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Extracellular Vaccinia Virus Envelope Glycoprotein Encoded by the A33R Gene

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With the aid of three monoclonal antibodies (MAbs), a glycoprotein specifically localized to the outer envelope of vaccinia virus was shown to be encoded by the A33R gene. These MAbs reacted with a glycosylated protein that migrated as 23- to 28-kDa and 55-kDa species under reducing and nonreducing conditions, respectively. The protein recognized by the three MAbs was synthesized by all 11 orthopoxviruses tested: eight strains of vaccinia virus (including modified vaccinia virus Ankara) and one strain each of cowpox, rabbitpox, and ectromelia viruses. The observation that the protein synthesized by ectromelia virus-infected cells reacted with only one of the three MAbs provided a means of mapping the gene encoding the glycoprotein. By transfecting vaccinia virus DNA into cells infected with ectromelia virus and assaying for MAb reactivity, we mapped the glycoprotein to the A33R open reading frame. The amino acid sequence and hydrophilicity plot predicted that the A33R gene product is a type II membrane protein with two asparagine-linked glycosylation sites. Triton X-114 partitioning experiments indicated that the A33R gene product is an integral membrane protein. The ectromelia virus homolog of the vaccinia virus A33R gene was sequenced, revealing 90% predicted amino acid identity. The vaccinia and variola virus homolog sequences predict 94% identical amino acids, the latter having one fewer internal amino acid. Electron microscopy revealed that the A33R gene product is expressed on the surface of extracellular enveloped virions but not on the intracellular mature form of virus. The conservation of this protein and its specific incorporation into viral envelopes suggest that it is important for virus dissemination.

Vaccinia virus (VV) was the orthopoxvirus used for vaccination in the worldwide eradication of smallpox. Currently, VV is widely used as a mammalian cell expression vector as well as a tool for exploring various aspects of virus-host interactions and immunology (26). Poxviruses are attractive candidates for human and animal recombinant vaccines, and some of these vaccines have now reached clinical and field trials (8–10, 16). Although the basic events of the VV life cycle are known (25) and the entire genome of nearly 200 kb has been sequenced (15), many questions regarding the functions of the encoded proteins remain. Perhaps least is known about the mechanisms and proteins involved in viral entry, morphogenesis, and dissemination of VV particles.

VV generates two distinct types of infectious particles: the intracellular mature virion (IMV) and the extracellular enveloped virion (EEV). While IMVs released by freeze-thawing or sonicating infected cells are widely used for experimental infections, several lines of evidence indicate that the more important natural infectious particle is the less abundant, less studied EEV (3, 4, 7, 29, 43). The EEV is formed from the IMV. First, the double-membrane IMV is enwrapped by two additional membrane layers derived from the *trans*-Golgi network to form the four-membrane intracellular enveloped virion (17, 20, 31, 37). After transport to the cell periphery, the outermost membrane of the intracellular enveloped virion fuses with the plasma membrane, releasing the three-membrane EEV, which may remain attached to the infected-cell surface or be released into the milieu (5, 20, 24, 31, 37). Whether or not it remains attached to its parent cell, at this stage the extracellular viral form is competent for attaching to a new cell and spreading the infection.

Five genes which encode proteins specific to the EEV have been identified: the A56R gene encoding the 89-kDa hemagglutinin protein (32, 40), the F13L gene encoding a nonglycosylated 37-kDa protein (4, 18), the B5R gene encoding a 42-kDa protein (13, 21), the A36R gene encoding a 43- to 50-kDa protein (27), and the A34R gene (also identified as SalL4R) encoding a variably glycosylated 22- to 24-kDa protein (11, 23). Interference with the expression of any of these proteins by deletion or repression has dramatic impact on the ability of the virus to spread from cell to cell in tissue culture and form plaques (4, 11, 14, 19, 23, 27, 38, 44). For example, the F13L deletion mutant requires 7 days to produce plaques of the size formed in 1 day by wild-type virus (4). Yet mutants with deletions of the F13L, B5R, A36R, or A34R gene make infectious IMV in normal amounts (4, 14, 23, 27, 44), underscoring the concept that the IMV is not responsible for the cell-to-cell spread of infection (5). Deletion of EEV-specific protein genes can also attenuate the virus in vivo, indicating that EEVs are important in nature (14, 27, 44).

Further evidence attesting to the importance of the EEV comes from antibody studies. Several researchers have reported that antibodies to EEV proteins protect animals from viral challenge significantly more than antibodies to IMV (2, 3, 7, 29, 43). Despite the importance of the EEV, little is known regarding the functions of EEV-specific proteins. The protein interactions required for EEV formation, egress from the infected cell, attachment to an uninfected-cell surface, entry, and uncoating are all unknown. Identification and further charac-

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terization of the EEV proteins are crucial to a better appreciation of the process of the spreading infection.

The development of monoclonal antibodies (MAbs) to EEV-specific proteins allows for unambiguous characterization of the proteins without the problems of cross-reactivity and background inherent in polyclonal sera (30). MAb 4 reacts with a variably glycosylated protein very similar to that encoded by the A34R gene, previously characterized by use of polyclonal sera (11, 23). The MAb 4-reactive protein and the A34R-encoded protein are both EEV specific, have similar molecular weights, and are expressed predominantly late in infection and variably glycosylated (11, 23, 30), leading to the assumption that MAb 4 recognizes the A34R protein (37). The realization that this might not be the case arose from studies involving a recombinant VV expressing an epitope-tagged A34R gene product (35). We demonstrate here that MAb 4 reacts with the product of the A33R gene rather than the A34R gene and that this protein is expressed on the surface of the EEV. This brings to six the number of VV genes encoding EEV-specific proteins that have been identified.

MATERIALS AND METHODS

Cells and viruses. Cells were grown in Eagle minimum essential medium with 10% fetal bovine serum, and infections were carried out in the same medium with 2.5% serum. BS-C-1 cells were used routinely for plaque assays, immunostaining, immunoprecipitations, and Western blotting (immunoblotting). Virus was propagated in RK_{13} cells for purification of EEV. Virus stocks were grown in HeLa cells as previously described (12). VV strain WR (ATCC Vr119) was used in most experiments. The recombinant VV (VV-A34Tag) expressing an epitope-tagged A34R gene product is described elsewhere (35). Ectromelia virus (EV) (Moscow strain) was the kind gift of G. Karupiah (The Australian National University, Canberra).

Antibodies. Mouse MAbs 4, 66, 105, and 20 were produced as previously described (30). An antibody to the 14-kDa IMV protein (mouse MAb C3) was kindly provided by M. Esteban (34). Mouse MAb 12CA5 recognizing the influenza virus hemagglutinin epitope tag was purchased from BABCO (Berkeley Antibody Company, Richmond, Calif.). Horseradish peroxidase-labeled antibodies were from Amersham (Arlington Heights, Ill.). Polyclonal rabbit sera raised against bacterially expressed A34R were the kind gift of G. L. Smith (University of Oxford, Oxford, England) (11). Polyclonal rabbit sera were raised against two peptides supplied by J. Coligan (National Institute of Allergy and Infectious Diseases [NIAID], Rockville, Md.) from the sequence of A34R. The A34R1 peptide sequence is QYDKHCYLDTNIKMSTDNAVYQCRK (residues 53 to 77), and the A34R2 peptide sequence is YKDYWVSLKKTNDKWLDINNDKD (residues 98 to 120). These peptides were coupled to keyhole limpet hemocyanin with an Imject activated immunogen conjugation kit (Pierce, Rockford, Ill.). A total of five injections were given, and the rabbits were bled to prepare antisera.

Immunoprecipitation and Western blotting. For immunoprecipitation, 2×10^6 BS-C-1 cells were infected for 6 h at a multiplicity of infection of 10. Growth medium was replaced with methionine-free medium, 2% dialyzed calf serum, and 100 μCi of [^{35}S]methionine (Amersham) per ml for 18 h. The medium was discarded, and the cells were lysed in 0.2 ml of cold buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, and 1% Triton X-100. Nuclei were removed by spinning the lysates at $1,800 \times g$ (Sorvall RT 6000B), and the cytoplasm was cleared by centrifugation at $14,000 \times g$ for 10 min at 4°C. Cleared cell lysates were incubated with protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) in phosphate-buffered saline (PBS). At the same time, protein A-Sepharose beads were incubated with 2 to 5 μl of antibody-containing ascitic fluid or serum in PBS. After both sets of reaction mixtures were rocked for 2 h at 4°C, all tubes were spun at $1,800 \times g$ for 5 min. Antibody-coated protein A-Sepharose beads were washed and incubated with the cell lysates for an additional 2 h at 4°C. After a thorough washing in the lysis buffer, the beads were boiled in loading buffer containing 2% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, and 10% glycerol in the presence or absence of 2% 2-mercaptoethanol, and the released proteins were loaded onto 15% polyacrylamide or 4 to 20% polyacrylamide gradient gels (Integrated Separation Systems, Natick, Mass.) and electrophoresed until the 21.5-kDa marker (Amersham) neared the bottom of the gel. For analysis of radioimmunoprecipitated proteins, after polyacrylamide gel electrophoresis (PAGE), the gels were fixed in 10% acetic acid in methanol-water (1:1) for 30 min to overnight, incubated in Amplify (Amersham), dried, and exposed to film.

For Western blots, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Mass.) for 2 h at 200 mA. The membranes were blocked in 0.1% Nonidet P-40–1% powdered milk–0.14 M NaCl–25 mM Tris (pH 7.4) for 30 min and then incubated with primary antibody at 1:500 to 1:1,000 dilutions for 1 h to overnight. The membranes were washed and incubated with a 1:1,000 dilution of alkaline phosphatase-labeled anti-mouse or anti-rabbit secondary antibody (Promega, Madison, Wis.) for at least 45 min. The membranes were washed five times in blocking buffer and incubated with Western Blue (Promega) substrate solution. The reaction was stopped by rinsing the membranes in water.

Immunostaining of cells. Cell monolayers were infected for 16 to 24 h, the medium was removed, and the cells were fixed with methanol-acetone (50:50) for 3 min, rinsed in PBS, incubated with primary antibody for at least 30 min, rinsed, incubated with horseradish peroxidase-labeled anti-mouse or anti-rabbit antibody (Amersham) for 30 min, and rinsed. Subsequently, 2 ml of the following substrate solution was added: 10 μl of 30% hydrogen peroxide and 200 μl of dianisidine-saturated ethanol (Sigma) in 10 ml of PBS. The brown horseradish peroxidase staining was stopped by rinsing in water.

Cosmid and plasmid DNA and PCR cloning. An overlapping cosmid library representing almost the entire genome of VV strain WR (42) was the kind gift of R. Condit (University of Florida, Gainsville). Construction of plasmid pA64 containing the 14.2-kb *Bam*HI fragment from near the center of *Hin*dIII-A was described previously (1). The 6.3-kb *Sal*I fragment of pA64 was subcloned into pGEM 3zF (9a). The A33R coding sequence and promoter were cloned by PCR into the TA cloning vector of Invitrogen (San Diego, Calif.) according to the manufacturer's instructions. The 5′ sense primer sequence was ATAATTATAT TTACAGTTAC, and the 3′ antisense primer sequence was TTTATTAATGTA CAAAAATA. PCR was carried out for 25 cycles by standard procedures using *Taq* polymerase (Boehringer Mannheim Biochemicals) with a 40°C annealing temperature. These primers amplified a 703-bp DNA including 122 bp upstream of A33R containing the native promoter sequences intergenic with the A32L gene, 555 bp of A33R coding sequence, and 26 bp intergenic with the A34R gene. White colonies were picked and grown in ampicillin-containing broth for plasmid purification with a Wizard Miniprep (Promega).

Infection and transfection. For gene mapping, 2×10^6 BS-C-1 cells were infected at a multiplicity of infection of 10. Transfections were prepared by incubating 2.5 μg of purified VV DNA, cosmids, or plasmid DNA with DOTAP transfection reagent (Boehringer) for 10 min according to the manufacturer's guidelines. The DNA-DOTAP mixture was added to the cells 1 to 2 h after infection. Infected and transfected cells were incubated at 37°C for 16 to 28 h and fixed for immunostaining or lysed for immunoprecipitation.

Sequencing of viral DNA. Virus-infected cell DNA was prepared for PCR by incubation in 1% Nonidet P-40–1% Triton X-100–proteinase K for 30 min at 56°C. After heat inactivation of the proteinase K, PCR was performed by standard procedures using *Taq* polymerase (Boehringer) with a 40°C annealing temperature for 25 cycles. Primers were based on the VV sequence (15). The 5′ sense primer sequence was AAAATATGGAAAACTAGGTCG, and the 3′ primer antisense sequence was ATACTAAACAATACTCTCAGATGT. Products from three separate reaction tubes were used as sequencing templates. The above primers were employed for sequencing in addition to the following three primers: GCGTCTAGCACTACACAATATGATCAC (sense), TGGTTGAGAGTAT CATCATTATGGCG (antisense), and AATGTAATATATAACAAGAACCC TGG (antisense). Approximately 70 ng of PCR product and 3.2 pmol of primer were used for each sequencing reaction with a Prism Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) and analyzed on the 373 DNA Sequencer (Applied Biosystems) with the kind assistance of J. Sisler (NIAID).

Triton X-114 partitioning of integral membrane proteins. BS-C-1 cells were infected at a multiplicity of infection of 10 for 24 h. The medium was removed, and cells were extracted in cold buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2% Triton X-114 (Pierce). Following a 15-min incubation, lysates were centrifuged for 7 min at $14,000 \times g$ to remove insoluble matter. Supernatants were underlaid with 6% sucrose, warmed to 37°C, and centrifuged for 10 min at $1,000 \times g$ to separate the upper aqueous phase from the lower detergent phase. The detergent phase was collected and again centrifuged over sucrose. Samples were analyzed by Western blotting.

Virus purification. EEV and IMV were separated and purified on the basis of their differential buoyant densities in CsCl as described previously (28). Briefly, RK_{13} cells were infected at a multiplicity of infection of 10 for 48 h. For EEV, supernatants were harvested and centrifuged twice at $1,000 \times g$ to remove cells. Then supernatants were centrifuged at $70,000 \times g$ in a Beckman L8-70M centrifuge for 80 min to pellet virus. For IMV, cells were scraped into 10 mM Tris (pH 9.0) and Dounce homogenized. After a low-speed centrifugation, virus was pelleted through a 36% sucrose cushion. Afterwards, both the EEV and the IMV were purified on a CsCl gradient (layered densities of 1.3, 1.25, and 1.2 g/ml) by centrifugation at $126,000 \times g$ with slow acceleration for 60 min in a Beckman L8-70M centrifuge.

Electron microscopy. Formvar carbon-coated grids were placed on a drop of purified IMV or EEV for 10 min, washed in 0.2% glycine in PBS, and blocked in 0.1% bovine serum albumin in PBS. The grids were washed and placed on a drop of 1:1,000-diluted primary antibody for 30 min. Next, the grids were rinsed and incubated on gold (10-nm-diameter)-labeled protein A for 30 min, washed in bovine serum albumin in PBS, and fixed in 2% paraformaldehyde. Finally, virus particles were stained in 4% uranyl acetate for 10 min. The grids were washed, dried, and viewed on a Philips CM 10 electron microscope with the kind assistance of E. Wolffe (NIAID).

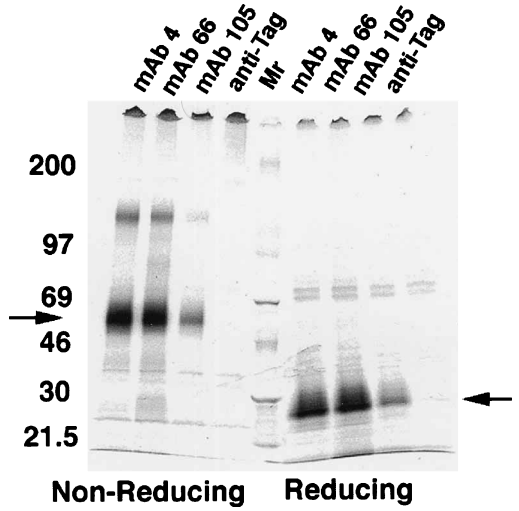

FIG. 1. SDS-PAGE analysis of metabolically labeled proteins from VV-infected cells. Wild-type-VV-infected BS-C-1 cells were labeled with [^{35}S]methionine for 18 h. Lysates were immunoprecipitated with MAbs 4, 66, and 105 and an irrelevant antibody (anti-Tag MAb 12CA5). Antibodies used for precipitation are indicated above the gel. Protein samples were prepared without reducing agents (left) or in the presence of 2% 2-mercaptoethanol (right) for gel electrophoresis in a 4 to 20% polyacrylamide gel. An autoradiogram is shown. The 55-kDa and 23- to 28-kDa species (arrows) are indicated. Protein standards were run in lane Mr (molecular weights, in thousands, are shown on the left).

Virus neutralization. Approximately 100 PFUs of CsCl-purified EEVs or IMVs were incubated with various antibodies (with a maximum concentration of a 1:100 dilution) in 50 to 100 μl for 1 h at 4°C. The mixture of virus and antibody was then applied to 2×10^6 cells grown in monolayers in a final volume of 1.1 ml. At 24 to 48 h after infection, the cells were fixed and stained with crystal violet for enumeration of plaques.

Datum presentation. Figure data were scanned with an Arcus Plus Image Scanner (Agfa-Gevaert NV) and prepared for presentation by using Adobe Photoshop (Adobe Systems, Mountain View, Calif.).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned accession no. U37059.

RESULTS

MAbs 4, 66, and 105 recognize the same protein. MAb 4 was previously identified by its reaction with the EEV form of VV but not with the IMV (30). Here, we describe the characterization of two additional MAbs: MAbs 66 and 105. To investigate the reactivities of these MAbs, cells were infected with VV strain WR and metabolically labeled with [^{35}S]methionine, and lysates were immunoprecipitated with MAbs 4, 66, and 105. Gel electrophoresis of the immunoprecipitated proteins followed by autoradiography revealed a broad protein band with an apparent molecular mass of 23 to 28 kDa under reducing conditions (Fig. 1). Under nonreducing conditions, a major, 55-kDa species (previously noted with MAb 4 [30]) and a minor, 150-kDa species were detected. It was discovered that MAbs 4, 66, and 105 have a strong preference for binding nonreduced blotted proteins; therefore, all samples for blotting with these antibodies were prepared in the absence of reducing agents with analysis of the 55-kDa species. As all three MAbs showed the same immunoprecipitation patterns, sequential immunoprecipitation and Western blotting were performed to determine if the three MAbs recognized the same protein. Figure 2A shows that MAb 66 bound the 55-kDa protein immunoprecipitated by MAbs 4, 66, and 105. Similar data were obtained when MAbs 4 and 66 were used for Western blotting of the immunoprecipitated proteins (data not shown), suggesting that all three MAbs recognize the same unidentified EEV protein.

MAbs 4, 66, and 105 do not react with the A34R gene-encoded protein. One possibility was that the three MAbs were directed to the previously characterized A34R protein. To determine if this protein was the A34R gene product, the reactivities of MAbs 4, 66, and 105 were sequentially compared with those of four antibodies that recognize the A34R gene product: two rabbit polyclonal antipeptide sera described above (A34R1 and A34R2), a rabbit polyclonal serum raised against bacterially expressed A34R (anti-A34R) (11), and the 12CA5 anti-Tag MAb specific for an epitope-tagged A34R gene product expressed by VV-A34Tag to be described in detail elsewhere (35). By sequential immunoprecipitation and Western blotting with 30 combinations of the seven antibodies (some antibodies did not bind blotted proteins well), antibodies that recognized the same proteins were grouped together. Representative Western blots are shown in Fig. 2, with a summary of results in Table 1. MAbs 4, 66, and 105 all recognized the same unidentified protein, but not protein immunoprecipitated by antibodies to A34R protein, whereas antibodies to the A34R protein bound to the A34R protein but not to the target of the MAbs. Figure 2A shows that MAb 66 bound proteins immunoprecipitated by MAbs 4, 105, and 66 but not proteins immunoprecipitated by the anti-Tag MAb from cells infected with VV-A34Tag. As shown in Fig. 2B, the anti-Tag MAb did not bind to blots of proteins immunoprecipitated with MAb 66 but did bind to blots of the tagged A34R proteins immunoprecipitated by the anti-Tag MAb, which appeared as a triplet of

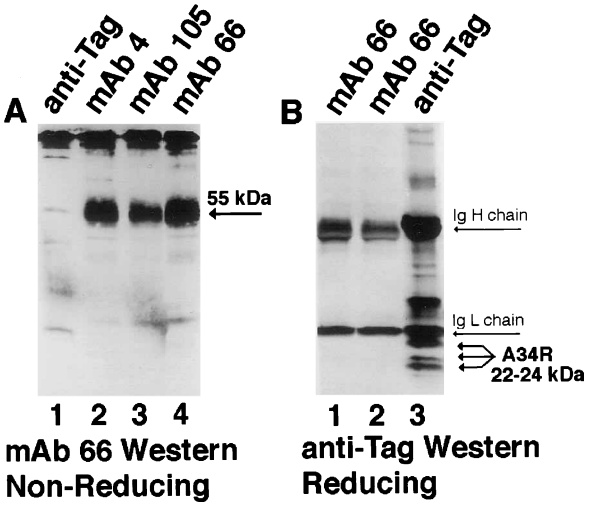

FIG. 2. Sequential immunoprecipitation and Western blotting of VV-infected BS-C-1 cell lysates. Cell lysates were immunoprecipitated with the MAbs indicated above the gel lanes. Proteins were boiled in 2% SDS in the presence or absence of 2% 2-mercaptoethanol, electrophoresed on 4 to 20% polyacrylamide gels, and transferred to membranes for blotting with the MAbs indicated below the gel. To explore the relationship of the MAbs and the A34R protein, the VV-A34Tag recombinant virus expressing the epitope-tagged A34R protein was used whenever the anti-Tag antibody was employed (lanes A1, B2, and B3). (A) Western blot analysis with MAb 66 of samples prepared under nonreducing conditions. Lane 1, VV-A34Tag-infected cell lysates immunoprecipitated with anti-Tag MAb; lanes 2 to 4, wild-type VV-infected-cell lysates immunoprecipitated with the indicated antibodies. The 55-kDa species is indicated. (B) Western blot analysis with the anti-Tag MAb of samples prepared under reducing conditions. Lane 1, uninfected-cell lysates immunoprecipitated with MAb 66; lane 2, VV-A34Tag recombinant virus-infected-cell lysates immunoprecipitated with MAb 66; lane 3, VV-A34Tag recombinant virus-infected-cell lysates immunoprecipitated with anti-Tag MAb. The tagged A34R gene product (21 to 24 kDa) is indicated. The immunoglobulin heavy (Ig H) and light (Ig L) chains are also shown.

TABLE 1. Sequential immunoprecipitation and Western blotting determination of antibody reactivity[a]

IP antibody[a]	Reactivity with Western blot antibody:				
	Anti-Tag[b]	Anti-A34R	MAb 4	MAb 66	MAb 105
Anti-Tag	+	+	−	−	−
A34R1	+	+	−	−	−
A34R2	+	+	−	−	−
MAb 4	−	−	+	+	+
MAb 66	−	−	+	+	+
MAb 105	−	−	+	+	+

[a] Immunoprecipitation (IP) with IP antibodies was followed by SDS-PAGE and Western blotting with a second antibody.
[b] A recombinant VV expressing an epitope-tagged A34R protein was used with the anti-Tag MAb.

22- to 24-kDa bands, as previously described (11, 23). The ability of this panel of antibodies to differentiate between these two proteins indicates that there is not an interaction between the proteins that withstands immunoprecipitation conditions. Parenthetically, because the antibodies used in the immunoprecipitation and the blot are both mouse antibodies, the heavy and light chains are visible on the gel prepared under reducing conditions and developed with anti-mouse secondary antibody (Fig. 2B). Collectively, these data indicated that there were two open reading frames that encoded EEV-specific glycoproteins with similar molecular weights, one corresponding to A34R and the other encoding the protein reactive with MAbs 4, 66, and 105.

Identification of an orthopoxvirus that does not encode a protein reactive with MAb 4 or MAb 105. To identify the gene encoding the protein recognized by MAbs 4, 66, and 105, a transfection approach was chosen. Since cellular transcription systems do not recognize VV promoters, our strategy involved transfection of VV DNA into cells infected with another orthopoxvirus able to transcribe and translate VV DNA. However, to implement this strategy, it was essential to identify a poxvirus that did not encode a protein that reacted with all three MAbs. In such a system, the gene encoding the putative new EEV protein could be identified by transfecting selected pieces of VV DNA into the appropriate virus-infected cells and screening for MAb reactivity. To find a suitable helper virus, an immunostaining survey was performed with MAbs 4, 66, and 105. Cells infected with cowpox, rabbitpox, and vaccinia virus strains WR, IHD-J, Copenhagen, NYCBH, Lister, Wyeth, and Ankara or modified Ankara vaccinia viruses reacted with all three of the MAbs and were therefore unsuitable for these experiments. However, cells infected with EV, Moscow strain, reacted with MAb 66 but not MAb 4 or 105. The lower panels of Fig. 3 shows an example of the typical strongly positive MAb 4, MAb 66, and MAb 105 horseradish peroxidase immunostaining of 24-h VV plaques. A VV plaque stained only with secondary antibody is shown as the negative control. EV does not form large plaques on BS-C-1 cells at 24 h. However, the numerous EV-infected cells stained very strongly with MAb 66, but not with MAb 4 or 105 (Fig. 3, upper panels). Western blotting showed that all 11 of the poxviruses (including EV) tested synthesized a MAb 66-reactive protein with an apparent molecular mass of 55 kDa under nonreducing conditions (data not shown). The data suggest that EV expresses a homologous protein that does not display epitopes reactive with MAb 4 or MAb 105 and, furthermore,

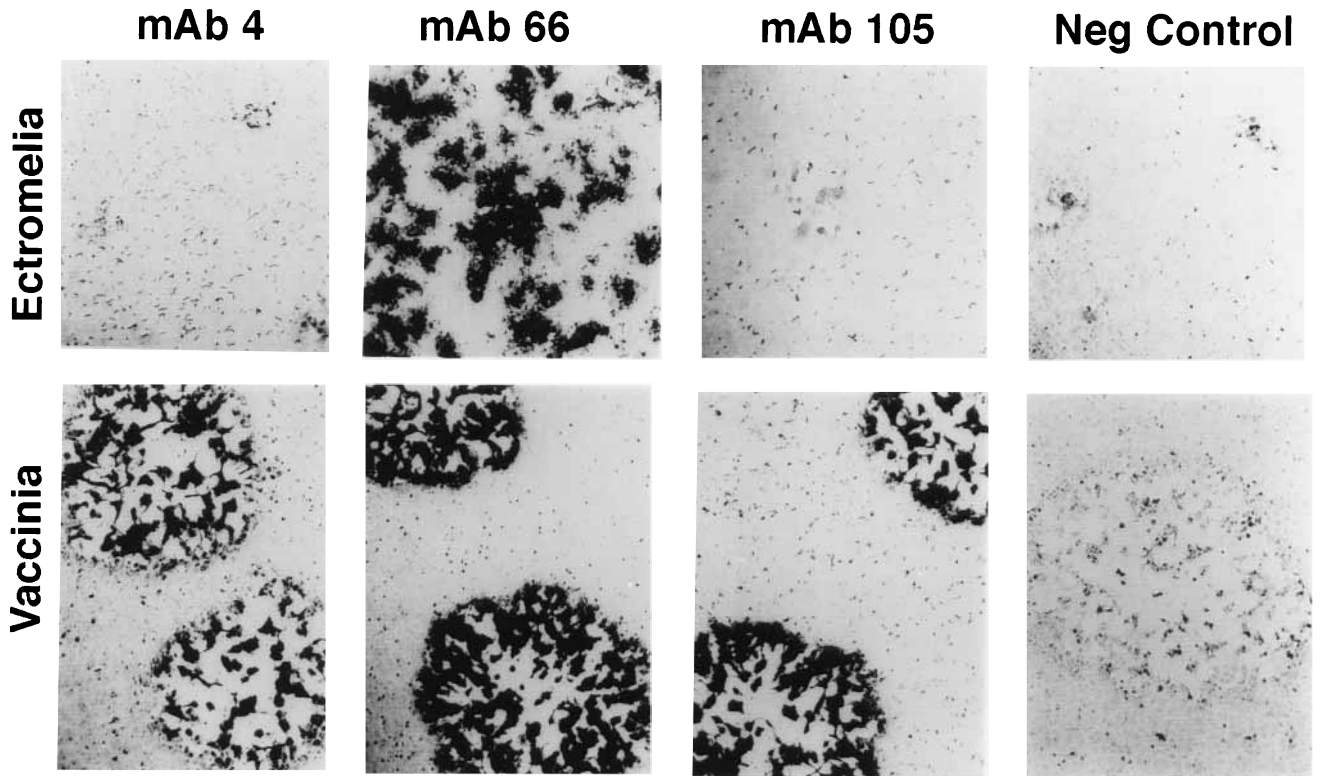

FIG. 3. Reactivity of EV and VV-infected cells stained with MAb 4, 66, or 105. Quadruplicate BS-C-1 monolayer wells were infected at a multiplicity of infection of <1 for 24 h, fixed, and immunostained with the noted primary MAbs and then incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin. The negative (Neg) controls were performed in the absence of primary antibody. The photographs are all at the same magnification.

FIG. 4. Expression of the MAb 4-reactive epitope after transfection of EV-infected BS-C-1 cells with cosmids and plasmids containing VV DNA. EV-infected cells were transfected with purified VV WR DNA or recombinant cosmids or plasmids containing segments of VV strain WR DNA. Numbers following WR indicate the approximate positions of the VV genome segments (in kilobases) within the cosmids. The Sal L plasmid contains 6.3 kb of DNA from the right center of *Hin*dIII-A. After 24 h, cells were fixed and immunostained with MAb 4.

that the MAb 66 epitope must be distinct from that of MAb 4 or 105.

Marker transfer of the MAb 4-reactive epitope. Having determined that EV does not synthesize a protein reactive with MAb 4 or MAb 105, the next step was to determine if such expression could be mediated by transfected VV DNA. When EV-infected cells were transfected with whole VV DNA, expression of the epitope reactive with MAb 4 was detected (Fig. 4). Uninfected cells that were transfected and EV-infected cells that were not transfected served as negative controls. These results confirmed previous observations that EV could transcribe VV DNA and translate it into VV proteins (36).

The next step was to determine which DNA sequence encoded the MAb 4-reactive protein. Individual overlapping cosmids from a library encompassing almost the entire genome of WR DNA (42) were transfected into EV-infected cells, which were then subjected to immunostaining. Figure 4 shows that three cosmids, each including sequences from kb 133 to 143 of the WR genome, allowed expression of the protein reactive with MAb 4, whereas cosmids with sequences from kb 93 to 130 and 143 to 178 did not. This mapped the gene encoding the MAb 4-reactive protein to a DNA segment near the middle of the *Hin*dIII A fragment of VV. Additionally, pA-64 (1), encompassing a 14.2-kb fragment also near the middle of *Hin*dIII-A, was positive for rescue of MAb 4 reactivity (data not shown).

Next, a subclone of pA-64 containing the 6.3-kb *Sal*I fragment was found to be positive in the marker transfer experiments (Fig. 4). This narrowed the candidate field to the eight VV genes from A31R to A38R. At this time, the plasmid was digested with restriction enzymes to remove segments of WR DNA, recircularized, and transfected into EV-infected cells. The immunostaining results of these experiments (data not shown) left three candidate genes: the A33R, A34R, and A35R genes. The A34R gene is the gene previously described to encode an EEV-specific protein with a similar molecular weight and biochemical properties (11, 23) but with distinct antibody reactivity (Fig. 2; Table 1). PCR was used to amplify the A33R gene, including its natural promoter elements, for cloning. Transfection of the cloned A33R DNA into EV-infected cells allowed expression of the protein that reacted with MAb 4, while transfection of vector DNA and cloned A35R and A34R did not (Fig. 5). Similar results were obtained with MAb 105 (data not shown). These data mapped MAb 4 and MAb 105 reactivity to the product of the A33R gene.

MAbs 4 and 105 immunoprecipitate a 55-kDa protein from EV-infected cells transfected with A33R. To further establish that the A33R gene was the gene encoding the authentic protein target of MAbs 4, 66, and 105, radioimmunoprecipitations of lysates of EV-infected or VV-infected cells were performed with the MAbs. In agreement with immunostaining data (Fig. 3), VV-infected cells synthesized a protein (55 kDa under nonreducing conditions) that reacted strongly with all three MAbs, whereas the EV-encoded protein reacted only with MAb 66 (Fig. 6A). However, when EV-infected cells were transfected with VV A33R DNA, MAbs 4 and 105 immunoprecipitated a 55-kDa protein similar to that immunoprecipitated from VV-infected cells (Fig. 6B). Under reducing conditions, the protein precipitated by MAbs 4 and 105 from EV-infected, A33R-transfected cells appeared as a 23- to 28-kDa protein (data not shown). Thus, the A33R gene encodes the 23- to 28-kDa monomer and the 55-kDa presumptive dimer that react with the EEV-specific MAbs.

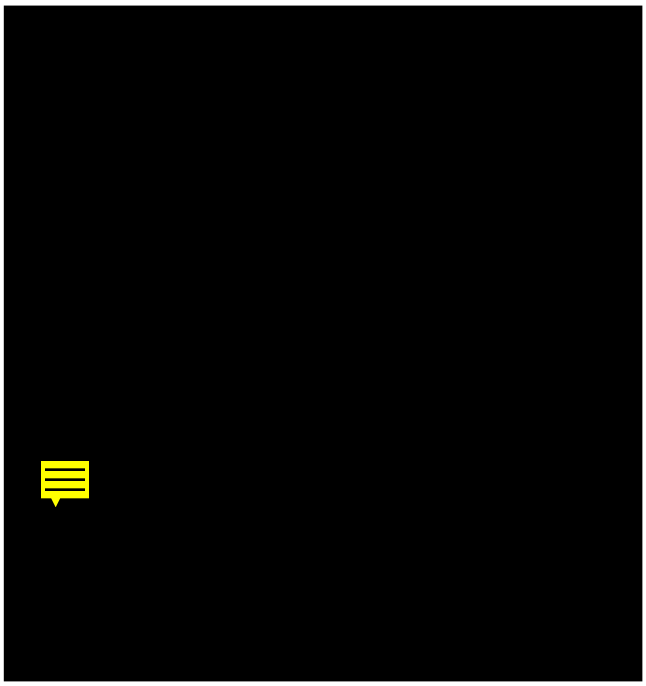

FIG. 5. Expression of the MAb 4-reactive epitope after transfection with the A33R gene. EV-infected BS-C-1 cells were transfected with vector DNA or cloned A33R, A34R, and A35R genes. After 24 h, cells were fixed and immunostained with MAb 4.

The similar electrophoretic mobilities of the EV and VV proteins recognized by MAb 66 (Fig. 6A) suggested that the EV protein is probably an A33R homolog rather than another viral protein with a coincidental cross-reactive epitope. However, the EV protein must differ in sequence from the VV protein, since it lacks the epitopes reactive with MAbs 4 and 105. Additionally, our data showed that the EV-encoded protein immunoprecipitated by MAb 66 is slightly larger than the VV-encoded protein both under reducing (data not shown) and nonreducing (Fig. 6A) gel conditions. This raised the possibility that the EV gene may have a longer coding sequence or more sites for glycosylation.

Sequence of the EV A33R gene. To determine if EV encodes an A33R homolog, EV DNA was amplified with primers specific for VV DNA flanking the A33R gene. A single EV PCR product the same size as the DNA amplified from VV was obtained. This DNA was then sequenced. Figure 7 displays the 635-nucleotide sequence, including 51 bp upstream, the 558-bp open reading frame, and 26 bp downstream of the EV A33R gene, with VV strain Copenhagen sequence differences noted below the EV sequence. Computer analysis with the FASTA program (33) revealed 94.2% DNA identity between VV and EV A33R open reading frame homologs. The VV late-gene transcription start site TAAAT motif was also conserved 18 bp upstream of the ATG in the EV sequence. Furthermore, both sequences predict proteins of 185 amino acids.

Figure 7 also shows the amino acid sequence of the EV A33R homolog in comparison with those of VV and variola virus. The VV A33R gene sequence predicts that 94.1% of amino acids are identical with the variola virus A36R gene product (10 differences) and that 90.3% are identical with the EV A33R homolog (18 amino acid differences). The variola virus A36R gene product has 27 amino acid differences relative to the EV gene product, with 85% identity. The A33R gene products of VV strains WR (41) and Copenhagen (15) have identical amino acid sequences. Variola virus strains Bangladesh, Harvey, and India are also identical to each other in the predicted 184 amino acids of the A33R homolog, A36R (39). There is one less amino acid in the corresponding variola virus protein than in the VV and EV proteins.

Disulfide bonds can be important structural features of proteins, and in the A33R gene-encoded protein, six cysteines are conserved in variola virus, VV, and EV. There are also two asparagine-linked NXS/T glycosylation sites at amino acid residues 125 and 135 in the VV and EV A33R gene products and only one in variola virus (position 134). Differences in amino acid composition and predicted molecular weights (20,505 for VV and 20,680 for the EV A33R homolog) may account for the slight difference in electrophoretic mobility between the VV and EV homologs (Fig. 6).

Hydrophilicity plots (Fig. 7) predict that the VV and EV A33R genes encode a type II integral membrane protein with a 31-amino-acid amino-terminal cytoplasmic domain, a stretch of hydrophobic amino acids from residues 32 to 57 which could serve as a combination signal sequence-membrane anchor, and a 128-amino-acid exterior carboxy terminus. The carboxy terminus is predicted to be on the outside surface of infected cells and on the outside surface of the EEV. The encoded N-linked glycosylation sites occur in the predicted extracellular-extraviral domain of the A33R protein.

The A33R gene encodes an integral membrane protein. To determine whether the A33R protein behaves as an integral membrane protein as predicted, Triton X-114 partitioning was performed (6). Uninfected and VV-infected cells were lysed in Triton X-114 detergent, separated into aqueous and detergent phases, electrophoresed, and transferred to membranes for Western blotting. The membranes were probed for the A33R gene product with MAb 4, and antibodies to the 94-kDa RNA polymerase-associated protein (RAP 94) were used as a control. As shown in Fig. 8, the polyclonal sera to RAP 94 detected protein solely in the aqueous phase, as expected for a soluble protein. However, MAb 4 recognized a protein in the aqueous phase as well as the Triton X-114 detergent phase of the extraction, as expected for an integral membrane protein.

A33R protein is on the outer envelope of EEV. Since the VV A33R protein behaves as an integral membrane protein and

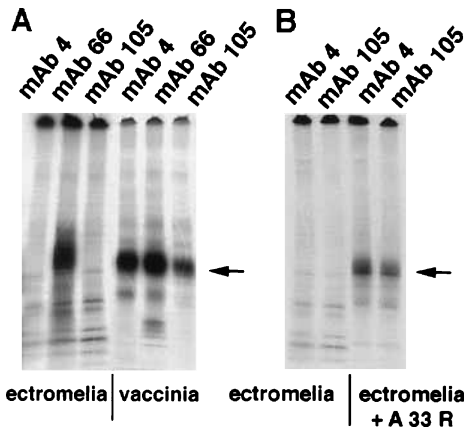

FIG. 6. SDS-PAGE analysis of metabolically labeled proteins immunoprecipitated from infected- and transfected-cell lysates. (A) BS-C-1 cells were infected with VV or EV, metabolically labeled, and lysed, and proteins were immunoprecipitated with the MAbs indicated above the gel lanes. Samples were prepared and electrophoresed under nonreducing conditions. (B) EV-infected cells, either untransfected or transfected with a plasmid containing the A33R gene, were similarly immunoprecipitated. The 55-kDa species (arrows) is indicated.

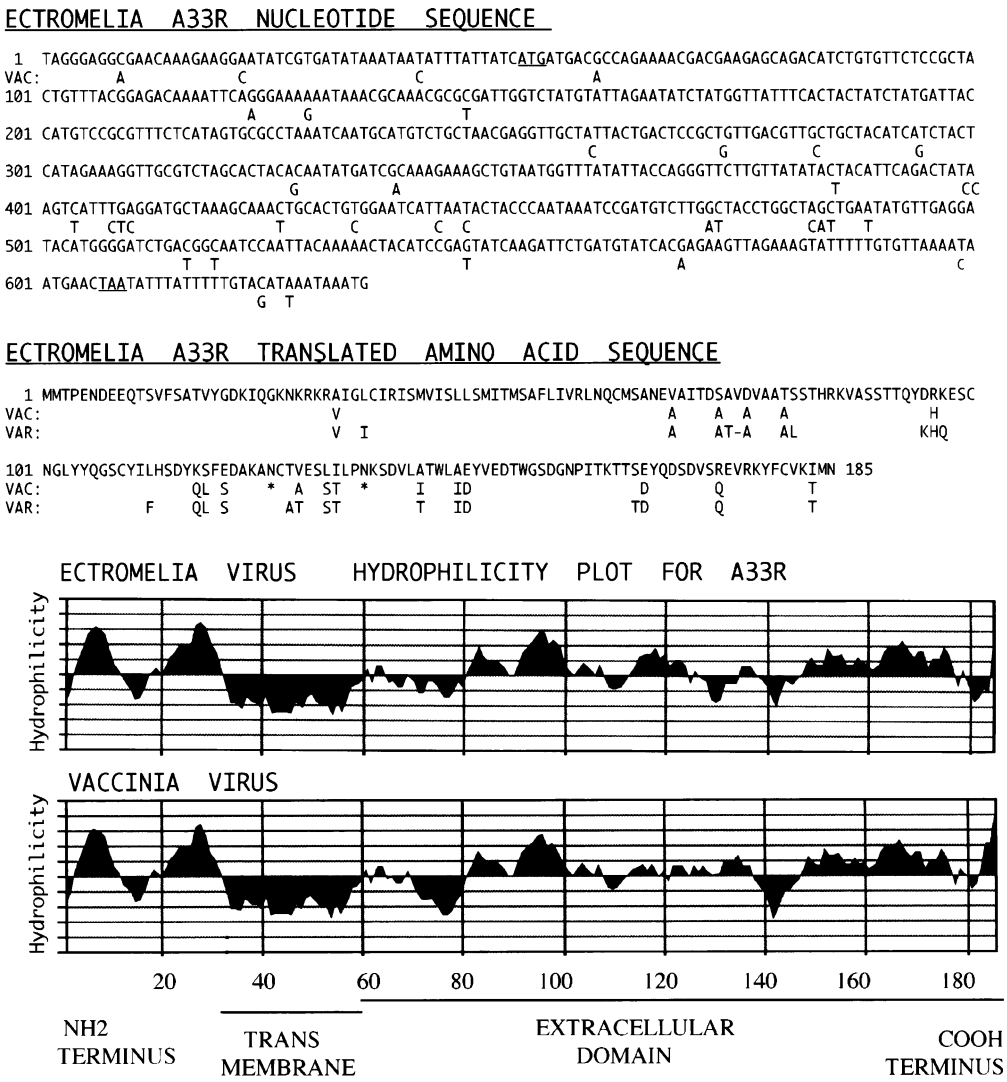

FIG. 7. Analysis of the nucleotide and predicted amino acid sequences of the A33R gene. EV A33R DNA sequence is shown, with VV differences noted below (VAC). Translation start and stop codons are underlined. The amino acid sequence of EV is also shown, with the differences in VV and variola virus (VAR) indicated below. Two asparagine-linked glycosylation sites (NXS/T) motifs (asterisks) and the variola virus deletion at amino acid 75 (dash) are indicated. Hydrophilicity plots of EV and VV A33R gene-encoded proteins are also shown, with the predicted transmembrane and extracellular domain indicated.

MAb 4 was reported to immunoprecipitate an EEV-specific protein (30), the presence of the A33R-encoded protein on the surface of EEV was investigated. EEVs and IMVs were purified in CsCl density gradients and immunogold stained for viewing by electron microscopy (Fig. 9). Antibodies previously characterized as EEV or IMV specific were used as controls. MAb 20 reacts with a 42-kDa protein encoded by the B5R gene and is EEV specific (21, 30). MAb C3 recognizes the 14-kDa protein product of the A27L gene and is IMV specific (34). MAbs 4, 66, and 105 all bound the protein on the surface of purified EEVs. As many as 80 gold grains on a single EEV were counted, whereas only occasional grains were associated with IMV (Fig. 9). Thus, the A33R gene product is exposed on the surface of the EEV. Furthermore, these data suggest that all three MAbs map to the 128-amino-acid hydrophilic portion of the protein that is predicted to be external.

Neither the EEV-binding protein nor the cell surface receptor has been identified, and the functions of the EEV-specific proteins have not been elucidated. Since the MAb 4-reactive

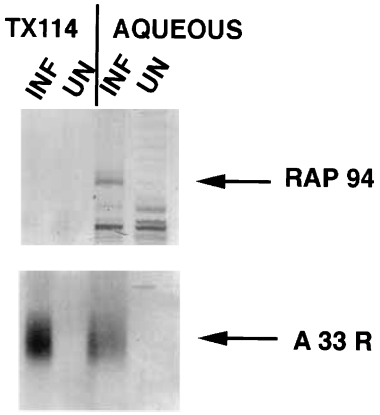

FIG. 8. Triton X-114 partitioning of the A33R protein. VV-infected (lanes INF) and uninfected (lanes UN) BS-C-1 cells were lysed in Triton X-114, and proteins were separated into aqueous and detergent (TX-114) phases. These proteins were electrophoresed and Western blotted with MAb 4 or polyclonal rabbit antisera to the 94-kDa protein RAP 94.

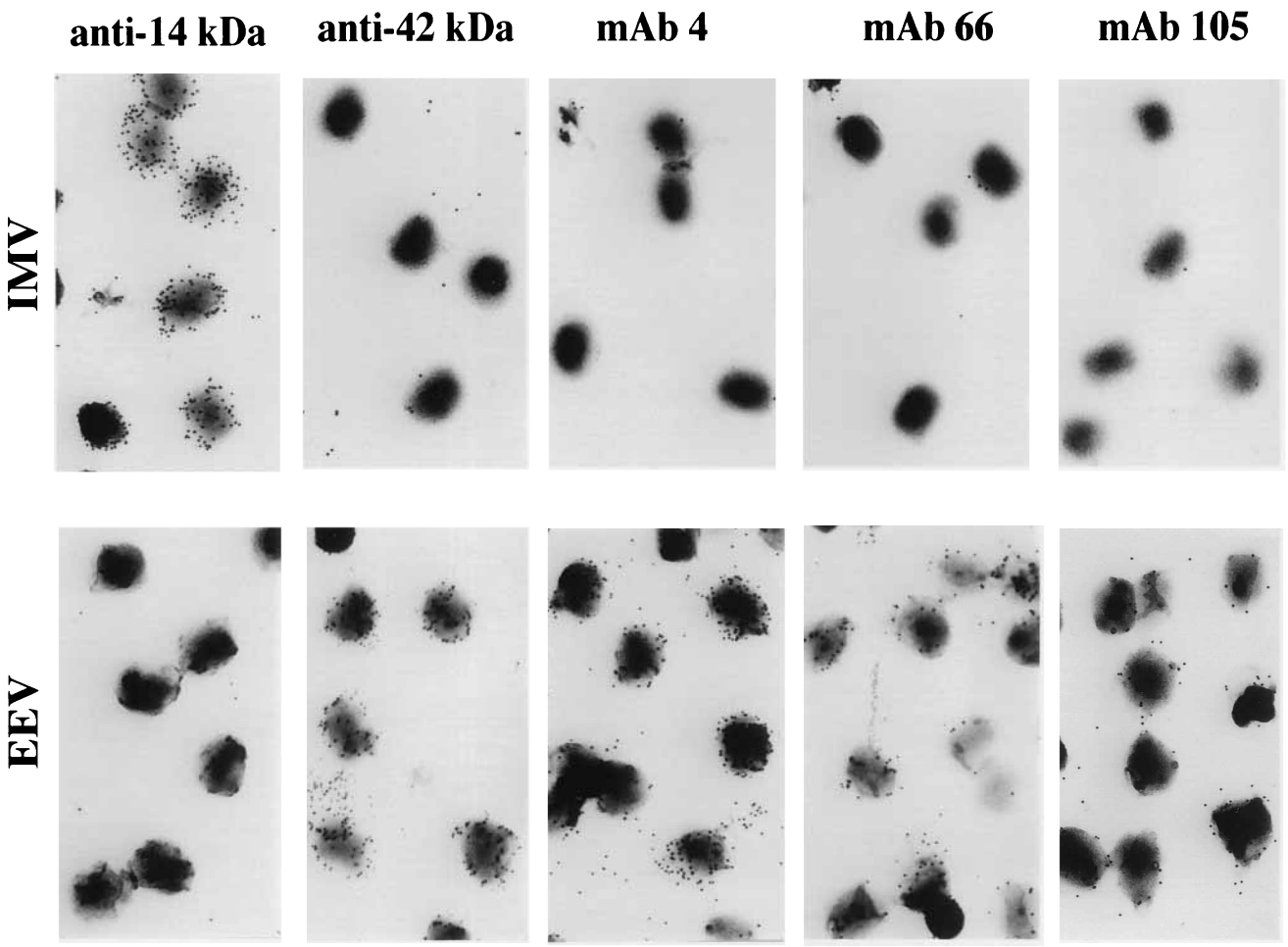

FIG. 9. Immunogold electron microscopy of VV particles. CsCl density centrifugation-purified EEV and IMV were adsorbed to grids and incubated with MAbs 4, 66, and 105 and then with protein A-gold (10-nm-diameter gold particles). For controls, the IMV-specific anti-14-kDa protein (A27L gene) antibody and the MAb 20 EEV-specific anti-42-kDa protein (B5R gene) antibody were used.

A33R-encoded protein is on the surface of the EEV, it may have a function in viral attachment or entry into the cell. As the EEV can be neutralized by polyclonal sera, we tested the MAbs for their ability to neutralize EEV infectivity. EEVs were CsCl gradient purified and incubated with MAbs 4, 66, and 105 at concentrations as high as 1:50 for 2 h, and subsequently the MAb-virus mix was used to infect cells. No neutralization of EEVs was found (data not shown). Note, however, that if the EEV membrane was disrupted, exposed IMV proteins could mediate infection, obscuring EEV-neutralizing activity of the MAbs.

DISCUSSION

This study provides the first evidence that the A33R gene encodes a protein specific to the outer membrane of the EEV. Payne (30) had shown that MAb 4 reacts with a group of 23- to 28-kDa glycoproteins that were specifically associated with the EEV. Subsequently, Duncan and Smith (11) reported that antiserum raised to a fusion protein containing A34R sequence reacted with three glycoprotein species of 22 to 24 kDa and made the reasonable conclusion that they were probably the same proteins. Having no reason to doubt this, Schmelz et al. (37) published data under this assumption. On the basis of the present work, we now know that both the A33R and the A34R genes encode proteins that are EEV specific, expressed late in infection, and multiply glycosylated and have similar molecular weights. Reinterpretation of the report of Schmelz et al. in light of the new data indicates that the protein encoded by the A33R gene is localized in the Golgi complex, the cell surface, and the outer viral envelope. Therefore, characterization of the A34R protein in these respects remains to be done.

Under reducing and nonreducing conditions, the A33R protein migrates through a polyacrylamide gel as a diffuse 23- to 28-kDa band and as a 55-kDa species, respectively. The predicted molecular weight of the polypeptide is 20,505. The difference between the predicted 21 kDa and the observed 23 to 28 kDa can be explained by glycosylation of the protein. The predicted amino acid sequence contains two N-linked glycosylation sites. Also, previously it was shown that viral growth in the presence of either tunicamycin or monensin decreased the apparent M_r of the MAb 4-reactive protein band, indicating that this protein is both N and O glycosylated (30). When the drugs were used in combination, a 21,000-molecular-weight protein band was precipitated from VV-infected cells, in good agreement with the predicted M_r of the A33R open reading frame.

It is likely that the 23- to 28-kDa band is the monomeric form of the protein and that the 55-kDa species represents a

dimer, as previously suggested (30). The monomeric A33R protein might form a homodimer or complex with another protein of similar size, such as the A34R protein. The data indicate, however, that the A33R and A34R proteins do not form heterodimers that withstand immunoprecipitation, as protein precipitated with antibodies to one protein did not react with antibodies to the other protein in a Western blot (Table 1). The putative dimerization interaction may be mediated by disulfide bonds, as reducing agents disrupt the dimer. The A33R sequence predicts six cysteine residues in the protein, so one or more of these cysteines could be available for the intermolecular bonding evidenced by the 55-kDa species.

Several lines of evidence indicate that the three MAbs bound products encoded by the A33R gene. MAbs 4, 66, and 105 all reacted with a 55-kDa protein under nonreducing conditions and with a 23- to 28-kDa protein in the presence of reducing agents. All three MAbs bound proteins precipitated by the others and reacted with the A33R protein on the surface of the EEV. Nevertheless, the epitope recognized by MAb 66 is distinct from the others, since MAb 66 immunostained EV-infected cells, whereas MAb 4 and MAb 105 did not. Indeed, this difference allowed us to map the gene encoding the MAb 4 and 105 determinant(s) by marker transfer. EV-infected cells that were transfected with the cloned A33R gene could be immunostained with MAbs 4 and 105. Furthermore, proteins of the expected molecular weight were immunoprecipitated from such transfected cells. Additional studies showed that the EV protein reacting with MAb 66 is a homolog of the VV protein. The MAbs should be of further use in characterizing the intracellular trafficking and processing of the EEV glycoprotein.

All 11 orthopoxviruses tested synthesized a protein recognized by MAb 66 in Western blots. The published VV and variola virus homologs exhibit 94% amino acid identity (22). We sequenced the EV homolog of the VV A33R gene and found that the overall amino acid identity between the two is 90%. However, the amino-terminal portion of the variola virus, VV, and EV A33R homologs is more highly conserved than the remainder of the protein (Fig. 7). The amino-terminal 67 amino acids of the EV-encoded protein is 99% identical to that of VV, while the carboxy two-thirds is 85% identical, suggesting that there may be an increased selective pressure to maintain sequence in the amino-terminal portion of the molecule (the amino acids in and around the transmembrane domain). Although the transcriptional regulatory sequences have not been characterized, the initiation codon of the A33R gene is 129 nucleotides away from that of the A32L gene, so it seems unlikely that the A33R coding sequence is constrained for A32L expression. Perhaps the amino-terminal coding residues are highly conserved because of A33R-encoded protein interactions with other conserved cellular or viral proteins for the appropriate localization of A33R in the viral envelope. Without such constraints, the carboxy terminus may tolerate more variation. Indeed, mutations in the exterior portion of the protein may confer selective advantages on the viruses in interacting with their specific hosts in vivo, e.g., variola virus and EV are restricted to humans and mice, respectively. While greater sequence variation is allowed in the extracellular portion of the protein, the presence of this domain is conserved. The variola virus A33R homolog has a 3-bp deletion corresponding to codon 75 of the VV and EV genes and is consistent with selective pressure to preserve expression of the entire protein in vivo.

The function of the A33R gene product remains to be elucidated. We attempted to engineer an A33R deletion mutant of VV by inserting an antibiotic resistance gene. Despite searches for small plaques, successive rounds of selective enrichment, and attempts at limiting-dilution cloning, no recombinant virus in which the A33R gene was deleted could be isolated. If the A33R gene is essential, it may have a function in addition to EEV formation, as mutants unable to form EEVs have been previously isolated by these methods (4, 44). However, there have been previous examples of genes which ultimately were deleted despite initial failure. Work is under way to create a repressible A33R mutant virus to study the phenotype in the absence of normal A33R protein levels.

Remarkably, three of the six known genes encoding EEV-specific proteins (A33R, A34R, and A36R) are clustered in 2.4 kb of the VV genome, and five reside within a 20-kb area of the nearly 200-kb genome. Whether the A35R gene product is also an EEV protein remains to be determined. Although A33R, A34R, and A36R have similar-length open reading frames, the very low level of sequence similarity between these genes indicates that they are not recent products of gene duplication. It is notable that the clustered A33R, A34R, and A36R genes reside in the most highly conserved region between variola virus strain DNA sequences (A33L to A49R) (39). This region of DNA has diverged from the VV sequence but seems to be preferentially conserved among different strains of variola virus, suggesting that the EEV may be important for the life cycle or virulence of variola virus (22, 39).

Six VV genes have now been reported to encode EEV-specific proteins. Following the identification of any remaining EEV genes, the focus will shift to determining the functions and interactions of these proteins with one another as well as other viral and cellular proteins. In time, this will lead to a basic understanding of how poxviruses assemble, leave the parent cell, and spread, biological questions with wide relevance.

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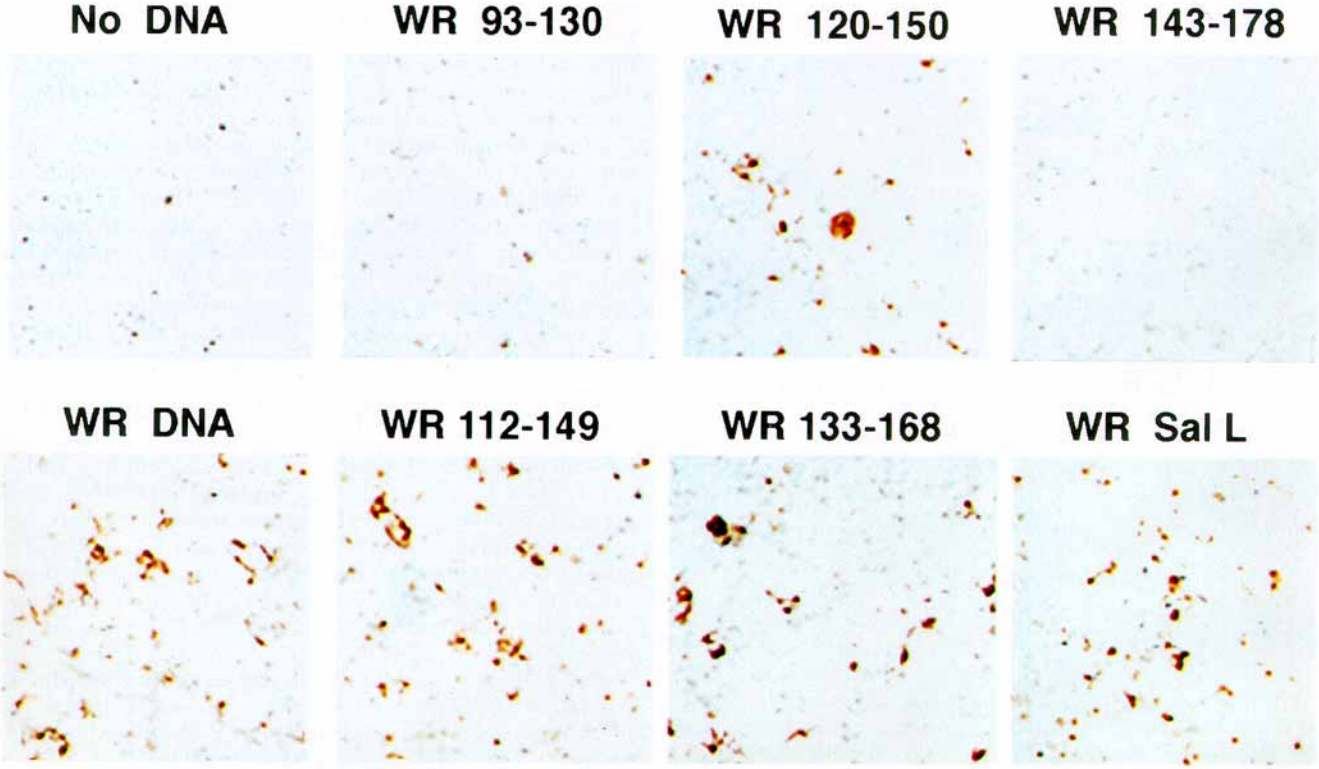
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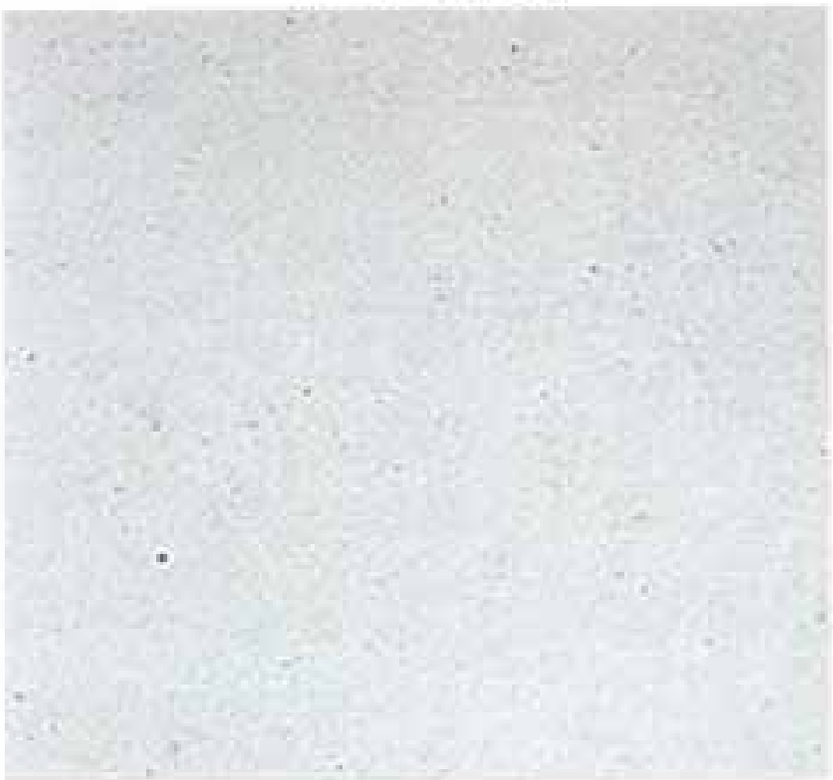

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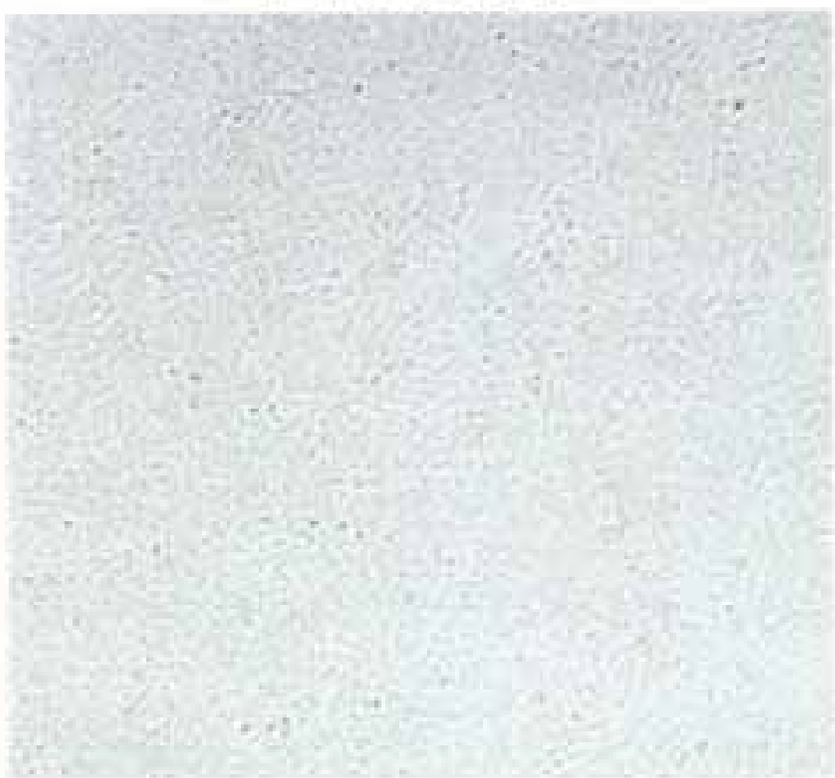

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