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Structural and Functional Studies of a 39,000-M_r Immunodominant Protein of Vaccinia Virus

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Little is known about the nature of poxvirus proteins involved in the host immune response. Screening a lambda gt11 expression library of genomic rabbit poxvirus DNA with hyperimmune rabbit anti-vaccinia virus serum and selection of monospecific antibodies identified a highly antigenic viral protein of about 39,000 molecular weight (39K protein). The same-size protein of vaccinia virus was also identified with a monoclonal antibody (MAb B6) obtained from hybridomas generated after fusion of hyperimmunized mouse spleen cells with mouse myeloma cells. Structural analysis revealed that the 39K protein is an acidic polypeptide, that it can exist in two molecular forms because of intramolecular disulfide linkages, and that it is part of the virus core. This protein shares antigenic determinants with a cytoplasmic component(s) from uninfected cells. Functional studies revealed that the 39K protein is synthesized at late times postinfection and appears to be required for virus assembly. This protein is highly conserved in members of the *Orthopoxvirus* group, but in cowpox virus, a 41K virion protein was specifically recognized by antibodies that reacted against the vaccinia virus 39K protein. Significantly, during long-term passages of Friend erythroleukemia cells persistently infected with vaccinia virus, some virus mutants were found to increase or decrease by about 2 kilodaltons the size of the 39K protein. Mapping analysis localized sequences encoding the 39K protein in a rifampin-sensitive gene cluster between the two major core-associated viral polypeptides, 4a and 4b. The fact that the 39K core protein of vaccinia virus elicits strong humoral immune response, induces antibodies that react against a host component(s), and is subjected to genetic variability suggests that this protein has important biological functions.

Vaccinia virus is an effective and potent immunogen. A vaccine prepared with live vaccinia virus was used in the first example of a worldwide immunization program that successfully eradicated a human disease, smallpox. One main feature of smallpox eradication is that vaccinia virus elicited a strong and long-lasting immunity, activating both humoral and cell-mediated immune responses (1, 4). New strategies based on the ability of vaccinia virus to express foreign genes (11, 17) may permit even wider use of vaccinia virus as a vaccine; however, little is known about the nature of viral proteins involved in the host immune response. The large, 185-kilobase, double-stranded genome of vaccinia virus codes for about 200 genes (14). About 274 different polypeptides are induced during virus infection (2), and about 100 polypeptides are associated with the virion (6). Because live vaccinia virus may potentially be used as a polyvalent vaccine against a broad spectrum of animal and human diseases (for a review, see reference 10), it becomes important to understand the molecular basis for immunogenicity of this virus. In this paper, we describe the identification and properties of an important immunogenic protein of vaccinia virus.

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MATERIALS AND METHODS

Virus and cells. The wild-type strain WR of vaccinia virus was propagated in HeLa S_3 cells and purified as previously described (9). Rabbitpox and cowpox viruses were grown in rabbit kidney cells and were kindly provided by J. A. Holowczak. Shope fibroma virus was kindly provided by B. G. T. Pogo. Vaccinia virus mutants were obtained from persistently infected Friend erythroleukemia (FEL) cells as previously described (16). Virus titration was done using monkey kidney (BSC-40) cells.

Antibody screening of lambda gt11 rabbitpox DNA library. A library prepared from randomly generated rabbit poxvirus DNA fragments contained within the expression vector lambda gt11 (13) was kindly provided by R. W. Moyer. Before its use in screening, the polyclonal rabbit anti-vaccinia serum was preadsorbed with 150-mm-diameter nitrocellulose filters containing *Escherichia coli* Y1090 cells infected with wild-type lambda gt11 bacteriophage. Screening was done by a modified procedure previously described (7, 20). Briefly, phage (1.2×10^6) were allowed to infect an overnight culture of *E. coli* Y1090 for 15 min at 37°C, plated in 150-mm-diameter dishes at 2×10^4 phage per plate in 0.7% soft agar, and incubated at 42°C for 3 h. Plates were overlaid with nitrocellulose paper presoaked in 10 mM isopropyl-β-D-thiogalactopyranoside and incubated at 37°C for 18 h. Thereafter, filters were washed with phosphate-buffered saline (PBS) and blocked for 2 h at room temperature with 5% nonfat dry milk (BLOTTO) in PBS. Filters were then incubated from 3 to 15 h with preadsorbed anti-vaccinia rabbit polyclonal antibodies. Filters were washed three times, 10 min each, first with PBS and then with a solution of 0.5% Triton X-100 in PBS twice. Immunopositive plaques were detected with ^{125}I-labeled protein A diluted in 5% BLOTTO and incubated with filters for 2 h at room temperature. Filters were washed as described above and air dried, and positive plaques were detected after autoradiography.

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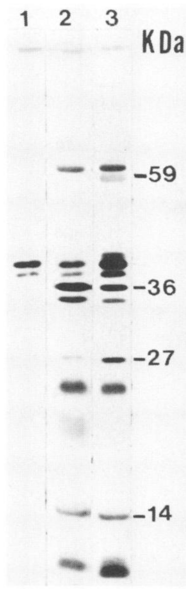

FIG. 1. Reactivity of monospecific and polyclonal antibodies of rabbit and mouse origin against structural polypeptides of vaccinia virus. Western blots of purified vaccinia virus (30 μg per lane) were reacted with antibodies (m2) selected from a recombinant poxvirus DNA phage (lane 1), with polyclonal anti-vaccinia serum generated in rabbits with disrupted virus (lane 2) and with antibodies generated after 2 weeks of immunization in mice inoculated intraperitoneally with live vaccinia virus (10^6 PFU per mouse) (lane 3). Molecular sizes in kilodaltons (KDa) are given for some virus polypeptides.

Plaques were picked up and rescreened until homogeneously positive. High-titer stocks of each positive phage were prepared on *E. coli* Y1090 (7).

Selection of monospecific antibodies from lambda gt11 recombinant phage. Monospecific antibodies were selected on nitrocellulose filters prepared as described in the preceding section. After incubation for 3 to 5 h with preadsorbed rabbit anti-vaccinia antiserum, filters were washed with PBS and then with a solution of 0.5% Triton X-100 in PBS twice, 10 min each time. Filters were then cut into small pieces and transferred to a capped tube containing 3.5 ml of 0.2 M glycine hydrochloride, pH 2.5, for 2 min with shaking. Thereafter, 1.75 ml of 1.0 M K_2HPO_4, pH 9.0, was added to the tube, and the solution was diluted with 1 volume of 2% bovine serum albumin in water (7). The eluted antibodies were tested for specificity on Western blots (immunoblots) of purified virus.

Monoclonal and polyclonal antibodies. Monoclonal antibodies against structural proteins of vaccinia virus were generated as previously described (19). Polyclonal antibodies were prepared from rabbits immunized by three intramuscular injections of purified viral envelopes or cores (100 μg of protein per injection). The first injection was in Freund complete adjuvant, and two other injections were administered at 15-day intervals but with incomplete adjuvant. One week later, rabbits were bled from the ear vein and serum was collected.

Preparation of envelopes and cores of vaccinia virus. Viral envelopes were prepared from 5 mg of purified virus suspended in 1 ml by sonication (Brownill Biosonik; three times for 5 s each at 50 W) in 50 mM Tris hydrochloride (pH 8.5)–10 mM $MgCl_2$–10 mM dithiothreitol–0.5% Triton X-100. Addition of a reducing agent facilitates separation of the virus lateral bodies from the core (4). After incubation at 37°C for 30 min, the extract was layered on top of a 36% sucrose cushion in the same buffer and cores were pelleted by centrifugation at 20,000 × *g* for 60 min at 4°C. The upper phase, containing the viral envelopes, was dialyzed extensively against PBS, and cores were suspended with sonication in PBS. Most of the cores remained intact as determined by electron microscopy of negatively stained preparations.

Immunoblotting. Protein (20 to 40 μg per well) from purified virions was applied to wells and fractionated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels. The proteins were transferred to nitrocellulose, and immunoblots were developed as described previously (19), except that 5% BLOTTO (nonfat dry milk) was used instead of fetal calf serum. Virions and cells were disrupted by boiling for 3 min in sample buffer (30 mM Tris hydrochloride, pH 6.8; 2% SDS; 5% glycerol; 0.012% bromophenol blue) in the absence or presence of the reducing agent β-mercaptoethanol.

Immunofluorescence. Cells growing in cover slips (24 wells) were infected with vaccinia virus (5 PFU per cell), and at various times postinfection (p.i.) cells were washed three times with PBS and fixed under conditions that make the cells nonpermeable (4% formaldehyde, 0.1% glutaraldehyde in PBS at 4°C for 45 min) or permeable (cold acetone at room temperature for 15 min). Subsequent treatments were done as previously described (21). Briefly, cells were washed, incubated for 60 min with a monoclonal antibody (MAb B6), washed, and then incubated for 30 min with affinity-purified goat anti-mouse immunoglobulin G conjugated with Rhodamine (Cooper Biomedical, Inc.) at 1:10 dilution in 1% bovine serum albumin. Cells were washed, cover slips were mounted with glycerol-PBS (1:1 [vol/vol]) on a microscope slide, and cells were visualized and photographed in a Zeiss fluorescence microscope.

Cloning and mapping. Isolation of phage and plasmid DNA, restriction site mapping, Southern blots, and subcloning were done by procedures previously described (20).

RESULTS

Identification of a 39K immunodominant protein of vaccinia virus. Two approaches were used to identify immunodominant proteins of vaccinia virus. The first is based on the use of a lambda gt11 expression library of rabbitpox virus DNA, and the second is based on the use of mouse hybridomas. The lambda gt11 expression library was screened with hyperimmune rabbit anti-vaccinia virus serum, and recombinant phage giving a strong signal with ^{125}I-labeled protein A were selected. These phage were used independently to infect *E. coli* cells, cell lysates were transferred to nitrocellulose paper, and monospecific antibodies were selected after incubation with rabbit polyclonal antiserum, followed by washings and elution (as described in Materials and Methods). Immunoblots of one-dimensional SDS-polyacrylamide gel electrophoresis of structural polypeptides of vaccinia virus reacted with recombinant phage-selected antibodies were used to identify the antibody specificity. The results obtained with one such recombinant (m2) are presented in Fig. 1. The virion protein recognized by the monospecific antibody (lane 1) was compared with the virion proteins that reacted with hyperimmune polyclonal antiserum from rabbits inoculated with disrupted vaccinia virus (lane 2) and with serum from primary immunized mice inoculated with live vaccinia virus (lane 3). It is evident that the phage-selected monospecific antibodies (m2) reacted

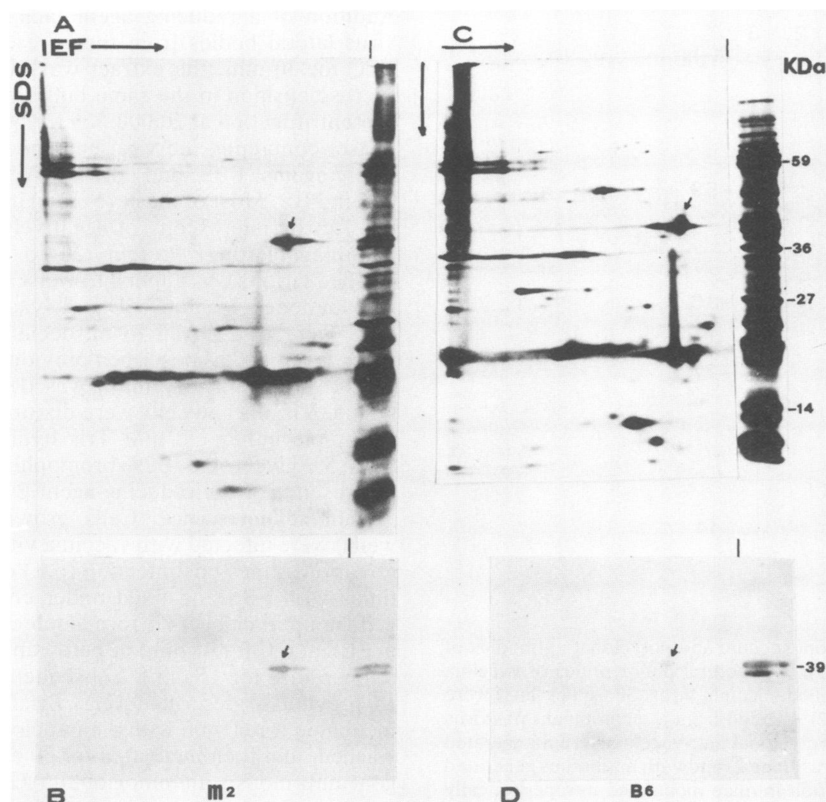

FIG. 2. Identification of a 39K immunodominant protein of vaccinia virus. Immunoblots of structural polypeptides of vaccinia virus (40 μg of protein) separated by two-dimensional SDS-polyacrylamide gel electrophoresis were reacted with monospecific (m2) and with monoclonal (MAb B6) antibodies. Panels A and C show autoradiograms of ^{35}S-labeled vaccinia virus polypeptides. Panels B and D show the immunoperoxidase staining of the same filters (A and C) reacted with m2 (panel B) and with MAb B6 (panel D), respectively. The migration of the 39K immunoreactive polypeptide is indicated by an arrow in each panel. One-dimensional run of vaccinia virus structural polypeptides is shown to the right of each panel. The first (isoelectric focusing) and second (SDS) runns are indicated. Molecular sizes in kilodaltons (KDa) of some vaccinia polypeptides are given.

strongly against a protein of about 39,000 molecular weight (39K protein) and weakly to a protein of about 37K. Similar-size proteins were strongly recognized by antibodies from immune sera of rabbits and of mice. Because the 39K protein is recognized similarly by the two different antisera, this protein might be a prototype of an immunodominant viral protein. Thus, we focused our studies on the functional significance of this protein. To provide evidence that the 39K protein is immunodominant, we immunized mice with vaccinia virus and prepared hybridomas as previously described (19). The hybridoma supernatants were tested in immunoblots for their ability to recognize a 39K protein of vaccinia virus. Assays similar to that shown in Fig. 1 identified a monoclonal antibody (MAb B6) that reacted specifically against a 39K protein. To show that m2 and MAb B6 do recognize the same-size protein, we performed immunoblot analysis of virion proteins separated by two-dimensional SDS-polyacrylamide gel electrophoresis. The complex pattern of ^{35}S-labeled vaccinia virus proteins is shown in the upper part of Fig. 2 (panels A and C). When Western blots were reacted with either m2 (Fig. 2B) or MAb B6 (Fig. 2D), a single protein was recognized by the two antibodies. This protein is acidic, with a pI of about 5. By superimposing the ^{35}S-labeled proteins (panels A and C) with the corresponding immunoblots (panels B and D), we confirmed that m2 and MAb B6 recognize the same-size 39K protein. From the results of Fig. 1 and 2, we conclude that we have identified an immunodominant protein of vaccinia virus of 39K.

39K protein of vaccinia virus is part of the virus core. Since vaccinia virus is an envelope virus and proteins of the envelope are thought to play a major role in host immune responses (4), it was of interest to determine the localization of the 39K protein in the virion. Thus, purified virions were treated with detergent and β-mercaptoethanol, and the envelope and core fractions were separated by centrifugation. The distribution of the 39K protein between these two fractions was examined by immunoblots of one-dimensional SDS-polyacrylamide gel electrophoresis. The bulk of the 39K protein was found as part of the virus core (Fig. 3).

Synthesis of the 39K vaccinia virus core protein occurs late in infection. Vaccinia virus gene expression is coordinately regulated (14). Thus, it was of interest to define the time course of synthesis for the 39K protein during virus infection. This was examined by immunoblot analyses. Cells were infected with vaccinia virus, in the absence or presence of specific inhibitors of virus DNA synthesis and of virus assembly, and at various times p.i. cell extracts were prepared and examined by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose paper, the paper was reacted with MAb B6, and the antigen-antibody complex was visualized with ^{125}I-labeled protein A after autoradiography (Fig. 4). It is clear that the 39K protein belongs to the late class of viral genes. The protein was not

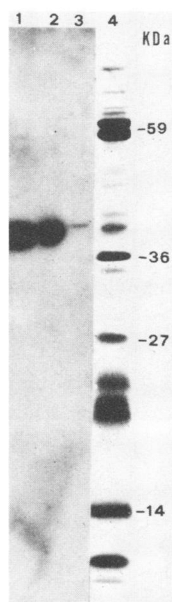

FIG. 3. Distribution of the 39K protein between envelope and core components of vaccinia virus. Western blots of about 20 μg of core (lane 1), whole virus (lane 2), and envelopes (lane 3) were reacted with MAb B6, and the immunoreactive protein was detected with ^{125}I-labeled protein A after autoradiography. Lane 4 represents ^{35}S-labeled polypeptides of purified vaccinia virus run in parallel. KDa, Kilodaltons.

synthesized in the presence of cytosine arabinoside (lane 3) and accumulated at late times (lane 10) p.i. Accumulation of this protein begins at about 8 h p.i. (data not shown). Significantly, synthesis of the 39K protein was completely blocked in infected cells treated with rifampin (lane 13), but it was synthesized after rifampin was removed from cultures (lanes 15 to 18). Rifampin is a specific inhibitor of vaccinia virus assembly (15). Upon drug removal, the 39K protein accumulated and by 18 h reached the same levels as in the control (compare lanes 14 and 15). This protein was not detected when cycloheximide (100 μg/ml) was added after rifampin reversal and cell extracts were analyzed up to 8 h later. This indicates that there is no precursor-product relationship for the 39K protein. With longer exposures of the autoradiograms, we detected some cross-activity of MAb B6 with 70K, 30K, and 16K cellular proteins (data not shown). The 39K protein accumulation after drug removal correlated with a gradual increase in virus yields (see legend of Fig. 4). From the results of Fig. 4, we conclude that the 39K protein of vaccinia virus is synthesized at late times p.i. On the basis of its inhibition of synthesis by rifampin and resumption of synthesis and of virus yields after removal of the drug, it suggests a role for this protein in the process of virus assembly.

Subcellular distribution of the 39K protein of vaccinia virus. To demonstrate appropriate subcellular distribution of the vaccinia virus 39K protein, uninfected and infected cells of various origins (mouse, monkey, and human) were examined by immunofluorescence. Representative findings with mouse L cells are shown in Fig. 5. The most striking observation is that in permeabilized cells MAb B6 cross-reacted with uninfected cells (panels A and B). Fluorescence in uninfected cells was observed only in the cytoplasm (panel B). Despite alterations in cellular morphology, in virus-infected mouse cells (Fig. 5, panels C and D) fluorescence was confined to the cytoplasm and was greater in infected than in uninfected cells. Control experiments revealed no fluorescence in nonpermeabilized uninfected cells (data not shown), and some degree of fluorescence occurred in virus-infected cells late in infection (panels E and F).

From the results of Fig. 5, we conclude that the 39K protein of vaccinia virus is distributed in the cytoplasm and cross-reacts with a cellular component(s).

39K protein of vaccinia virus forms intramolecular disulfide linkage. Immunogenicity of a protein may be determined by the extent of its aggregation. It is known that a number of the structural proteins of vaccinia virus can form aggregates held by disulfide bonds (8). Since we have identified a 14K envelope protein that forms covalently linked trimers and elicits neutralizing antibodies (19, 21), it was of interest to examine whether the 39K protein could also form covalently bound aggregates. Purified ^{35}S-labeled virions were disrupted in the presence of increasing concentrations of β-mercaptoethanol, and the Western blot of one-dimensional SDS-polyacrylamide gel electrophoresis was reacted with MAb B6 (Fig. 6A). While in the presence of high concentrations of the reducing agent, there is a single 39K band (lane 2); as the concentration decreases (lanes 4 and 6), a prominent 37K band appears instead. This result suggests intramolecular linkage in the 39K protein. This was further documented by a diagonal immunoblot analysis of two-dimensional SDS-polyacrylamide gel electrophoresis using purified ^{35}S-labeled virus. The first dimension was run under either reducing (Fig. 6B) or nonreducing (Fig. 6C) condi-

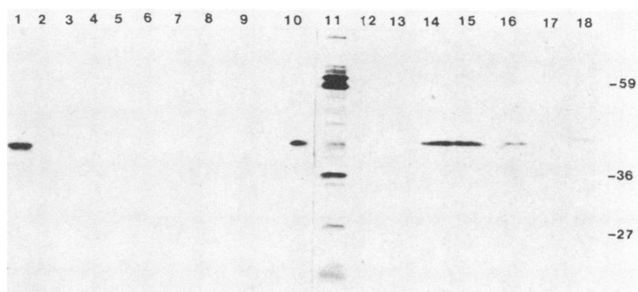

FIG. 4. Accumulation of the 39K protein in the course of vaccinia virus infection of cells. BSC-40 cells (10^6) grown in 24-well Limbro dishes were infected with 5 PFU of vaccinia virus per cell in the absence or presence of cytosine arabinoside (40 μg/ml) or rifampin (100 μg/ml). At appropriate times p.i., cells were collected and lysed in sample buffer. Samples (70 μg of protein) were examined by SDS-polyacrylamide gel electrophoresis, Western blots were reacted with MAb B6, and the immunoreactive protein was detected with ^{125}I-labeled protein A after autoradiography. Two independent experiments are shown (lanes 1 to 10 and 11 to 18). Lane 1 represents 20 μg of purified vaccinia virus. The time course of virus infection is indicated as follows. Lanes: 2, uninfected cells; 3, cells infected in the presence of cytosine arabinoside; 4, zero time p.i.; 5, 2 h p.i.; 6, 4 h p.i.; 7, 5 h p.i.; 8, 6 h p.i.; 9, 7 h p.i.; 10, 18 h p.i.; 11, ^{35}S-labeled vaccinia virion proteins; 12, uninfected cells; 13, rifampin-treated, infected cells at 18 h p.i.; 14, untreated, infected cells at 18 h p.i. In lanes 15 to 18, infected cells were treated with rifampin for 18 h, the drug was removed by washing, and cells were collected at various times after drug removal: 18 h (lane 15), 6 h (lane 16), 4 h (lane 17), and 2 h (lane 18). The virus yields (PFU per milliliter) were respectively: 4×10^4 (18 h in the presence of rifampin); 1.04×10^6 (2 h after drug reversal); 1.8×10^6 (4 h after drug reversal); 4.6×10^6 (8 h after drug reversal); 1.2×10^7 (18 h after drug reversal). Numbers at the right indicate molecular sizes in kilodaltons.

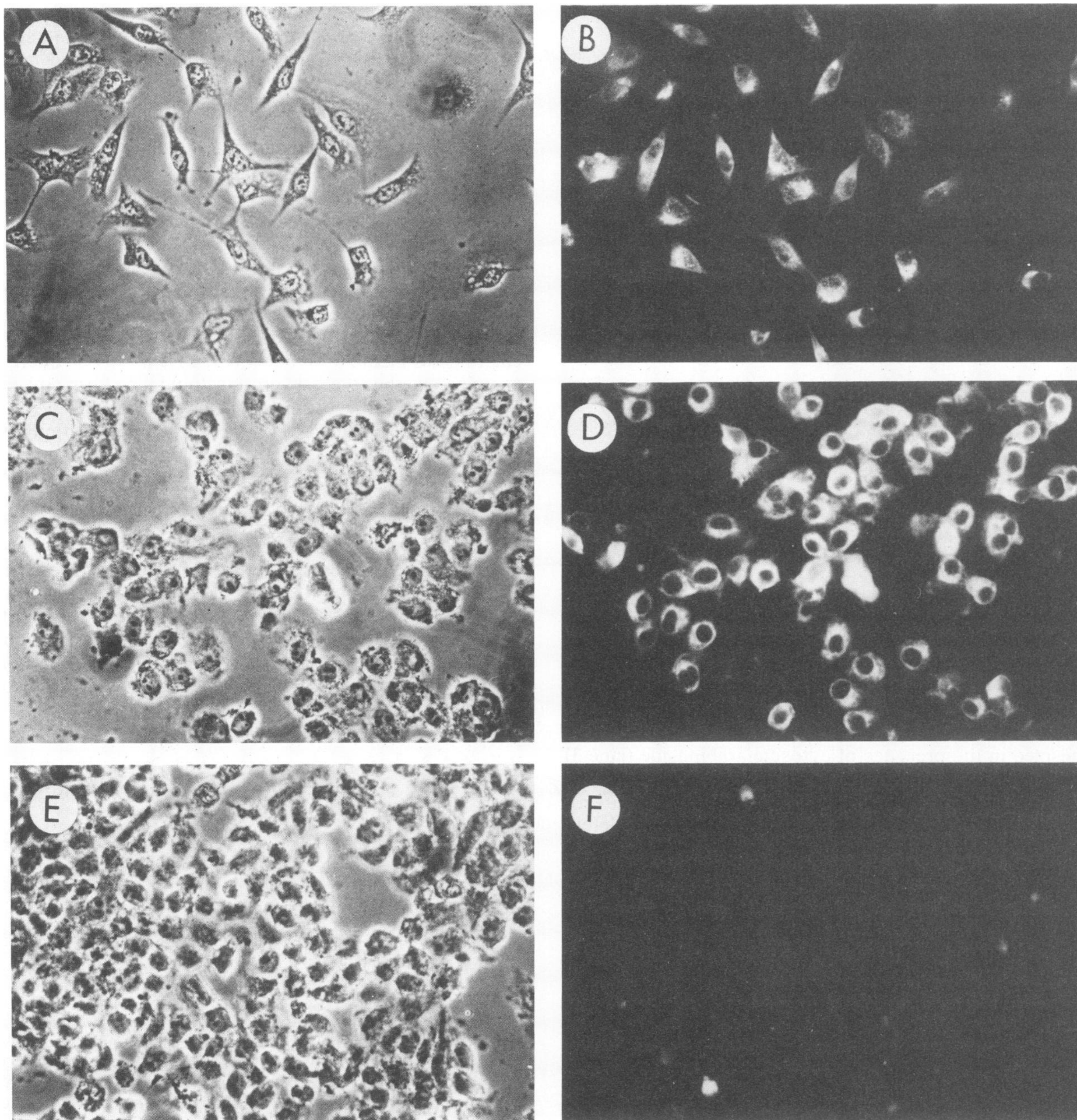

FIG. 5. Subcellular localization of the 39K protein in vaccinia virus-infected cells. Mouse L cells grown in cover slips were infected with 5 PFU of vaccinia virus per cell, and at 18 h p.i., cells were fixed and treated with MAb B6 at a 1:10 dilution, and the cover slips were processed for indirect immunofluorescence staining as described in Materials and Methods. Photographs represent phase-contrast and fluorescent images of permeabilized uninfected cells (A and B), permeabilized virus-infected cells at 18 h p.i. (C and D), and nonpermeabilized infected cells at 18 h p.i. (E and F).

tions, and the second dimension was run under standard conditions. The immunoblots were reacted with MAb B6 to visualize the 39K protein, and the same blot was also exposed to X-ray film to visualize the virion proteins after autoradiography. From the autoradiogram, it is evident that a number of virion proteins form aggregates under nonreducing conditions (panel C) as compared with reducing conditions (panel B). Among these aggregates, there is a 39K protein. By superimposing the autoradiograms of panels B and C with corresponding immunoblots (not shown), we established the position of the 39K protein under reducing and nonreducing conditions as the one indicated by the arrows.

From the results of Fig. 6, we conclude that the 39K protein of vaccinia virus is present in the virion as a single polypeptide and can exist in two molecular forms as a result of intramolecular disulfide linkage.

39K protein is conserved in members of the *Orthopoxvirus* group but is modified in size in cowpox virus. To document relatedness of the 39K protein, it was of interest to examine whether the 39K protein was conserved among poxviruses. Thus, Western blots were done with several purified viruses

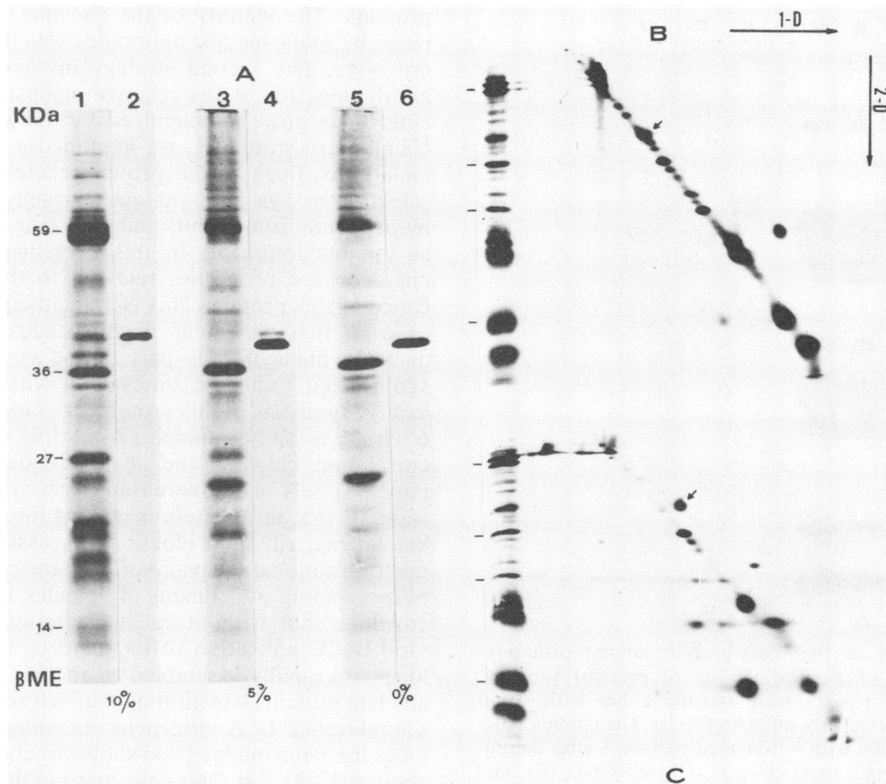

FIG. 6. State of aggregation of the 39K protein in vaccinia virions. (A) Western blots of one-dimensional SDS-polyacrylamide gel electrophoresis of ^{35}S-labeled purified vaccinia virus disrupted in the presence of the reducing agent β-mercaptoethanol (βME) at 10% (lanes 1 and 2), 5% (lanes 3 and 4), and 0% (lanes 5 and 6). Western blots in lanes 2, 4, and 6 were reacted with MAb B6, and the 39K protein was detected by ^{125}I-labeled protein A. Panels B and C show autoradiograms of a diagonal two-dimensional SDS-polyacrylamide gel electrophoresis procedure using [^{35}S]vaccinia virion proteins separated by size under reducing (panel B) and nonreducing (panel C) conditions. The purified virus was disrupted with (panel B) or without (panel C) β-mercaptoethanol and run in the first dimension; thereafter, the lanes were cut, soaked in buffer with 10% β-mercaptoethanol and run in the second dimension under standard conditions. The same blot was reacted with MAb B6, and the migration of the immunoreactive 39K protein is indicated with arrows in the autoradiogram.

of the *Orthopoxvirus* group (vaccinia virus, rabbitpox virus, and cowpox virus) and of the *Leporipoxvirus* group (Shope fibroma virus). The blots were reacted either with polyclonal antibodies or with MAb B6. The results are shown in Fig. 7 for cowpox, rabbitpox, and vaccinia viruses. Immunoblots reacted with hyperimmune rabbit polyclonal antibodies (panel A) revealed a similar pattern for the three viruses, except for a highly immunogenic protein of about 39K in vaccinia and rabbitpox viruses and of about 41K in cowpox virus. There was no reactivity of polyclonal antibodies with Shope fibroma virus proteins (data not shown). Significantly, MAb B6 reacted not only with a 39K protein in vaccinia and rabbitpox viruses but with a 41K protein in cowpox virus. Again, there was no reactivity of the monoclonal with Shope fibroma virus proteins (data not shown). The immunoreactivity and appearance of a protein doublet (39K and 37K) in Fig. 7 suggests that similar sequences and intramolecular linkages exist for the 39K and 41K proteins in the three viruses.

From the results of Fig. 7, we conclude that the 39K protein is highly conserved in members of the *Orthopoxvirus* group and is modified in size in cowpox virus.

39K protein of vaccinia virus is modified in size during virus persistence. Modifications in size of a structural protein between vaccinia and cowpox viruses suggest mutational changes in the viral genome. These mutational changes could have occurred spontaneously as a result of multiple passages of the two viruses. Because vaccinia virus can establish persistent infections in FEL cells (16, 18), this virus-cell system offers the means to analyze whether modifications in size of the 39K protein could have taken place during continuous virus growth in culture cells. Figure 8 shows an immunoblot of two representative virus mutants isolated after long-term passages from persistently infected FEL cells and reacted with polyclonal and with m2 antibodies. The most striking observation is to find modifications in size of the 39K protein in mutants from persistently infected cells. One of the mutants (65-16) increased the size of the 39K immunoreactive protein by about 2 kilodaltons, while another mutant (101-14) decreased its size by about 2 kilodaltons. Similar results were obtained when the immunoblots were reacted with MAb B6 (data not shown). Analyses similar to those of Fig. 6 provided evidence that molecular weight differences between mutants, 65-16 and 101-14, and wild-type virus are due to changes in size of the 39K protein (data not shown). Because of the specificity of antibodies (m2 and MAb B6), we conclude that alterations in size of the 39K protein of vaccinia virus are the result of mutational changes introduced in the virus population during virus persistence.

Physical map of the 39K-encoding gene. To localize in the viral genome the sequences encoding the 39K protein, we performed Southern blot hybridization analyses. Vaccinia virus DNA was digested with various restriction enzymes,

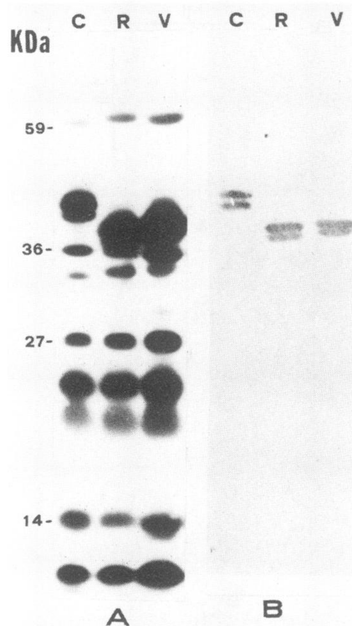

FIG. 7. Conservation of the 39K protein among orthopoxviruses. Western blots of purified (40 μg of protein) vaccinia, rabbitpox, and cowpox viruses were reacted either with rabbit anti-vaccinia virus serum (A) or with MAb B6 (B). The immunoreactive proteins were detected with ^{125}I-labeled protein A after autoradiography. The virus origin was: cowpox (C), rabbitpox (R), and vaccinia (V). Molecular sizes, in kilodaltons (KDa), of some proteins of vaccinia virus are given. The pattern of proteins for the three viruses stained with amido black has been described previously (19).

blotted, and hybridized to various nick-translated probes (Fig. 9). From the hybridization data and from the known restriction maps of vaccinia virus DNA (5, 22, 27), it was found that sequences encoding the 39K protein mapped to the left of vaccinia virus *Hin*dIII-A DNA. Finer mapping localized the 39K-encoding sequence within the two genes that encode the two major core polypeptides 4a and 4b (Fig. 9). This is a region in which a polypeptide of about 38K has been detected with late viral mRNAs after hybrid selection and cell-free translation (22).

DISCUSSION

Vaccinia virus has been used in the past as an effective vaccine against smallpox (1); however, little is known about the nature of viral proteins that are involved in the host immune response (4, 14). These proteins are of particular relevance in view of the possible novel use of vaccinia virus as a recombinant vaccine (11, 17). This is because genes of human and veterinary importance can be introduced into vaccinia virus DNA, can be expressed faithfully under the control of the virus regulatory signals, and can elicit both humoral and cell-mediated immune responses that fully protect inoculated animals that have been challenged with infectious agents (for a review, see reference 10).

To identify viral proteins involved in host immune response, we have used two complementary strategies. The first involved screening a lambda gt11 expression rabbitpox DNA library with rabbit-polyclonal anti-vaccinia virus serum and selection of recombinant phage that induced strong immunoreactive fusion proteins. Monospecific antibodies were then selected from recombinant phage-induced fusion proteins. The identity of the vaccinia virus protein recognized by monospecific antibodies was determined by Western blots. The second strategy involved screening mouse hybridomas for MAbs reactive against the same-size vaccinia virus protein recognized by monospecific antibodies. Using these strategies, we identified a 39K protein of vaccinia virus. Because this protein reacted strongly in the two selection assays and was also strongly recognized by immune serum from rabbits and mice (Fig. 1), it can be defined as immunodominant. In this investigation, we have also characterized properties relevant to the functional significance of this protein. This is an acidic polypeptide that can exist in two molecular forms because of intramolecular disulfide linkage. It is part of the virus core, and is not synthesized until late times after virus infection. Significantly, synthesis of this protein was blocked by the inhibitor of virus assembly, rifampin. After drug removal, there was a correlation between the accumulation of this protein in infected cells with increased virus yields (Fig. 4). This suggests that this protein is needed for virus assembly. It is known that rifampin blocks virus assembly by preventing posttranslational cleavage of a major core polypeptide and by preventing attachment of spicules to the bilayer of the envelope, but without causing alterations in the mode of viral DNA replication. After the drug is washed out, envelopes are rapidly assembled to enclose the DNA plus early and late proteins constituting the mature virion (for a review, see reference 4). A virus gene encoding a rifampin-sensitive locus has been mapped and shown to encode a late protein of about 63K (23, 24). This gene maps to the right of the *Hin*dIII D-A junction and is near the major core polypeptide 4b, the cleavage of which is blocked by rifampin (22, 27). This gene cluster is expressed during infection in a noncoordinated fashion (25). Of significance is that we have found that the 39K-encoding sequence maps between the viral genes encoding the two major core polypeptides 4a and 4b (Fig. 9). In

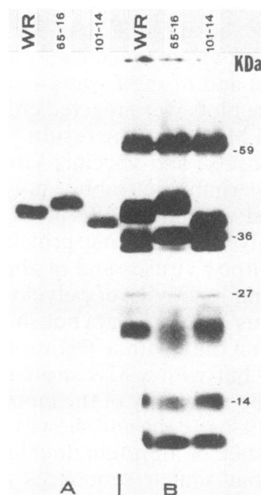

FIG. 8. Alterations in size of the 39K protein of vaccinia virus during virus persistence. Establishment of virus persistence in FEL cells was as described previously (16). Two representative virus mutants isolated at passages 65 and 101 are shown. Western blots of purified (40 μg of protein) vaccinia virus wild type (WR) and mutants (65-16 and 101-14) were reacted either with monospecific antibodies (m2) (A) or with rabbit anti-vaccinia serum (B). The immunoreactive proteins were detected with ^{125}I-labeled protein A after autoradiography. Molecular size markers are indicated, with sizes in kilodaltons (KDa).

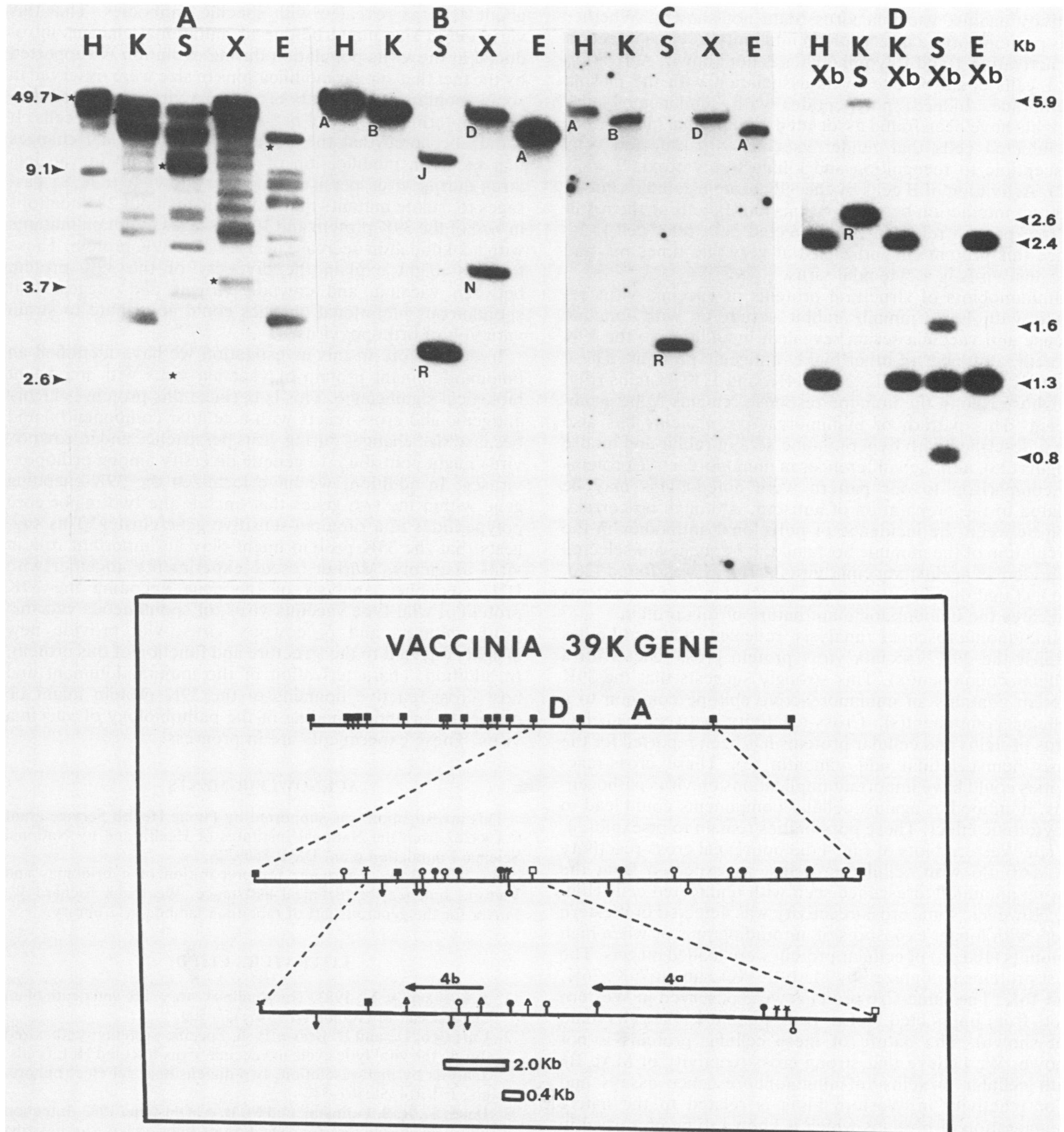

FIG. 9. Physical mapping of sequences encoding the 39K protein on vaccinia DNA. The *Eco*RI inserts of 2 and 0.4 kilobases from two recombinant phages (m2 and m3) of rabbitpox DNA used in the selection of monospecific antibodies to the 39K protein were subcloned in pUC19. The plasmid DNA was labeled with ^{32}P by nick translation and was hybridized to Southern blots of various restriction fragments of vaccinia DNA. The probes were total vaccinia virus DNA (A), 2-kilobase *Eco*RI insert (B and D), and 0.4-kilobase insert (C). Restriction maps of vaccinia virus DNA and the localization of the 39K-encoding sequences from two recombinant phages are shown. The nomenclature of restriction fragments of vaccinia DNA is from DeFilippes (5). Restriction enzymes: *Hin*dIII (H,); *Sal*I (S,); *Bam*HI (B,); *Xho*I (X,); *Pvu*I ( ); *Xba*I (Xb,); *Kpn*I (K,); *Eco*RI (E, ★). Kb, Kilobases.

view of this association, it is possible that the 39K-encoding gene is regulated at the transcriptional or posttranscriptional level by a gene(s) from the rifampin-sensitive gene cluster. This gene cluster is likely to play an important role in virus assembly (23). The exact mechanism by which structural polypeptides of vaccinia virus are affected by rifampin remains to be elucidated.

Although the 39K protein evoked a strong humoral immune response, this protein did not elicit neutralizing antibodies. Neither MAb B6 nor m2 antibodies had neutralizing

activity against vaccinia virus (data not shown). Whether this protein plays a direct role in immunity to virus infection by activating T and B lymphocytes is not known. Activation of T cells could be due to its association with the plasma membrane. Indeed, polypeptides with similar molecular weights have been found associated with plasma membranes of infected cells that render vaccinia virus-infected cells susceptible to recognition and killing by cytotoxic T cells (12). Activation of B cells by the 39K protein could be due to a direct interaction between virions and B cells or the result of free protein released from infected cells after cell lysis, since this protein is synthesized at very late times p.i. and vaccinia virus is a cytocidal virus.

Immunoblots of structural proteins of vaccinia virus reacted with hyperimmune rabbit serum or with immune mouse anti-vaccinia serum revealed, in addition to the 39K protein, a number of other highly antigenic proteins. These are 62K, 36K, 32K, 27K, 21K, 14K, and 11K proteins (Fig. 1) whose role in the immune response remains to be established. This pattern of immunoreactive proteins has also been described with hyperimmune sera of rabbit and mouse origins (26), although differences in immunoreactive proteins in comparison to our pattern were noted. This may be related to the preparation of antisera. Although no correlation between the incidence of polyclonal antibodies in the circulation of the immune host and the frequency of selected hybridomas against vaccinia virus antigens was found (26), the fact that the 39K protein was selected in the two systems indicates the immunodominant nature of this protein.

Immunofluorescence analysis revealed that antibodies against the 39K vaccinia virus protein cross-react with a cellular component(s). This strongly suggests that the 39K protein contains an immunoreactive epitope common to a cellular component(s). Cross-reactivity between vaccinia virus proteins and cellular proteins has been reported for the virus hemagglutinin and vimentin (3). These cross-reactivities could have important implications on virus pathogenesis, if antibodies against cellular components could lead to a cytotoxic effect. These possibilities remain to be explored.

The Western blots of Fig. 4 did not reveal cross-reactivity of MAb B6 with cellular proteins, as expected from the strong immunofluorescence seen with uninfected cells (Fig. 5). However, some cross-reactivity was detected in Western blots with longer exposures of autoradiograms or when high amounts (100 μg) of cellular protein were loaded on gels. The host proteins recognized by MAb B6 were about 70K, 26K, and 16K. The same-size proteins were observed in Western blots from uninfected HeLa, BSC-40, and L-929 cells (data not shown). The nature of these cellular proteins is not known. We believe that strong cross-reactivity of MAb B6 with cellular proteins in immunofluorescence assays and poor reactivity in Western blots is related to the native configuration of proteins which is known to be preserved in the former assay as opposed to the later assay. Similar observations have been reported with other MAbs (26).

Another important finding reported in this investigation concerns genetic variability and evolution of orthopoxviruses. Although there are many members in the poxvirus family, little is known about their genetic variability. It is generally believed that these viruses are well conserved throughout evolution. With the exception of terminal heterogeneity at both ends of the viral genome, strong sequence conservation has been found within a central region of the DNA (14). In this investigation, we have found that a 39K core protein changed in size in orthopoxviruses. The size of the 39K protein in vaccinia virus changed in cowpox virus to about 41K, as revealed with specific antibodies. That this variation in size might be due to mutational changes introduced in the virus population during evolution is supported by the fact that similar modifications in size were observed in some spontaneous mutants of vaccinia virus generated during long-term passages of persistently infected FEL cells. It should be noted that the frequency of mutational changes that result in modification of the 39K protein in vaccinia virus during virus persistence is very low. It took 65 passages to isolate mutants that have gained about 2 kilodaltons in size of the 39K protein and 101 passages to obtain mutants with a 2-kilodalton decrease in size of the 39K protein. These findings might explain the diversity of the 39K protein between vaccinia and cowpox viruses and suggest that spontaneous mutational changes could contribute to strain variation of orthopoxviruses.

In conclusion, in this investigation we have identified an immunodominant protein of vaccinia virus with important biological significance. This is because this protein is highly antigenic and cross-reacts with a cellular component(s) and because size changes during virus persistence and in cowpox virus might contribute to genetic diversity among orthopoxviruses. In addition, we have localized the 39K-encoding gene within the two genes that encode the two major core polypeptides in a rifampin-sensitive gene cluster. This suggests that the 39K protein might play an important role in virus assembly. Marker rescue experiments, together with DNA sequence analysis of the gene encoding the 39K protein of wild-type vaccinia virus, of spontaneous vaccinia virus mutants, and of cowpox virus, will provide new insights relevant to the structure and function of this protein. In addition, characterization of the immunodominant and host cross-reactive domains of the 39K protein might be crucial to an understanding of the pathobiology of vaccinia virus. These experiments are in progress.

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