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Vaccinia Virus Glycoprotein A34R Is Required for Infectivity of Extracellular Enveloped Virus

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The vaccinia virus strain Western Reserve (WR) A34R gene encodes a C-type lectin-like glycoprotein, gp22-24, that is present in the outer membrane of extracellular enveloped virus (EEV) with type II membrane topology (S. A. Duncan and G. L. Smith, J. Virol. 66:1610–1621, 1992). Here we show that a WR A34R deletion mutant (WRΔA34R) released 19- to 24-fold more EEV from infected cells than did WR virus, but the specific infectivity of the released virions was reduced 5- to 6-fold. Rupture of the WRΔA34R EEV outer envelope by freeze-thawing increased virus infectivity by five- to sixfold, because of the release of infectious intracellular mature virus. All other known EEV-specific proteins are incorporated into WRΔA34R EEV, and thus the loss of gp22-24 is solely responsible for the reduction of EEV specific infectivity. The WRΔA34R virus is highly attenuated in vivo compared with WR or a revertant virus in which the A34R gene was reinserted into WRΔA34R. This attenuation is consistent with the known important role of EEV in virus dissemination and virulence. Vaccinia virus strain International Health Department-J (IHD-J) produces large amounts of EEV and forms comets because of an amino acid substitution within the A34R protein (R. Blasco, R. Sisler, and B. Moss, J. Virol. 67:3319–3325, 1993), but despite this, IHD-J EEV has a specific infectivity equivalent to that of WR EEV. Substitution of the IHD-J A34R gene into the WR strain induced comet formation and greater release of EEV, while coexpression of both genes did not; hence, the WR phenotype is dominant. All orthopoxviruses tested express the A34R protein, but most viruses, including variola virus, have the WR rather than the IHD-J A34R genotype. The A34R protein affects plaque formation, EEV release, EEV infectivity, and virus virulence.

Vaccinia virus (VV) is an orthopoxvirus and the live vaccine used to eradicate smallpox. It replicates in the cell cytoplasm, encodes many enzymes and virulence factors, and produces two infectious forms termed intracellular mature virus (IMV) and extracellular enveloped virus (EEV) (for reviews, see references 11, 25, and 38). EEV represents only a small minority of infectious progeny (≤1%) with most VV strains, such as the Western Reserve (WR) strain, but with the International Health Department-J (IHD-J) strain, 30% of progeny virus is EEV (28). EEV is distinguished from IMV structurally by the possession of an additional lipid envelope that is derived from the trans-Golgi network (32), antigenically by the possession of at least 10 polypeptides that are absent from IMV (4, 27, 28), and biologically by being the form of virus that mediates long-range virus dissemination and comet formation (9, 29). Immunization with EEV proteins confers protection against orthopoxvirus challenge, whereas immunization with inactivated IMV does not (3, 8, 29, 43).

Five genes are known to encode proteins present in the EEV outer envelope. These are A56R, encoding the virus hemagglutinin gp86 (28, 36); F13L, encoding a 37,000-molecular-weight protein (37K protein), p37 (19); A34R, encoding a triplet of glycoproteins, gp22-24 (13); B5R, encoding a 42K glycoprotein, gp42 (14, 21); and A36R, encoding a 45K to 50K protein, p45-50 (26). These account for the majority of proteins detected in the outer EEV envelope (28). A preliminary understanding of the functions of these proteins has been deduced by analyses of virus mutants which have specific amino acid substitutions or in which one of the genes either is repressed by the *Escherichia coli lac* operator/repressor or is deleted. Thus, it has been demonstrated that deletion or mutation of F13L (5, 33) or B5R (15, 23, 44) greatly reduces plaque size and EEV formation, while deletion of A36R produces a modest reduction in plaque size and a fivefold reduction in EEV formation (26). In contrast, loss of A56R does not reduce plaque size but causes cell fusion (20), and repression of A34R causes a small-plaque phenotype (13). The A34R protein also affects the release of cell-associated enveloped virus (CEV) from infected cells, since a K-151→E (K151E) mutation in the IHD-J virus strain enables the release of larger amounts of EEV (7). None of the EEV proteins affect the production or infectivity of IMV, and hitherto no EEV protein has been shown to affect EEV infectivity. The cellular molecules to which EEV binds are unknown.

Here we report the further characterization of the A34R gene function by the construction of an A34R null mutant (WRΔA34R) and a VV WR strain which expresses either the IHD-J A34R gene alone or both the IHD-J and WR A34R genes upon addition of IPTG (isopropyl-β-D-thiogalactopyranoside). The data presented demonstrate that the A34R protein has multiple functions and controls plaque size, comet formation, EEV release, EEV infectivity, and virus virulence.

MATERIALS AND METHODS

Cells and viruses. RK-13, B-SC-1, and TK-143 cells were grown in modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS). VV strains WR and IHD-J were propagated in B-SC-1 or RK-13 cells. Buffalopox virus, camelpox virus, cowpox virus (strain Brighton Red), elephantpox virus, rabbitpox virus, and VV strains Copenhagen, Dairen, IHD-W, King Institute, Lister, Patwadangar, Tashkent, Tian Tan, USSR, and Wyeth were obtained and grown as described previously (2, 41).

Construction of recombinant viruses. (i) WRΔA34R. The VV WR A34R gene was inactivated by transfecting CV-1 cells, which had been infected with WR at 0.01 PFU per cell, with plasmid pSAD8, which contains a version of the A34R

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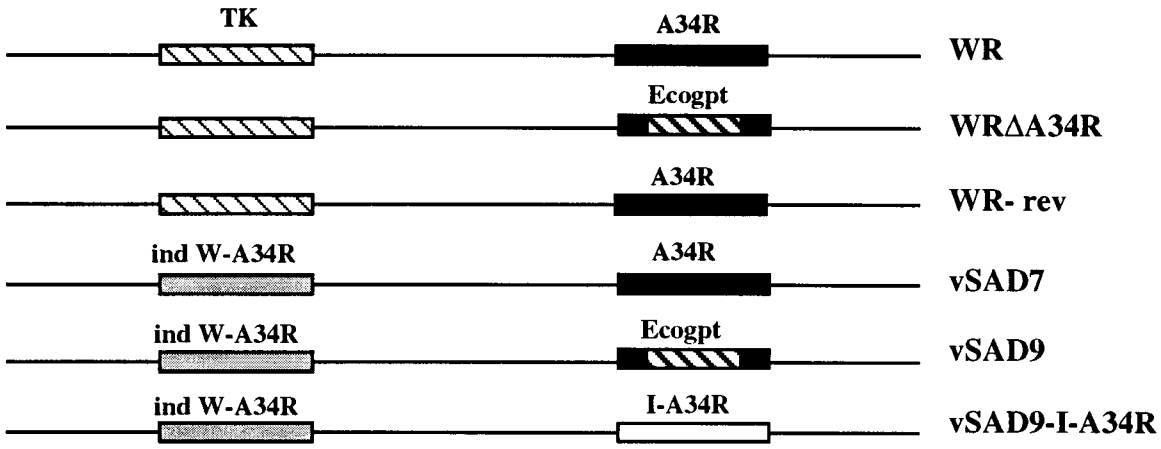

FIG. 1. Schematic representation of recombinant virus genomes. The VV strain WR genome is shown with the thymidine kinase (TK) (▨) and A34R (■) genes highlighted. In WRΔA34R the A34R gene has been replaced with the Ecogpt gene (▨), and in WR-rev this has been restored to WT. Viruses vSAD7 and vSAD9 have been described previously (13) and contain an IPTG-inducible (ind) version of the WR A34R gene (▨) in the TK locus. vSAD9 also has the natural A34R gene replaced with Ecogpt. Virus vSAD9-I-A34R was derived from vSAD9 by insertion of the IHD-J A34R gene (□) into the A34R gene locus.

gene with 80% of the coding sequence deleted and replaced by the *E. coli* xanthine guanine phosphoribosyltransferase (Ecogpt) gene driven by the VV 7.5K promoter (13). Virus isolates expressing Ecogpt were selected by plaque assay in the presence of mycophenolic acid (MPA) (10, 17). Initially, wild-type (WT)-sized MPA-resistant plaques which had resulted from the insertion of plasmid vSAD8 into the VV genome by a single recombination event and which still expressed the A34R gene were selected. Such a virus was then allowed to resolve into either WT virus or a virus with an inactivated A34R gene (WRΔA34R) by recombination between the repeated regions of the A34R gene. The latter virus was identified as small plaques that developed after growth for 7 days with MPA selection. After two further rounds of plaque purification in the absence of MPA selection, a virus was amplified and its genome was analyzed by Southern blotting (40) and by PCR with oligonucleotide primers specific for the Ecogpt gene and sequences flanking the A34R gene. These analyses confirmed that WRΔA34R had the predicted genome structure.

(ii) WR-rev. A revertant virus in which the A34R gene was reinserted into the WRΔA34R genome was formed by marker rescue with a plasmid containing the whole A34R gene. CV-1 cells infected with WRΔA34R were transfected with a DNA fragment containing the WR A34R gene, and 2 days later the progeny virus was harvested and plated onto B-SC-1 cells. Plaques of WT size that had formed 2 days later and which lacked the Ecogpt gene were selected, plaque purified twice more, and then amplified. The genome of such a virus was analyzed by PCR analysis as described above, and the genome was shown to contain the WR A34R gene but not the Ecogpt gene.

(iii) vSAD9-I-A34R. A virus which inducibly expresses the WR A34R gene from the thymidine kinase locus and which expresses the IHD-J A34R gene under control of the A34R gene promoter at its natural locus was constructed from vSAD9 (13) by transfecting vSAD9-infected CV-1 cells with a PCR fragment containing the IHD-J A34R gene. A recombinant virus, vSAD9-I-A34R, which formed a WT-sized plaque in the absence of IPTG induction was selected.

PCR. The IHD-J A34R gene was amplified from IHD-J viral DNA by PCR with oligonucleotide primers 5′-GATGTATCACAAGAAGT-3′ and 5′-AATAACAAACGCGGCGT-3′. Cycles were (i) 1 min at 94°C, 1 min at 42°C, and 2 min at 72°C; (ii) 20 cycles of 1 min at 94°C, 1 min at 42°C, and 2 min at 72°C; and (iii) 1 min at 94°C, 1 min at 42°C, and 5 min at 72°C. A 507-bp fragment was sequenced by using fluorescent dideoxynucleotides and an ABI automated sequencing machine.

Growth curves. Virus stocks were propagated in B-SC-1 cells and used to infect RK-13 cells at 10 PFU per cell. After 90 min, the virus inoculum was removed and the cell monolayers were washed three times with phosphate-buffered saline (PBS) and then overlaid with MEM containing 2.5% FBS. The extracellular virus was titrated after an initial centrifugation of the culture supernatant at 2,000 rpm for 10 min in a Beckman GPR benchtop centrifuge to remove cell debris. For intracellular virus the infected cells were scraped from the tissue culture flask, collected by centrifugation at 2,000 rpm for 10 min in a Beckman GPR benchtop centrifuge resuspended in 1 ml of MEM containing 2.5% FBS, and then subjected to three rounds of freeze-thawing followed by sonication. Both extracellular and intracellular viruses were titrated in duplicate on B-SC-1 cells.

Cesium chloride (CsCl) density gradients. RK-13 cells were infected at 10 PFU per cell. The inoculum was removed at 1.75 h postinfection (hpi), and following three washes with PBS, the cells were incubated in MEM containing 2.5% FBS and [^{3}H]thymidine (5 μCi/ml). Extracellular virus was isolated at 24 hpi by clarifying the supernatant at 2,000 rpm for 10 min in a Beckman GPR benchtop centrifuge and then pelleting the virus at 14,000 rpm for 60 min in a Beckman SW28 rotor. The pelleted EEV was resuspended in 1 ml of 10 mM Tris-HCl (pH 9.0), layered onto a CsCl density gradient (1.2 g/ml [4.5 ml], 1.25 g/ml [3.5 ml], and 1.3 g/ml [2.5 ml]), and centrifuged at 25,000 rpm for 95 min in a Beckman SW41 rotor. Following centrifugation, gradient fractions were collected dropwise from the bottom of the tube, the radioactivity in an aliquot of each fraction was counted in a scintillation counter, and the density of each fraction was determined by refractometry. Those fractions with a density identified as corresponding to the density of EEV (1.23 g/ml) were pooled, and the infectivity was determined by plaque assay on B-SC-1 cells.

Western blotting (immunoblotting) of purified EEV. RK-13 cell monolayers were infected at 0.1 PFU per cell. After 3 days, the unlabeled EEV was purified by CsCl density gradient centrifugation as described above. Following dialysis, 1 mg of purified EEV protein was mixed with an equal volume of 2× protein sample buffer (22), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to a nitrocellulose membrane (42). To detect EEV-specific proteins, the membranes were incubated with rabbit antisera specific for B5R (diluted 1/1,000) (14), A56R (diluted 1/2,500) (20), F13L (diluted 1/100,000) (19), and A36R (diluted 1/250) (26). Bound immunoglobulin was detected with enhanced chemiluminescence (Amersham plc, Little Chalfont, United Kingdom) according to the manufacturer's instructions.

Measurement of virus virulence. Groups of five 6-week-old, female BALB/c mice were infected intranasally with 10^4, 10^5, 10^6, or 10^7 PFU of WR, WRΔA34R, or WR-rev in 20 μl. The input virus was titrated on the day of infection on B-SC-1 cells to confirm the dose of inoculated virus. Each mouse was weighed daily, and animals whose body weight was less than 30% of that on day zero were sacrificed by cervical dislocation.

RESULTS

A recombinant VV, vSAD9, which had the endogenous A34R gene deleted and an ectopic copy of the A34R gene regulated by the *E. coli lac* repressor was described previously (13). This virus formed a very small plaque when A34R expression was repressed but formed a normal-size plaque when expression of the A34R protein was induced by IPTG. The virus could not be used to assess the contribution of the A34R gene to virus virulence because of its thymidine kinase-negative phenotype, a mutation known to cause attenuation (12). To overcome this problem and to further investigate the role of the A34R protein in VV replication and dissemination, we constructed a recombinant VV, WRΔA34R, in which the A34R gene was replaced by the Ecogpt gene driven by the VV 7.5K promoter (see Materials and Methods). A revertant virus, WR-rev, in which the A34R gene was reinserted into WRΔA34R at its natural locus was also constructed to ensure that phenotypic differences between WR and WRΔA34R were due to the loss of the A34R gene and not to mutations else-

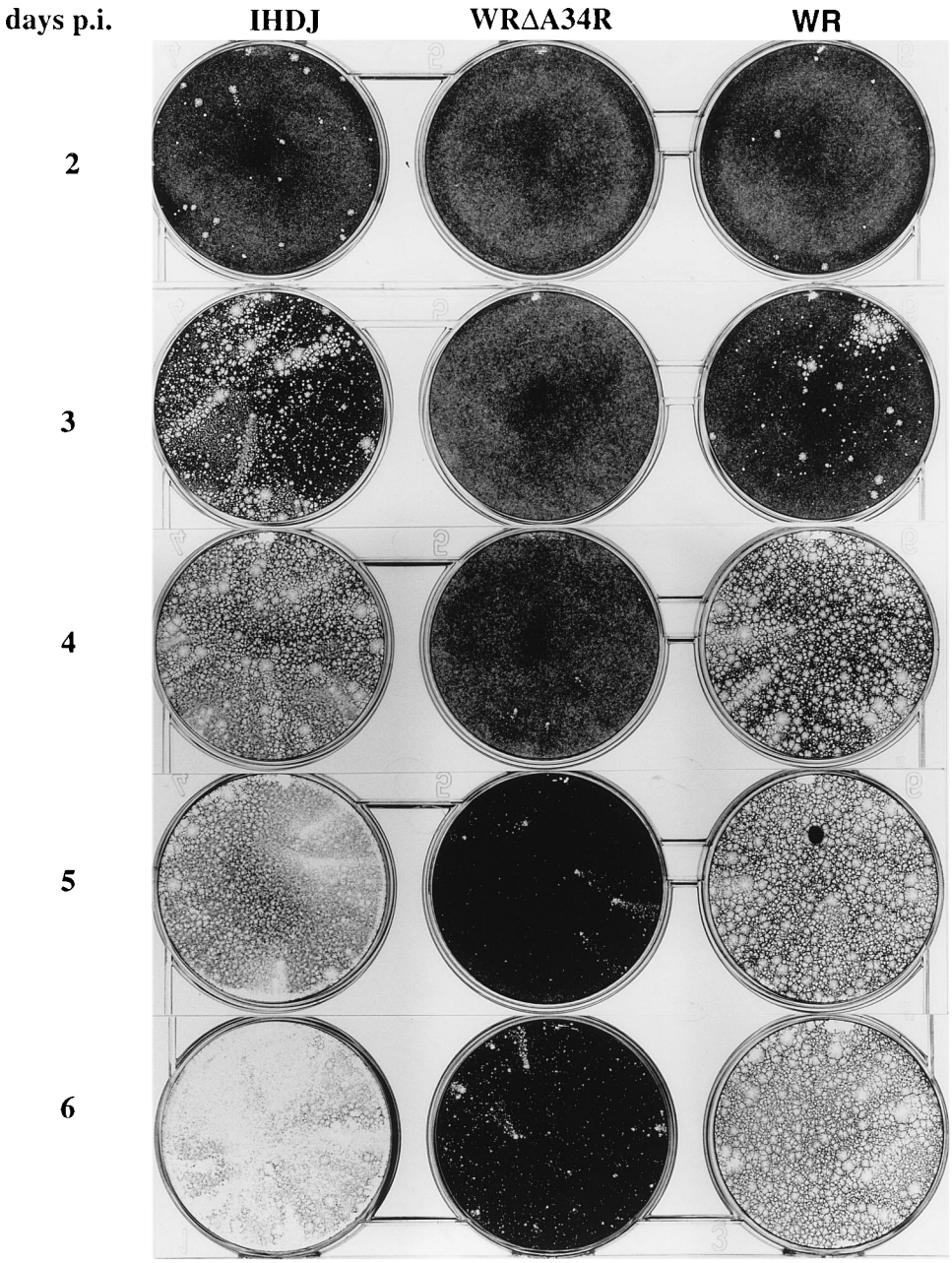

FIG. 2. Plaque phenotypes of WR, WRΔA34R, and IHD-J viruses. B-SC-1 cells were infected with each virus (by using IMV) and incubated for between 2 and 6 days under a liquid overlay to allow formation of comets. The cells were then stained with crystal violet.

where in the virus genome (see Materials and Methods). PCR and Southern blot analyses established that the genomes of these viruses were as predicted (Fig. 1; data not shown).

WRΔA34R forms small comets. Plaque formation by WR, WRΔA34R, and IHD-J was compared over 6 days under a liquid overlay (Fig. 2). After 2 to 3 days, normal plaques were formed by WR, whereas IHD-J produced the characteristic comets because of the release of greater amounts of EEV. Consistent with the small-plaque phenotype of vSAD9, WRΔA34R formed tiny plaques which became visible only by day 4, but upon longer incubation these plaques produced small comets (days 5 and 6). This confirmed that A34R is required for normal plaque formation, and in addition, the formation of comets suggested that EEV is released in the absence of A34R.

EEV formation by WRΔA34R. In view of the comet formation by WRΔA34R, the production of infectious intracellular and extracellular virus from RK-13 cells infected with WR, IHD-J, or WRΔA34R was measured by plaque assay. All three viruses produced equivalent IMV titers, showing that loss of the A34R gene did not affect IMV formation (Fig. 3). As expected, there was a much larger amount of extracellular virus produced by the IHD-J strain than by the WR strain, but the yields of infectious extracellular virus formed by WR and WRΔA34R were similar. This was surprising in view of the prior failure to observe enveloped virion formation by electron

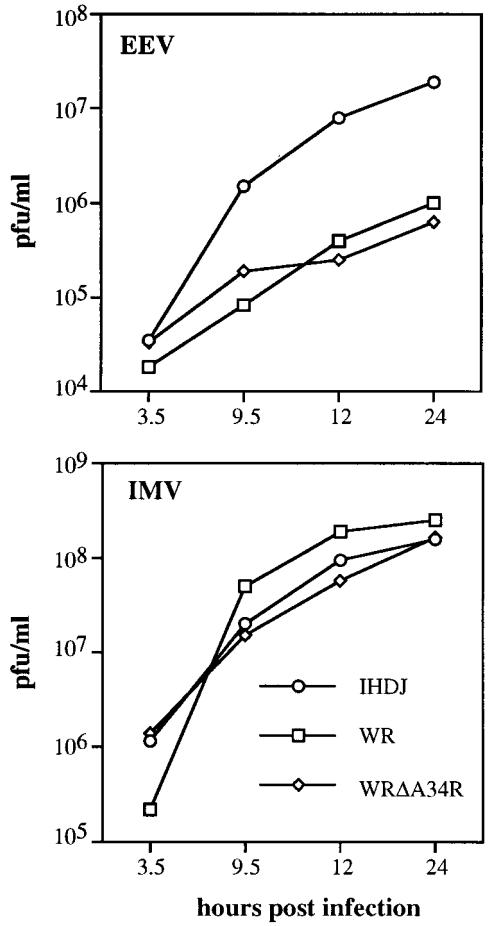

FIG. 3. One-step growth curves for WR, IHD-J, and WRΔA34R viruses. RK-13 cells were infected at 10 PFU per cell. At different times after infection, the culture supernatant was removed and centrifuged to remove detached cells. The clarified supernatant represented "EEV." The adherent cells were scraped from the flasks, recovered by centrifugation, and combined with detached cells. Virus present in these cells (IMV) was released by three cycles of freezing and thawing and brief sonication. EEV and IMV samples were then titrated in duplicate by plaque assay on B-SC-1 cells under 1.5% carboxymethyl cellulose in MEM with 2.5% FBS for either 2 days (WR and IHD-J) or 6 days (WRΔA34R). Plaques were stained with crystal violet and counted.

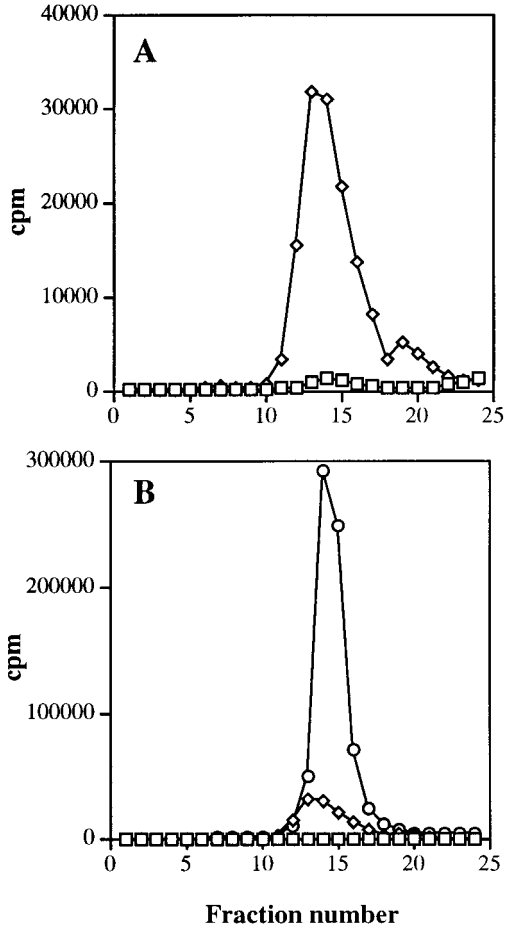

FIG. 4. Biochemical and infectivity measurements of EEV formation. RK-13 cells were infected with WR (□), WRΔA34R (◇), or IHD-J (○) virus and labeled with [^{3}H]thymidine. EEV that had been released by 24 hpi was purified on CsCl density gradients as described in Materials and Methods, and the radioactivity in the gradient fractions was determined. Panels A and B show comparisons of EEV formed by WR and WRΔA34R on different scales, since the WR peak is not apparent when plotted together with IHD-J EEV (B). Pooled gradient fractions representing the EEV peaks (density, 1.27 g/ml) were titrated in duplicate on B-SC-1 cells as described in the legend to Fig. 3. Data taken from this experiment are also presented in Table 1.

microscopy in cells infected with virus vSAD9 (13) and was therefore investigated further by both biochemical and infectivity measurements of EEV formation.

Infected RK-13 cells were labeled with [^{3}H]thymidine, and at 24 hpi EEV was purified from the cell supernatants by CsCl density gradient centrifugation and quantified by measurements of radioactivity and infectivity. The results of one experiment are expressed graphically in Fig. 4, and the data from this and a second experiment are shown in Table 1 and referred to below. For IHD-J, fractions from the CsCl gradients with densities corresponding to the density of EEV (1.27 g/ml) contained 138- or 135-fold more radioactivity than that for WR (Fig. 4B and Table 1). However, WRΔA34R-infected cells produced 24- or 19-fold more EEV than did WR-infected cells (Fig. 4A and Table 1). The level of EEV released by WRΔA34R was still 5.7- or 7.1-fold lower than that produced by IHD-J (Fig. 4B and Table 1). This much greater EEV production by WRΔA34R was not due to a less efficient infection with WR, since slightly more IMV was produced from WR- than from WRΔA34R-infected cells (Table 1). This result contrasted with the infectivity measurements (Fig. 3) and suggested a reduction in the specific infectivity of WRΔA34R compared with WR. This was confirmed by determining the infectivity of the EEV peaks (Table 1): WRΔA34R EEV had only 5.0- or 3.1-fold-greater infectivity than WR EEV, despite the 24- or 19-fold increase in EEV particle formation by WRΔA34R. Thus, the specific infectivity of WRΔA34R particles was reduced by 4.8- or 6.1-fold compared with that of WR. The revertant virus, WR-rev, was also included in the second experiment. This virus produced EEV at a level similar to that produced by WR, and these EEV particles had a specific infectivity sixfold greater than that of WRΔA34R (data not shown). This confirmed that the reduced specific infectivity of the WRΔA34R EEV was due to deletion of the A34R gene. Table 1 also shows that despite the alterations in the A34R protein of IHD-J (see below), the specific infectivity of IHD-J EEV particles is similar to that of WR.

The amounts of IMV and enveloped virus associated with cells at 24 hpi were also examined by CsCl density gradient centrifugation. This analysis, together with the amounts of

TABLE 1. Biochemical and infectivity measurements of EEV formation by WR, WRΔA34R, and IHD-J[a]

Strain	EEV cpm (ratio[b])		EEV PFU (ratio[b])		10^8 IMV PFU (ratio[b])	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
IHD-J	698,843 (138)	696,678 (135)	8.7×10^7 (128)	6.7×10^7 (124)	3.9 (0.60)	5.0 (0.77)
WRΔA34R	121,402 (24.1)	97,650 (18.9)	3.4×10^6 (5.0)	1.7×10^6 (3.1)	2.6 (0.40)	3.7 (0.57)
WR	5,046	5,175	6.8×10^5	5.4×10^5	6.5	6.5

[a] RK-13 cells were infected with IHD-J or WR at 10 PFU per cell and then labeled with [^{3}H]thymidine from 1.75 to 24 hpi. EEV in the supernatant was purified on CsCl density gradients, and the radioactivity in and density of each fraction were determined by scintillation counting and refractometry, respectively. The aggregated counts per minute for peak fractions with a density corresponding to the EEV density (1.23 g/ml) and the infectious virus titers of these fractions, determined by plaque assay on BS-C-1 cells, are presented. Experiment 1 is the same as shown in Fig. 4. The IMV titers from each experiment are also presented.

[b] Ratio of the value for the indicated strain to that for WR.

these viruses in the supernatants, (Fig. 4 and Table 1), showed that the total levels of enveloped virus produced by WR and WRΔA34R were similar (data not shown). Therefore, the enhanced levels of EEV observed in the supernatant of WRΔA34R-infected cells reflected enhanced release rather than greater envelopment of IMV.

The reduced specific infectivity of WRΔA34R EEV might have been directly due to the loss of A34R from EEV or indirectly due to the absence of other EEV proteins which require A34R for their incorporation into virions. The presence or absence of other EEV proteins was therefore investigated by immunoblotting WR and WRΔA34R EEVs with EEV-specific antibodies. Figure 5a shows that the A34R-encoded gp22-24 is present in WR but missing from WRΔA34R EEV. In contrast, the F13L, B5R, A56R, and A36R EEV proteins are present in both viruses (Fig. 5b to e, respectively). Thus, the processing and incorporation of other known EEV-specific proteins into EEV do not require A34R, and the reduced specific infectivity of WRΔA34R is due to the absence of A34R (or some other, as yet unidentified, EEV component). These data imply that the A34R protein is not required for EEV formation but is required for its normal infectivity.

Freeze-thawing WRΔA34R EEV increases virus infectivity. Since the A34R protein is present only in EEV (13) and does not affect IMV formation or infectivity (Fig. 3), disruption of the outer envelope of WRΔA34R EEV would be predicted to increase virus infectivity because of release of infectious IMV from within "noninfectious" EEV. This was tested by subjecting WR and WRΔA34R EEVs, purified from CsCl gradients, to increasing numbers of freeze-thaw cycles and then titrating virus infectivity on B-SC-1 cells (Fig. 6). Whereas freeze-thawing clearly had no effect on the infectivity of WR, it resulted in an increase in the infectivity of WRΔA34R, which was maximal (five- to sixfold) after two freeze-thaw cycles. The hypothesis that this increased infectivity was due to release of infectious IMV was supported by the observation that after freeze-thawing, an increased proportion of virus was neutralized by anti-14K protein monoclonal antibody (data not shown), an antibody that is able to neutralize IMV (31) but not EEV.

WRΔA34R is highly attenuated in vivo. The reduced specific infectivity of WRΔA34R EEV suggested that the WRΔA34R virus might be attenuated in vivo, since EEV is known to mediate virus dissemination. This was examined in a murine intranasal model (Fig. 7). Groups of five 6-week-old, female BALB/c mice were infected with WR, WRΔA34R, or the revertant virus, WR-rev, at doses of 10^4, 10^5, 10^6, or 10^7 PFU. Each group of animals infected with WR or WR-rev suffered a severe illness with substantial loss of body weight, and at doses of 10^5 PFU or greater, all animals were sacrificed (on days 5 to 7). In sharp contrast, none of the mice infected with WRΔA34R showed any visible signs of illness, and only at the highest virus dose was there a modest (10%), transient loss of body weight. Clearly, loss of the A34R gene results in a drastic decrease in virus virulence. The inclusion of the revertant virus WR-rev confirmed that the attenuation seen with the WRΔA34R deletion mutant was attributable to the loss of the A34R gene and not to other mutations in the virus genome.

The A34R protein is conserved in orthopoxviruses. To examine whether the A34R gene was conserved and expressed in other orthopoxviruses, cells were infected with camelpox virus, cowpox virus (strain Brighton Red), elephantpox virus (a cowpox virus strain), rabbitpox virus and buffalopox virus (strains

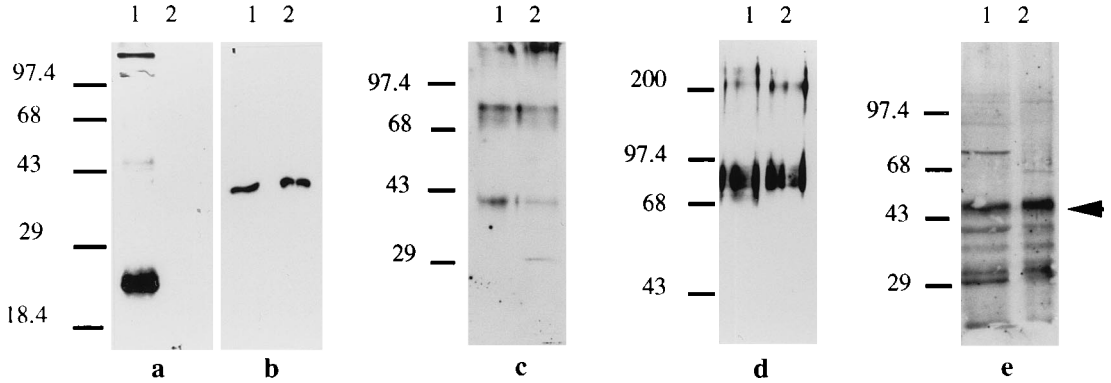

FIG. 5. Immunoblotting of EEV proteins. EEV was purified from WR (lanes 1)- or WRΔA34R-(lanes 2)-infected RK-13 cell supernatants on CsCl gradients. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed with antibodies against EEV-specific proteins as described in Materials and Methods (a) A34R (gp22-24); (b) F13L (p37); (c) B5R (gp42); (d) A56R (gp86 [hemagglutinin]); (e) A36R (p45-50). Positions of molecular mass markers (in kilodaltons) are indicated.

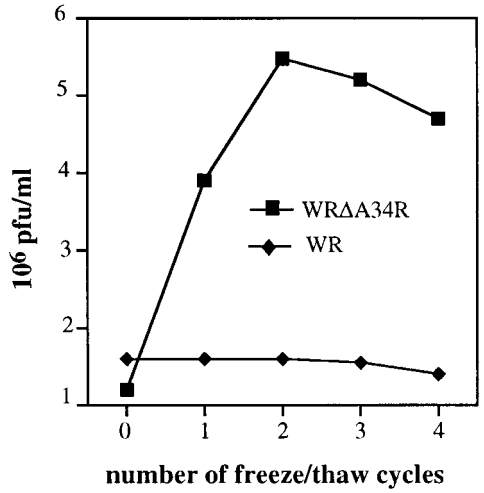

FIG. 6. Freezing and thawing of WRΔA34R EEV increases infectivity. WR and WRΔA34R EEVs were purified on CsCl gradients, and the infectivity was then titrated on B-SC-1 cells either directly or after the indicated number of freeze-thaw cycles.

of VV), and 12 other VV strains, and virus-infected cell extracts were analyzed by immunoblotting (Fig. 8A). Proteins with sizes similar to those of the WR A34R gene products (22 to 24 kDa) were detected in each of the 17 orthopoxviruses examined. The levels detected varied slightly between viruses and varied most notably for camelpox virus, which was markedly less abundant. A control antibody to the p37 EEV envelope protein also detected similar levels for all of the viruses except camelpox virus. Sequence data for three strains of variola virus further demonstrate the high degree of conservation of the A34R open reading frame (1, 24, 35).

Since VV strains IHD-J and WRΔA34R each produce more EEV than WR, the IHD-J A34R gene was sequenced and compared with that of WR. Six nucleotide changes were found in the coding region (T178C, T186C, C240T, G255T, G328A, and A451G, in which the first and last letters indicate the sequences of the WR and IHD-J genes, respectively). These changes cause only two amino acid alterations (D110N and K151E). Blasco et al. (7) had reported the same amino acid substitutions and had shown that the K151E mutation in IHD-J A34R was largely responsible for the increased EEV release by this virus. Fortuitously, this amino acid change results from a nucleotide alteration within an *Mse*I restriction endonuclease site, and thus the presence or absence of this site

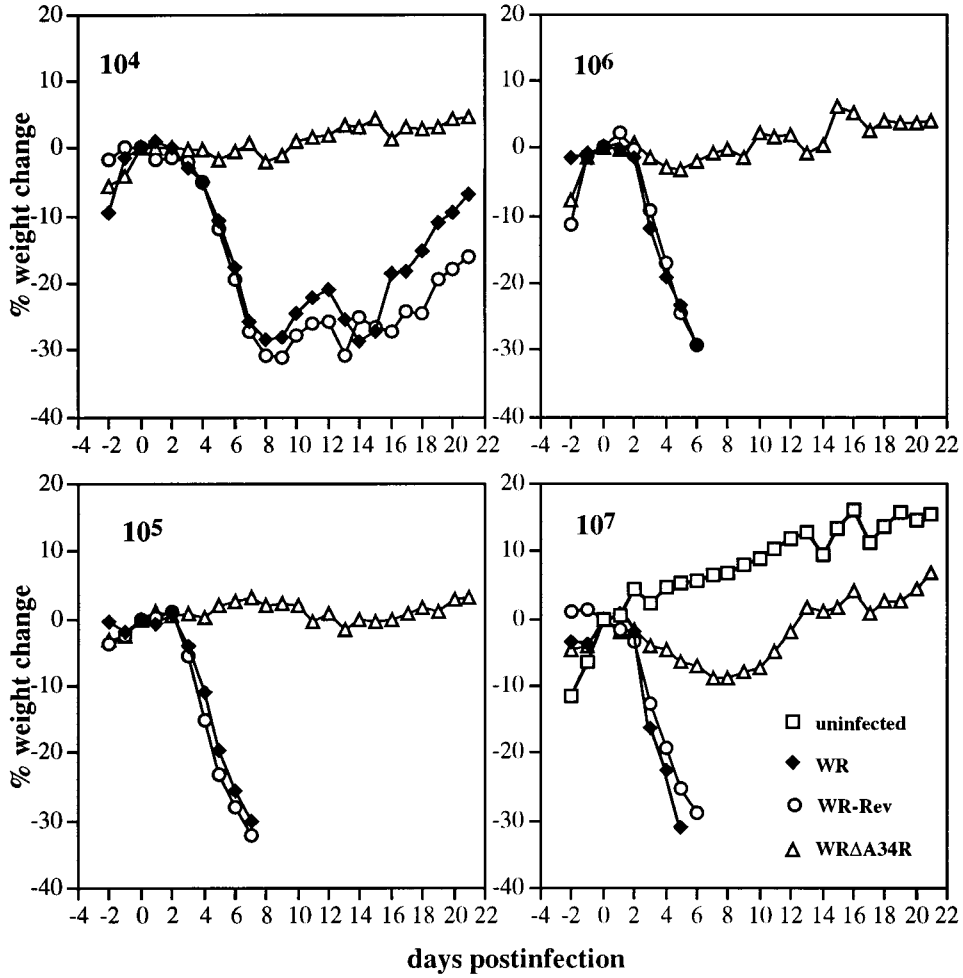

FIG. 7. Assay of virus virulence. Groups of five BALB/c mice were intranasally infected with the indicated doses (PFU) of WR, WRΔA34R, or WR-rev virus, and daily thereafter the weight of each group of animals was compared with that on the day of infection. All animals infected with $\geq 10^5$ PFU of WR or WR-rev virus were sacrificed.

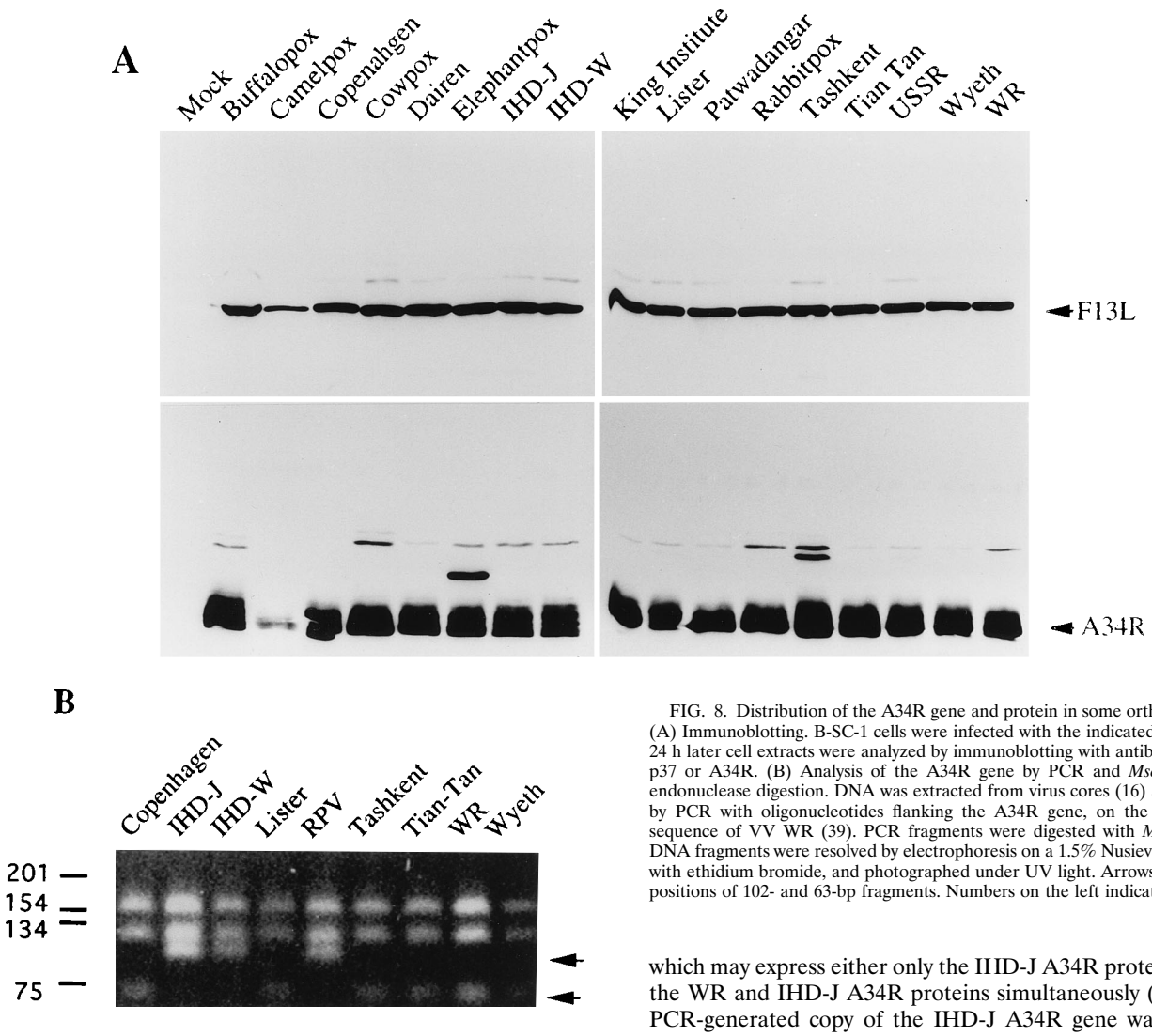

FIG. 8. Distribution of the A34R gene and protein in some orthopoxviruses. (A) Immunoblotting. B-SC-1 cells were infected with the indicated viruses, and 24 h later cell extracts were analyzed by immunoblotting with antibody to either p37 or A34R. (B) Analysis of the A34R gene by PCR and *Mse*I restriction endonuclease digestion. DNA was extracted from virus cores (16) and analyzed by PCR with oligonucleotides flanking the A34R gene, on the basis of the sequence of VV WR (39). PCR fragments were digested with *Mse*I, and the DNA fragments were resolved by electrophoresis on a 1.5% Nusieve gel, stained with ethidium bromide, and photographed under UV light. Arrows indicate the positions of 102- and 63-bp fragments. Numbers on the left indicate base pairs.

can be used to investigate whether the WR or IHD-J genotype is present in different orthopoxviruses. The A34R gene was amplified by PCR from eight of the viruses used in the experiments described above and then was digested with *Mse*I. Electrophoresis of the DNA fragments revealed a 102-bp fragment in viruses lacking the *Mse*I site spanning codon 151, while viruses containing this site had a 63-bp fragment (Fig. 8B). It can be seen that only IHD-J, IHD-W, and rabbitpox virus lack this *Mse*I site. Notably, these strains, but not the other five, form comets. PCR amplification and *Mse*I restriction of the A34R gene thus provide a rapid test to determine the genotype of the A34R gene (WR or IHD-J) and indicate if that strain of virus is likely to produce comets. This analysis also demonstrates that the majority of orthopoxvirus strains examined have the WR rather than IHD-J A34R genotype. Furthermore, the Harvey (1), Bangladesh-1975 (24), and India-1967 (34) strains of variola major virus also have the WR A34R genotype and would therefore be expected to produce relatively little EEV.

The WR A34R genotype is dominant over the IHD-J genotype when the A34R genes are coexpressed. To determine whether the WR or IHD-J A34R gene expresses the dominant phenotype, we constructed a recombinant virus, based on WR, which may express either only the IHD-J A34R protein or both the WR and IHD-J A34R proteins simultaneously (Fig. 1). A PCR-generated copy of the IHD-J A34R gene was inserted into the A34R locus of vSAD9 by transfection into vSAD9-infected cells and selection of a virus forming a large plaque. The resultant recombinant virus, vSAD9-I-A34R (Fig. 1), expresses only the IHD-J A34R gene in the absence of IPTG but both the WR and IHD-J A34R genes in the presence of IPTG.

Plaque formation by WR, vSAD9, vSAD9-I-A34R, and IHD-J was examined on RK-13 cells under a liquid overlay (Fig. 9). Characteristically, IHD-J formed comets whereas WR did not, and these phenotypes were unaffected by the presence of IPTG. As reported previously (13), vSAD9 formed plaques only in the presence of IPTG, when the A34R gene is expressed. With vSAD9-I-A34R, comets were formed in the absence of IPTG, (i.e., when only the IHD-J A34R gene is expressed), but in the presence of IPTG (i.e., when both A34R genes are expressed), the comet phenotype disappeared. This confirmed that the IHD-J A34R gene controls comet formation and demonstrated that the WR protein is dominant when both proteins are expressed simultaneously.

To determine the levels of EEV produced by vSAD9-I-A34R, we performed CsCl density gradient centrifugation on virus grown in the presence or absence of IPTG (Fig. 10). As usual, IHD-J produced very much more EEV than WR, but the phenotype of vSAD9-I-A34R was intermediate. In the absence of IPTG, EEV levels were similar to those produced

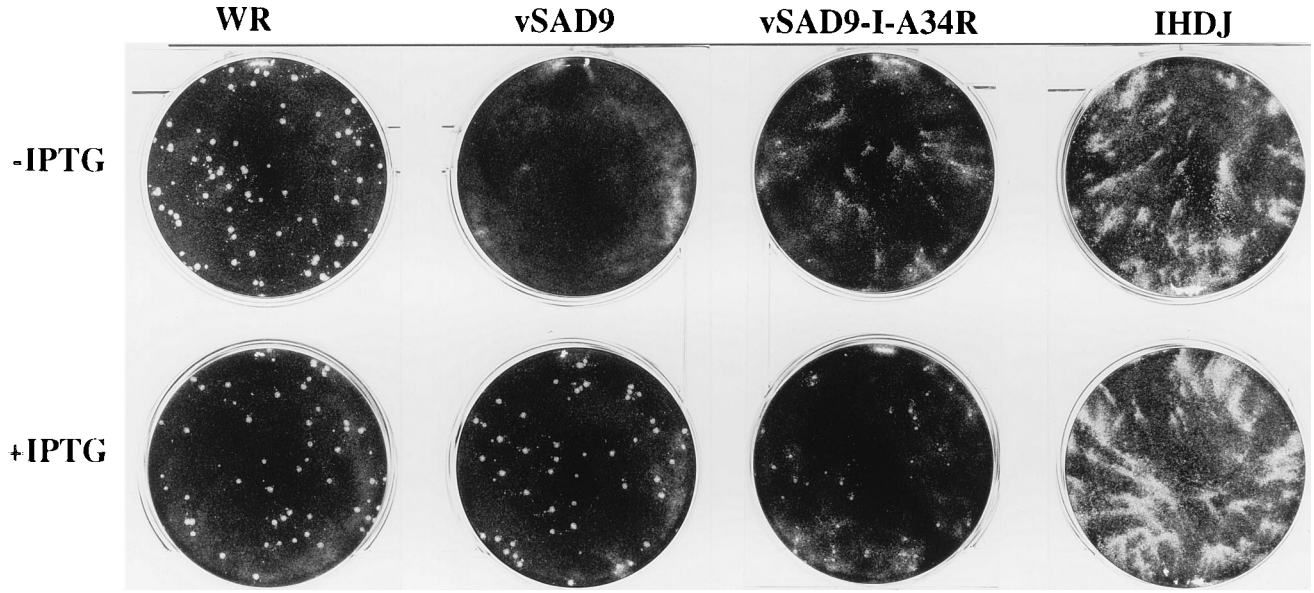

FIG. 9. Plaque phenotypes of WR, vSAD9, vSAD9-I-A34R, and IHD-J viruses. RK-13 cells were infected with the indicated viruses and incubated under a liquid overlay in the presence or absence of 5 mM IPTG. The cells were stained with crystal violet after 48 h.

by IHD-J, while in the presence of IPTG, the levels were reduced and closer to those with WR. The failure to release EEV at IHD-J levels when only IHD-J A34R was expressed is consistent with the data shown in Fig. 4 and Table 1, which indicated that deletion of the WR A34R gene does not induce production of IHD-J levels of EEV. This is presumably due to contributions of another IHD-J gene(s) to the IHD-J phenotype, as suggested by Blasco et al. (7). In the experiment whose results are shown in Fig. 10, the result may also be due to the slight leakiness of the IPTG-inducible WR A34R gene in the absence of IPTG (13). When both A34R proteins were expressed, the levels of EEV release were somewhat higher than those observed with WR, and this might be due to competition between the two forms of A34R incorporated into EEV. Notably, this level of EEV release was not sufficient to permit comet formation (Fig. 9). Taken together, these results demonstrate that the A34R gene influences the level of EEV release and thus the ability to form comets.

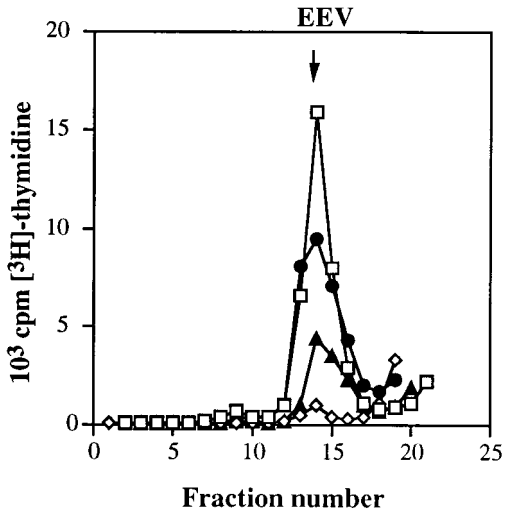

FIG. 10. Biochemical measurement of EEV formation. RK-13 cells were infected with WR (◇), IHD-J (□), or vSAD9-I-A34R and labeled with [^{3}H]thymidine. vSAD9-I-A34R was incubated in either the presence (▲) or absence (●) of 5 mM IPTG. At 24 hpi EEV was purified on CsCl density gradients as described in Materials and Methods, and the radioactivity in the gradient fractions was determined.

DISCUSSION

In this paper we provide evidence that the A34R gene product has multiple functions in the VV life cycle and that it is essential for the normal infectivity of EEV. A deletion mutant lacking the majority of the A34R gene (WRΔA34R) formed a very small plaque in vitro that was clearly visible only after prolonged incubation. This was consistent with the previous observation that repression of the A34R gene by the *E. coli lac* repressor resulted in a small-plaque phenotype (13). The plaques formed by WRΔA34R were, however, smaller than those formed by vSAD9 in the absence of IPTG (data not shown), and this is attributable to the slight leakiness of the A34R gene under the *E. coli lac* repressor. Unexpectedly, we observed that cells infected with the WRΔA34R virus released levels of infectious extracellular virus similar to or slightly higher than those released by cells infected with WR. This was inconsistent with the previous inability to observe enveloped virions by electron microscopy when the A34R gene was repressed (13). EEV formation by WRΔA34R was therefore examined biochemically and found to be 19- to 24-fold greater than that by WR, while the yields of IMV were similar. Despite this, the infectivity of WRΔA34R EEV purified from CsCl density gradients was only three- to fivefold greater than that of WR EEV, and thus the specific infectivity of WRΔA34R EEV was reduced five- to sixfold compared with WR EEV. Reinsertion of the A34R gene restored the specific infectivity to that of WR. The loss of A34R was directly implicated in the dramatic reduction in specific infectivity, since all other known EEV proteins were present in WRΔA34R EEV. While it is possible that the loss of some unknown component of EEV might explain the result, this seems unlikely, since (i) the genes

for the majority of the EEV-specific proteins detected by Payne (28) have been mapped and (ii) a mutation in the A34R protein is known to enhance the release of EEV from cells infected with the IHD-J strain of VV (7).

Whatever the exact defect in WRΔA34R that caused the reduced specific infectivity, it seemed likely that the IMV contained within the EEV envelope would still be infectious, since no EEV protein has yet been shown to affect the formation or infectivity of IMV. To test this hypothesis, WRΔA34R EEV was subjected to several cycles of freeze-thawing to rupture the outer envelope. This resulted in a five- to sixfold increase in infectivity, whereas there was no corresponding increase in the infectivity of WR EEV. This increase in infectivity was probably due to the release of IMV within the noninfectious EEV, because as the infectivity increased, the majority of virus became sensitive to neutralization with a monoclonal antibody directed against the IMV surface 14K protein.

The virulence of WRΔA34R was dramatically decreased compared with those observed for WR and a revertant virus, WR-rev. This was consistent with the results obtained for the deletion of other genes, encoding EEV proteins hemagglutinin, p37, gp42, and p45-50. In each of these cases, the virus was attenuated (15, 18, 26, 37, 44). The severe attenuation resulting from the loss of p37 (5) and gp42 (15, 44) was not surprising, because the loss of these genes dramatically reduced formation of EEV, the form of virus responsible for long-range virus dissemination. In contrast, the dramatic attenuation of the deletion mutant lacking p45-50 was accompanied by only a fivefold reduction in EEV formation and no loss of EEV specific infectivity (26). In this paper we have shown that loss of the A34R protein caused an increased release of EEV, but despite this, a dramatic attenuation was observed. This is explained by the five- to sixfold-reduced specific infectivity of EEV and the small-plaque phenotype. Taken together, these observations emphasize the important role of EEV in virus virulence.

The sequence of the A34R open reading frame from IHD-J revealed six nucleotide changes but only two amino acid changes from the WR open reading frame. These changes are as reported by Blasco et al. (7), who showed that a K151E mutation within the lectin-like domain of the A34R protein was responsible for the enhanced release of EEV by IHD-J. The role of the A34R protein in EEV dissemination was investigated here by constructing a WR-based recombinant virus (vSAD9-I-A34R) which expresses either the IHD-J protein only (in the absence of IPTG) or both the IHD-J and WR A34R proteins together (in the presence of IPTG). When only the IHD-J protein was expressed, the virus formed comets and released more EEV (a phenotype similar to that of IHD-J). This phenotype was reversed by inducing expression of the WR A34R protein with IPTG. These data confirmed that the A34R protein was largely responsible for the comet phenotype and in addition showed that the WR phenotype is dominant when the two forms of the protein are coexpressed.

PCR amplification of the A34R gene and restriction digestion with *Mse*I allowed the identification of virus strains which had the WR or IHD-J sequence at codon 151, thereby indicating which strains would be expected to release larger amounts of EEV and thus form comets. This analysis showed that all of the strains examined except rabbitpox, IHD-J, and IHD-W had the WR genotype, and consequently, this is likely to be the normal sequence at this position. Notably, the three viruses which did not exhibit the WR genotype are all known to form comets in vitro. Thus, the presence or absence of the *Mse*I restriction site at codon 151 allows a prediction of whether the majority of enveloped virus is shed from the cell surface as EEV or remains attached as CEV. This might be useful for those orthopoxviruses which cannot easily be grown because of a requirement for high-level containment facilities, as is the case for variola and monkeypox viruses, or when nucleotide sequence data are not available. It is surprising that most virus strains release little EEV, but this might be due to the absence of a receptor-destroying enzyme, such as the neuraminidase expressed by influenza virus. There is no reported advantage in having larger amounts of CEV for, for instance, increased cell-to-cell spread of virus, since the WR and IHD-J strains have widely differing amounts of CEV (6) and yet form similar-sized plaques.

It remains to be determined whether the A34R protein is the major EEV protein required for attachment to uninfected cells or whether it is required for some other aspect of the infectious process, such as cell fusion, but it is noteworthy that IHD-J EEV preparations have the same specific infectivity as WR EEV (Table 1). Thus, the K151E mutation, which allows greater release of EEV, does not reduce the infectivity of EEV. Alternatively, either the D110N mutation, which is also present in IHD-J A34R, or a change in some other IHD-J protein(s) may compensate for the K151E substitution.

Last, if the A34R protein is critical for EEV infectivity, as we propose here, it is surprisingly that neither a polyvalent antibody raised against the A34R protein from bacteria (13) nor monoclonal antibodies against this protein or other EEV glycoproteins (30) neutralize EEV infectivity. This might be due to the nature of the antibodies themselves or, alternatively, to the contamination of EEV with IMV. Since in most strains of virus IMV is ≥100-fold more abundant than EEV, there need be only a 1% IMV contamination to render 50% of the "EEV" resistant to EEV-neutralizing antibodies.

Investigation of the conservation of the A34R gene in different orthopoxviruses showed that each virus examined contained a similar-sized gene and expressed a similar-sized protein. This finding emphasizes the important roles of the A34R protein in virus plaque formation, EEV release, EEV infectivity, and virus virulence.

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