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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

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Vaccinia virus strains vary considerably in the amounts of extracellular enveloped virus (EEV) that they release from infected cells. The IHD-J strain produces up to 40 times more EEV than does the related WR strain and consequently generates elongated comet-shaped virus plaques instead of sharply defined round ones in susceptible monolayer cells under liquid medium. The difference in EEV formation is due to the retention of enveloped WR virions on the cell surface (R. Blasco and B. Moss, J. Virol. 66:4170–4179, 1992). By using WR and IHD-J DNA fragments for marker transfer and analyzing the progeny virus by the comet formation assay, we determined that gene A34R and at least one other gene regulate the release of cell-associated virions. Replacement of the A34R gene of WR with the corresponding gene from IHD-J increased the amount of EEV produced by 10-fold and conferred the ability to form distinctive comet-shaped plaques. Gene A34R encodes an EEV-specific glycoprotein with homology to C-type animal lectins (S. A. Duncan and G. L. Smith, J. Virol. 66:1610–1621, 1992). The nucleotide sequences of the A34R genes of WR and IHD-J strains differed in six positions, of which four were silent. One of the codon mutations (Lys-151→Glu), which is located in the putative carbohydrate recognition domain, was sufficient to transfer a comet-forming phenotype to WR virus. These data indicate that the A34R-encoded glycoprotein is involved, through its lectin homology domain, in the retention of progeny virus on the surface of parental cells and raise the possibility that the protein also has a role in virus attachment to uninfected cells.

Vaccinia virus, the representative orthopoxvirus, is a large DNA-containing virus that replicates in the cytoplasm of infected cells (24). Assembly of progeny virions begins in specialized areas of the cytoplasm termed viral factories, where crescent-shaped membrane structures arise and develop into spherical shells enclosing granular material (7). As maturation proceeds, the structures become electron dense, oval in shape, dispersed in the cytosol, and infectious. These particles are called intracellular naked virus (INV), even though they have a lipoprotein membrane. Some INV become wrapped by a double layer of cisternal membrane and transported to the cell periphery, where fusion of the outermost viral membrane with the plasma membrane results in their translocation into the extracellular space (20, 23, 29). The translocated particles contain an additional membrane relative to INV and may remain attached to the cell surface or be released into the medium; the two forms of virus have been referred to as cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV), respectively. The EEV envelope has been characterized biochemically (18, 25, 26, 28) and is presumably similar or identical to that of CEV. The major protein component is a palmitylated 37-kDa species encoded by gene F13L (19). In addition, there are several envelope glycoproteins, including the 89-kDa hemagglutinin encoded by gene A56R (33), a 42-kDa species encoded by gene B5R (14, 21), and 21- to 24-kDa proteins encoded by gene A34R (10). (The gene nomenclature used in this report is that of Goebel et al. [17] rather than that of the original authors.)

Several observations indicate that INV, despite their intrinsic infectivity, are inefficient for virus transmission in tissue culture cells and highlight the importance of the virus envelope. The drug N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazide (18, 29, 32) and deletion or repression of the genes encoding the 37-kDa (2), 42-kDa (36) and 21- to 24-kDa (10) EEV proteins or the 14-kDa INV protein (31) inhibit membrane wrapping of INV and reduce plaque size. EEV has been implicated in long-range virus spread in cell culture and in virus dissemination in vivo (5, 27, 30), whereas CEV can mediate cell-to-cell virus transmission (2, 3). The quantity of EEV produced by individual virus strains varies greatly (26, 27). With two related and widely used strains, WR and IHD-J, similar amounts of CEV are produced despite differences in EEV formation (3). In the present study, we demonstrate that a single nucleotide substitution in the A34R genes of WR and IHD-J is largely responsible for the differential retention of mature, enveloped virus at the plasma membrane.

MATERIALS AND METHODS

Cells and viruses. Vaccinia virus strain IHD-J was obtained from S. Dales, University of Western Ontario, London, Ontario, Canada; the WR strain is available from the American Type Culture Collection (ATCC VR1354).

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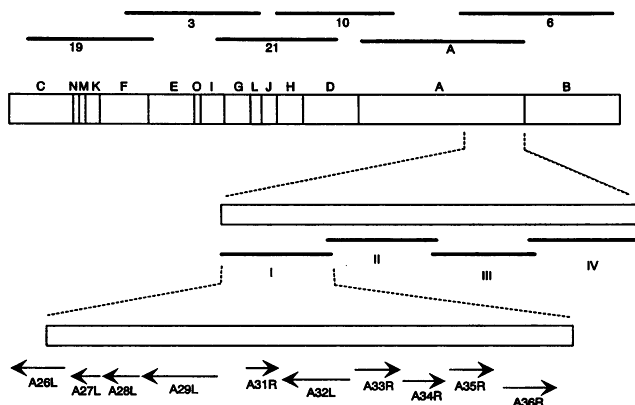

FIG. 1. Viral DNA fragments used for marker transfer. The *Hin*dIII restriction endonuclease map of the vaccinia virus strain WR genome (7) is shown as a segmented bar near the top. The lines above the genome map indicate the approximately 40-kbp WR DNA segments contained within Cos 19, 3, 21, 10, A, and 6 (22). Below the genome map, the DNA shared by Cos A and 6 has been expanded. The lines labeled I to IV indicate the segments of IHD-J DNA that were amplified by PCR. Arrows at the bottom indicate the locations, sizes, and directions of ORFs within segment I of the WR strain (34). The following ORFs were expected to be contained within the fragments: I, A26L partial through A37R partial; II, A37R partial through A44L partial; III, A44L partial through A50R partial; and IV, A50R partial through A57R partial.

Viruses were routinely propagated and titrated in BSC-1 cells. Plaquing, infection, and transfection were carried out according to standard protocols (11–13).

Marker rescue with cosmids. To increase the proportion of recombinants, a modification of the protocol of Fathi et al. (16) was used. Briefly, CV-1 monolayers (25 cm^2) were infected at a multiplicity of 1 with vRB10, an IHD-J mutant with a deletion in the VP37 gene (2). After adsorption for 1 h, the cells were transfected with a mixture of pRB21, a plasmid that contains an intact and functional VP37 gene (4), and DNA from a WR cosmid library (22). After 2 days, the cells were harvested, freeze-thawed three times, and sonicated. Progeny virus was analyzed by plaque assay on fresh BSC-1 monolayers (150 cm^2); after 36 h, the cells were stained with crystal violet.

Marker rescue with DNA fragments. Monolayers of CV-1 cells were infected at a multiplicity of 1 with vRB12, a WR mutant with a deletion in the VP37 gene (3), and subsequently transfected with a mixture of WR DNA and individual fragments (I to IV) generated by polymerase chain reaction (PCR) using IHD-J DNA as the template. The IHD-J DNA segments amplified by the primers correspond to the following DNA segments of the Copenhagen strain (17) of vaccinia virus: I (138,721 to 144,960); II (144,721 to 150,960); III, (150,721 to 156,601); and IV (156,361 to 162,601). The following primer pairs used for the amplification were synthesized with a model 394 DNA/RNA synthesizer (Applied Biosystems): I, 5′-AGCTCCTAATGGAGA TTT and 5′-TGGTACATCTCATTGTCA; II, 5′-TTTATAA AATTGAAGTAATAT and 5′-GTTTAATCTTCTATTGA TG; III, 5′-GTTCGTTTGAATGCCACA and 5′-TAGATG AGTAGATTCTTT; and IV, 5′-GACCAATTAACGATGA TT and 5′-TTTGCCAATATCACGCGA.

For fine mapping, subfragments of I were amplified by PCR and used for transfection. The DNA that gave a positive result in the marker rescue was a 600-bp subfragment amplified by using the primers 5′-GTTAGAAAGTATT TTTGTG and 5′-ACGTTAACGACTTATTAT.

DNA sequencing. Viral DNA, from purified virions or cytoplasmic extracts, was used as a template for PCR. The amplified DNA was purified by using magnetic beads as a solid support and sequenced by using fluorescent dideoxynucleotides and an Applied Biosystems model 373A automated sequencer as described in protocols provided by Applied Biosystems.

PCR mutagenesis. Primer oligonucleotides, synthesized with point mutations, were used for PCR with WR genomic DNA as the template, and the resulting products were gel purified. Then, two overlapping PCR products with mutations close to the ends were simultaneously assembled into a single DNA fragment and amplified by PCR. The latter DNA was used directly to transfect cells. The oligonucleotide primers, with underlined nucleotide substitutions relative to the WR sequence, were as follows: for mutation Asp-110→Asn, PCR 1, 5′-CTTGGAAACAATTCATAA and 5′-CTAACCATTTATTATTGGTCT, and PCR 2, 5′-AAAGAC CAATAATAAATGGT and 5′-TGGTACATCTCATTGT CA; for mutation Lys-151→Glu, PCR 1, 5′-CTTGGAAACA ATTCATAA and 5′-CATACTGTTTCAACCAGTTTT, and PCR 2, 5′-CTGGAAAACTGGTTGAAACAG and 5′-TGGT ACATCTCATTGTCA.

RESULTS

Mapping of a gene responsible for comet formation. Vaccinia virus is routinely titrated by plaque assay on susceptible cell monolayers under liquid medium. Under these conditions, some vaccinia virus strains have characteristic plaque morphologies that are related in part to the amount of released EEV. Vaccinia virus strain WR, in which less than 1% of the infectious progeny are EEV, gives rise to round, well-defined virus plaques. By contrast, up to 30% of the infectious progeny of strain IHD-J are EEV, and the plaques appear larger, with a diffuse elongated comet shape caused by the distribution of EEV-derived secondary plaques.

To localize the gene or genes responsible for the strain difference in EEV formation, a marker transfer assay was devised. We considered transfecting IHD-J DNA into cells infected with WR virus to produce recombinants that would form comet-shaped plaques or transfecting WR DNA into cells infected with IHD-J virus to produce recombinants that would form round plaques. Although we recognized that it would be easier to detect a small number of comets in a field of round plaques rather than the reverse, the availability of a complete set of overlapping cosmids of WR genomic DNA (22) led us to choose the less desirable approach for the initial screening. We used two markers in the transfection assay because the expected ratio of double recombinants to single recombinants is higher than the ratio of single recombinants to wild-type virus when a single marker is used (16). Therefore, to increase the proportion of desired recombinant viruses, we infected cells with a VP37 deletion mutant of IHD-J virus that is blocked in plaque formation (2) and transfected a plasmid containing a complete VP37 gene as a nonlinked selectable marker together with WR-derived cosmids. Therefore, all comet-shaped plaques should be products of at least one recombination event, between the IHD-J genome and the VP37 gene, whereas round plaques should be products of at least two recombination events, between the IHD-J genome and both the VP37 gene and DNA from one of the cosmids.

The experiments were carried out in two steps. First, the

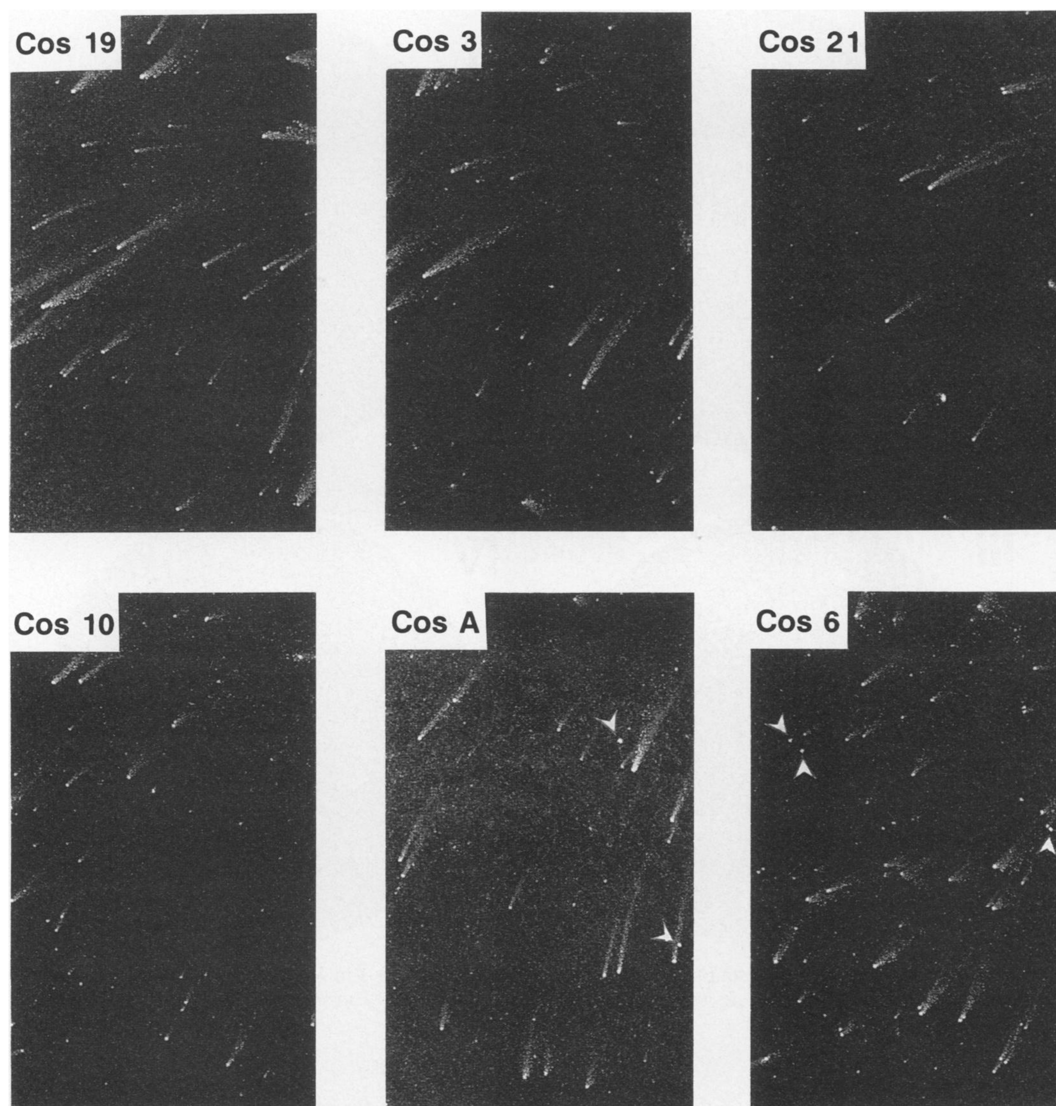

FIG. 2. Marker transfer mediated by cosmids containing strain WR DNA. Individual cosmids (Fig. 1) were transfected into cells infected with vaccinia virus strain IHD-J, and the cell-associated progeny were analyzed by plaque assay and stained with crystal violet. Representative round plaques, typical of vaccinia virus strain WR, are indicated by arrowheads.

cells were infected and transfected; after 2 days, the medium was discarded and the progeny virus was released by disrupting the cells. Then, the virus was diluted and applied to cell monolayers, which were subsequently stained with crystal violet. In our initial attempts, it was difficult to distinguish round primary plaques from the more numerous secondary plaques caused by comet-forming virus. For better separation of plaques, succeeding experiments were carried out in large flasks with cell monolayers of 150 cm^2 rather than in six-well plates and staining was performed after 36 rather than 48 h. With these modifications, candidate recombinant virus-produced round plaques could be distinguished from smaller secondary IHD-J plaques and were detected when either of two cosmids, Cos A and Cos 6, with overlapping sequences was transfected (Fig. 1, top; Fig. 2, arrowheads). The possibility that genes affecting comet size were also present in other cosmids could not be ruled out because of the relative insensitivity and subjectiveness of the assay.

As noted above, we anticipated that the detection of comets in a field of round plaques would be far easier than the procedure used. Therefore, having localized at least one candidate gene with the WR cosmid library, we changed strategies. The overlap region of Cos A and Cos 6 was divided into four segments (Fig. 1, I to IV). Using the vaccinia virus (Copenhagen strain) sequence (17) as a guide, oligonucleotide primers were made, and the four segments of IHD-J DNA were amplified by PCR. Cells were infected with a VP37 deletion mutant of vaccinia virus strain WR, vRB12 (3), and cotransfected with the individual PCR products and genomic WR DNA. Since a putative comet-forming recombinant would be expected to produce more extracellular virus than the parental WR virus, a step of EEV selection was carried out to increase the proportion of the desired recombinant virus. The selection was achieved by a second round of infection in which the virus was harvested from the medium instead of the cells. Transfection with fragment I produced many comets resembling those of

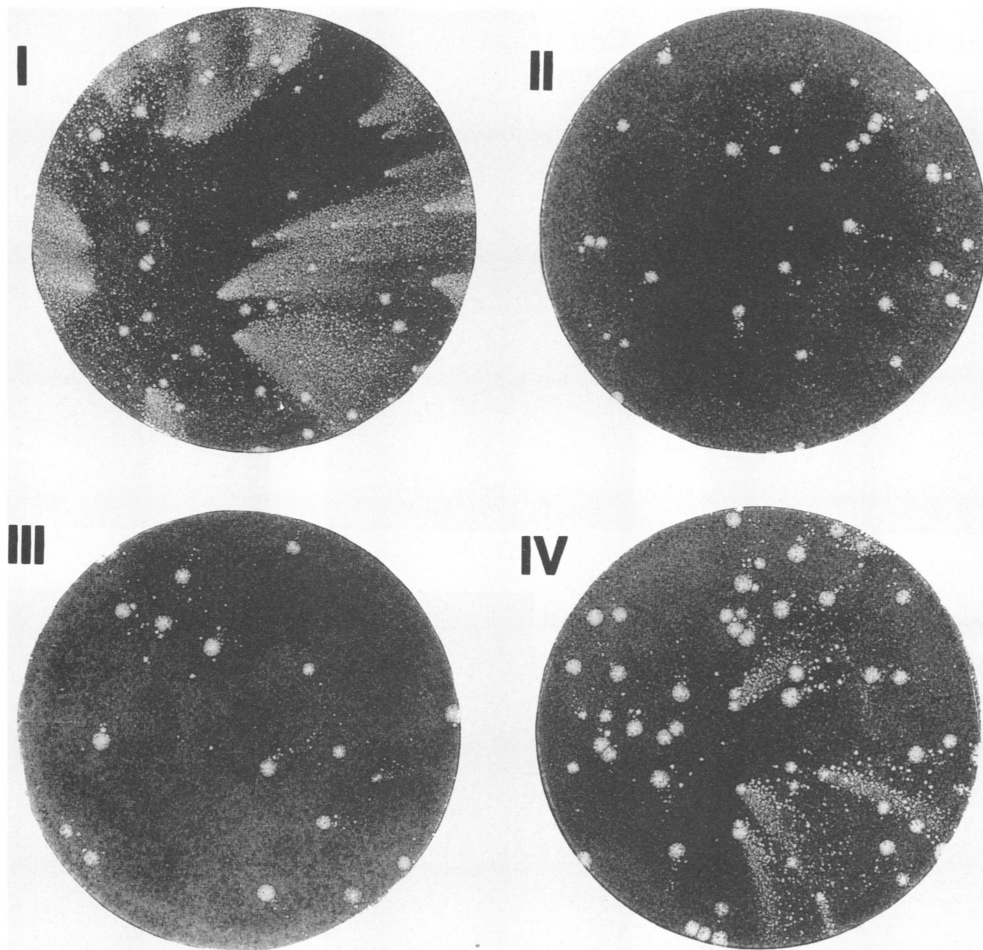

FIG. 3. Marker transfer mediated by PCR-amplified IHD-J DNA. DNA segments I to IV (Fig. 1), PCR amplified from a genomic IHD-J DNA template, were used to transfect cells infected with vaccinia virus strain WR. Extracellular virus was analyzed by plaque assay and stained with crystal violet.

IHD-J, whereas fragments II and III did not (Fig. 3). Transfection with fragment IV also yielded comets, but they had tails more narrow than those produced with fragment I. For the present study, we chose to map and characterize the marker DNA within fragment I because it produced large comets.

Characterization of a recombinant WR virus containing the A34R gene from IHD-J. If the IHD-J sequence is similar to that of WR, then fragment I should contain nine complete open reading frames (ORFs) (Fig. 1). Marker transfer assays were repeated with PCR fragments of IHD-J DNA predicted to contain one or more ORFs. Only one of these, a 600-bp fragment spanning the A34R gene, was able to confer the comet-forming phenotype to vaccinia virus WR (data not

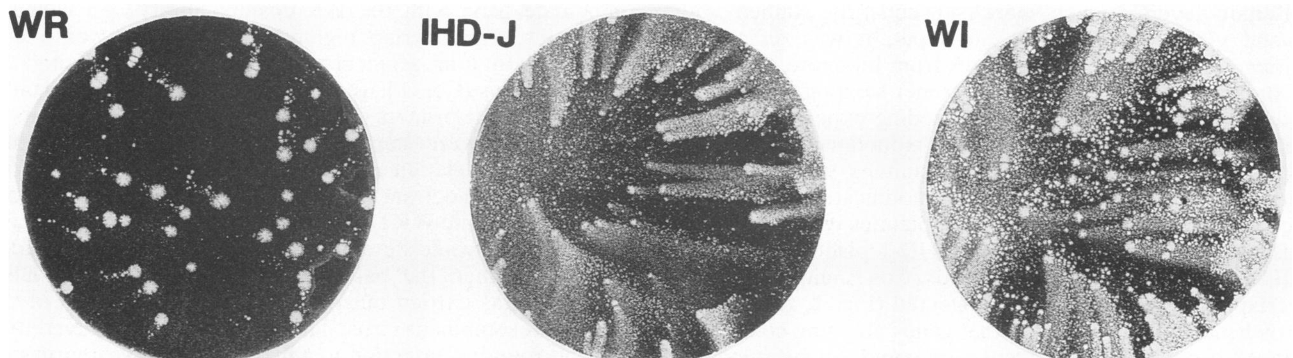

FIG. 4. Plaque formation by parental and recombinant vaccinia virus strains. Plaques formed by WR, IHD-J, and recombinant WI containing the IHD-J A34R gene in a WR background were stained after 48 h with crystal violet.

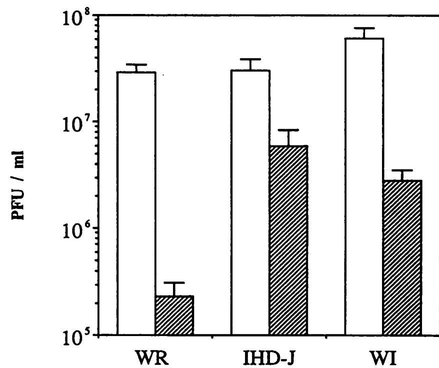

FIG. 5. Intracellular and extracellular virus yields obtained after infection with WR, IHD-J, and WI. Open bars, cell-associated virus; hatched bars, virus in the medium.

shown). A comet-forming virus derived by transfecting the A34R gene from IHD-J into WR virus-infected cells was isolated by two consecutive rounds of plaque purification. The resulting virus, termed WI, produced comet-shaped plaques on BSC-1 monolayers that were similar to those of IHD-J plaques (Fig. 4). In a one-step growth curve, WI released about 10 times more infectious virus into the medium than did WR, though this was still less than the amount released by IHD-J (Fig. 5). Therefore, other genes in IHD-J may also contribute to enhanced virus release. As judged from the marker transfer results (Fig. 3), one candidate is located in fragment IV (Fig. 1).

Sequence of the A34R gene of IHD-J. The sequence of the A34R gene of vaccinia virus WR has been reported (34), whereas that of IHD-J has not. To compare them, the 600 bp of coding and flanking sequences of the A34R genes used in the transfection assays were PCR amplified from WR and IHD-J virus genomic DNA and sequenced. The WR sequence was identical to the published one; the IHD-J sequence contained six nucleotide differences, all of which were located within the coding region (Fig. 6A). Four of the nucleotide substitutions were silent mutations, whereas the remaining two resulted in amino acid changes, Asp-110→Asn and Lys-151→Glu (Fig. 6B).

A single amino acid substitution in the A34R gene can transfer the comet-forming phenotype to vaccinia virus strain WR. We wished to determine whether one or both of the amino acid substitutions were necessary to confer the comet-forming trait to vaccinia virus strain WR. With that aim, we used a recombination PCR strategy to generate DNA fragments derived from the WR sequence in which either one or both of the mutations occurred. The results of marker transfer using those fragments are shown in Fig. 7. Only the introduction of the Lys-151→Glu mutation, alone or in combination with the other mutation, resulted in the appearance of comets. That those comet-forming viruses had incorporated the Lys-151→Glu mutation was confirmed by PCR genomic sequencing of three separate plaque-purified viral clones.

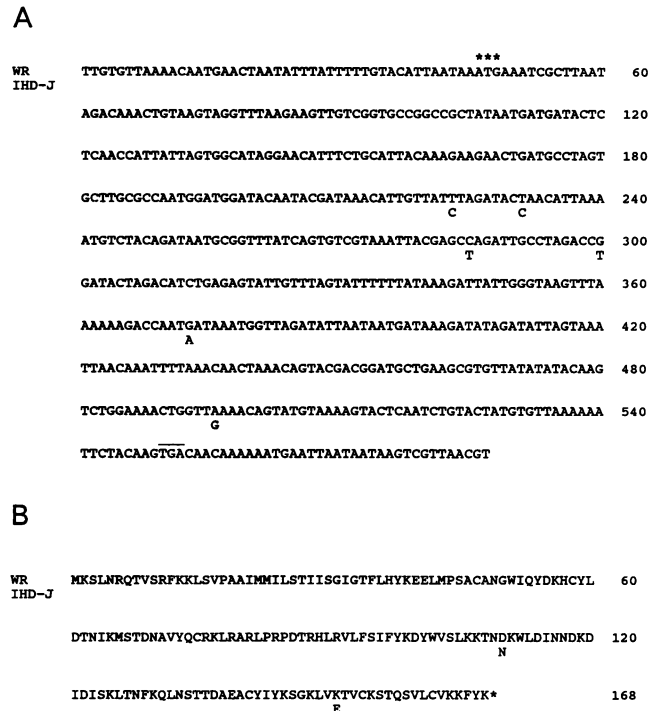

FIG. 6. Nucleotide and amino acid sequences of the A34R genes of WR and IHD-J. (A) The entire A34 sequence is shown for WR, and the six nucleotide differences in IHD-J are shown below. The ATG at the start of the ORF is indicated by asterisks. The TGA at the end of the ORF is indicated by a bar. (B) The deduced amino acid sequence of the A34 ORF of WR is shown, with the two amino acid differences in IHD-J shown below. The asterisk indicates the C terminus of the translated protein.

DISCUSSION

Genetics provides a powerful tool for unraveling the complex steps involved in the envelopment of vaccinia virions with intracellular membranes, the fusion of the outer viral and plasma membranes, and the detachment of the virus from the cell surface. In this study, we attempted to account for the past observation that different amounts of extracellular virus are liberated from cells infected with the closely related WR and IHD-J strains of vaccinia virus (27). Marker transfer procedures led to the conclusion that a mutation in gene A34R can regulate the release of progeny virions from the surface of parental cells.

The WR and IHD-J strains of vaccinia virus are derivatives of the New York City Board of Health strain (6), and both have been used extensively for laboratory studies. In a preceding investigation (3), we demonstrated that the low amount of EEV produced by the WR strain is not due to a defect in the membrane wrapping of INV or translocation of the enveloped virus. Indeed, electron micrographs showed that the surface of infected cells was studded with enveloped WR virions. Release of infectious virus, with the buoyant density of EEV and capable of causing comet-shaped plaques characteristic of IHD-J, could be effected by incubation of the WR-infected cells with trypsin. These experiments indicated that the lower EEV production by WR than by IHD-J is due to a greater retention of virions at the cell surface. Gene transfer experiments ruled out the possibility that VP37, the major protein constituent of the EEV membrane, was responsible for the variation in surface retention of virions. Rather than testing other candidate genes individually, we carried out comprehensive marker transfer experiments. Interestingly, the comet phenotype was acquired by transfection with two separate DNA fragments. However, one DNA fragment alone had a major effect, and further

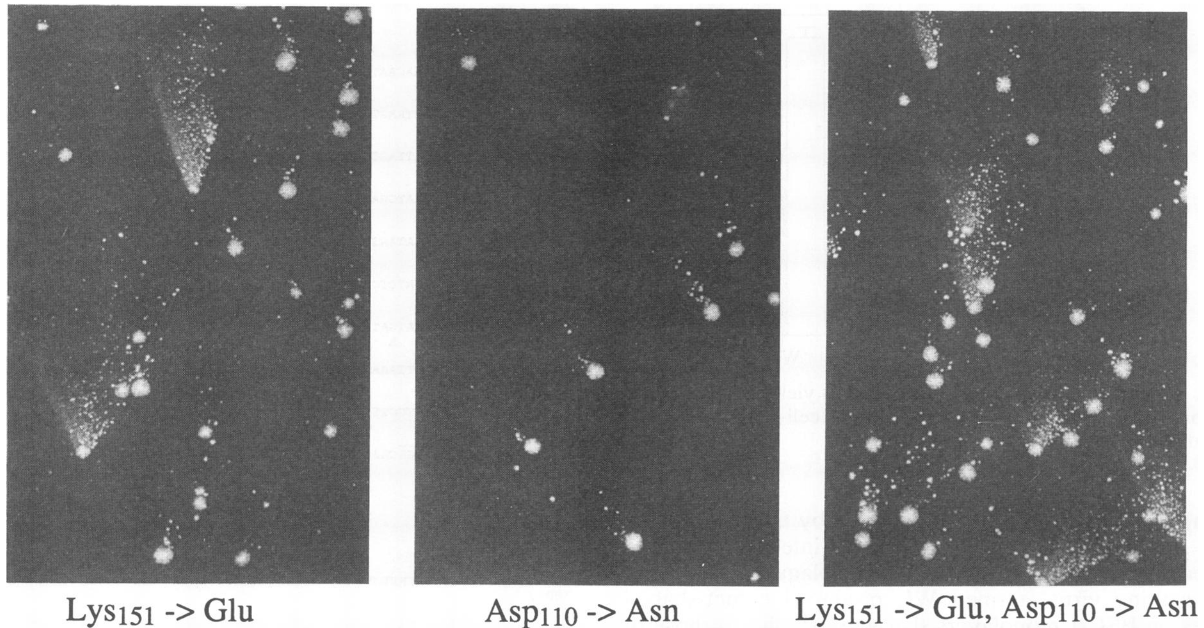

FIG. 7. Marker transfer of A34R genes of WR containing one or two nucleotide substitutions. A PCR strategy was used to construct A34R genes of vaccinia virus strain WR containing the single-nucleotide substitution Asp-110→Asn or Lys-151→Glu or both substitutions. The PCR-amplified DNA was used to transfect cells infected with vaccinia virus strain WR. Extracellular virus was analyzed by plaque assay and stained with crystal violet.

marker transfer experiments mapped the activity to the A34R ORF. Sequence analysis indicated only six nucleotide differences between the A34R genes of the two virus strains, and four were silent mutations in the coding region. Of the coding changes, the Lys-151→Glu substitution was necessary and sufficient to confer a comet phenotype on WR virus. The A34R gene sequence of the Copenhagen strain of vaccinia virus also has a Lys at position 151 (17) and forms round plaques. A Lys at position 151 is also present in the Harvey (1) and Bangladesh (15) strains of variola virus, further suggesting that IHD-J is unusual in having a Glu at this site.

The A34R gene, previously shown to encode an EEV-specific glycoprotein (10), is predicted to be a type II membrane protein with homology to C-type animal lectins (34). In this regard, the single amino acid change necessary to induce virus release and comet formation lies in the conserved carbohydrate recognition domain (9, 35). Intriguingly, alignments suggest that this position is occupied by an aspartic acid residue in several members of the lectin family (34); consequently, the glutamic acid in the IHD-J version of the protein could be a more conservative change than the lysine in the WR gene. Experiments to determine whether the WR and IHD-J versions of the A34R glycoprotein actually have carbohydrate and cell surface binding properties are needed.

Duncan and Smith (10) reported that repressed expression of the A34R gene by a recombinant vaccinia virus (strain WR) led to small-plaque formation. In addition, their electron micrographs indicated the absence of wrapped INV, as had been found when the 37-kDa EEV protein (2) and the 14-kDa INV protein (31) were deleted and repressed, respectively. On this basis, the authors (10) suggested a role for the A34R gene in INV wrapping. Our results suggest a different or second role for the glycoprotein encoded by A34R, namely, the association of enveloped virions with the plasma membrane. This idea could explain the observation that the relative amount of extracellular WR virus was increased when A34R expression was repressed (10).

The finding that a single amino acid substitution in the lectin homology region of the A34R envelope glycoprotein modified the binding of enveloped virus to the cell suggests some interesting possibilities. First, the glycoprotein may mediate the retention of virions at the plasma membrane by binding to a component that could be a carbohydrate. Furthermore, a similar interaction might be involved in the adsorption of enveloped virus to the uninfected cell during the initial stage of infection. If this is the case, the large amounts of EEV produced by IHD-J might be a consequence of decreased adsorption as well as increased virus release.

The A34R glycoprotein is not the only determinant of virus release from the cell membrane, since the recombinant strain produced less EEV than did IHD-J. Moreover, a second IHD-J DNA fragment by itself was able to transfer a small comet phenotype to the WR virus. The latter IHD-J fragment is predicted to contain seven ORFs, including one encoding the viral hemagglutinin, another glycoprotein constituent of the EEV membrane. Further marker transfer experiments should reveal whether mutations in the hemagglutinin or another gene enhance the release of EEV.

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