hof, G. van Meer, B. Moss, and G. Griffiths.** 1993. Assembly of vaccinia virus: role of the intermediate compartment between the endoplasmic reticulum and the Golgi stacks. J. Cell Biol. **121**:521–541.
39. **Takahashi-Nishimaki, F., S.-I. Funahashi, K. Miki, S. Hashizume, and M. Sugimoto.** 1991. Regulation of plaque size and host range by a vaccinia virus gene related to complement system proteins. Virology **181**:158–164.
40. **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.