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Expression of Aleutian Mink Disease Parvovirus Capsid Proteins by a Recombinant Vaccinia Virus: Self-Assembly of Capsid Proteins into Particles

DAHN L. CLEMENS,[1]† JAMES B. WOLFINBARGER,[1] SHIRO MORI,[1] BRADLEY D. BERRY,[1] STANLEY F. HAYES,[2] AND MARSHALL E. BLOOM[1]*

Laboratory of Persistent Viral Diseases[1] and Laboratory of Vectors and Pathogens,[2] Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840

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A portion of a cDNA clone containing coding sequences for both structural proteins (VP1 and VP2) of Aleutian mink disease parvovirus (ADV) was inserted into recombinant vaccinia viruses, VV:ADSP. Immunohistochemical staining of VV:ADSP-infected cells revealed that the ADV antigen was readily detected and localized in the nuclei of infected cells. Analysis of VV:ADSP-infected cell lysates indicated that both VP1 and VP2 were produced and comigrated with authentic VP1 and VP2 from ADV-infected Crandell feline kidney cells. These results suggested, therefore, that both VP1 and VP2 were synthesized from a single cloned transcript. CsCl density gradient centrifugation of partially purified VV:ADSP-infected cell lysates indicated that the majority of the antigen was located in a fraction with a density near 1.33 g/ml, indicative of empty ADV particles. Subsequent electron microscopic examination revealed the presence of 27-nm icosahedral virion-like structures at the same density, suggesting that the proteins self-assembled into empty virions. Furthermore, sera from eight of eight mice inoculated with VV:ADSP contained ADV-specific antibodies and two of these eight serum samples had neutralizing activity, indicating that the particles produced in VV:ADSP-infected cells were immunogenic. Finally, when lysates from VV:ADSP-infected cells were compared with standard ADV antigens in counterimmunoelectrophoresis assays, a similar pattern of specific reactivity was observed for sera from normal and infected mink.

Aleutian mink disease parvovirus (ADV) causes a severe disease in mink. The pathogenesis of ADV infections is influenced by the genetic background of the mink, the age at which the animal becomes infected, and the strain of virus (1, 20, 22). In seronegative neonatal mink, ADV causes a fatal acute pneumonia (1), whereas ADV infection in adult mink results in a persistent disease characterized by hypergammaglobulinemia, plasmacytosis, arteritis, and fatal immune-complex glomerulonephritis (22, 38, 40, 41).

Attempts to protect mink from ADV infection by vaccination with either killed or nonpathogenic, cell culture-adapted ADV have proven unsuccessful (10, 39). In fact, animals receiving such materials have developed more severe forms of disease upon challenge with pathogenic virus, presumably because the vaccination primes the animal's immune system to subsequent virus challenge. Nevertheless, neutralizing antibodies can be detected in the sera of infected mink (44), and it remains possible that a suitable vaccine might be developed. One means of exploring this possibility would be to express ADV proteins in a eukaryotic system and to study the usefulness of such materials as vaccine candidates.

Development of such a system is possible because the tissue culture-adapted strain of ADV, ADV-G, has been molecularly cloned, and the entire sequence has been determined (5, 6). In addition, the transcriptional map and program of ADV-G have been studied (2). As with other parvoviruses, ADV has a linear single-stranded DNA genome, approximately 5,000 bases in length, of negative polarity. Synthesis of a series of nonstructural proteins coded by the left half of the genome is directed by a promoter at map unit 4. The right half of the genome is transcribed under the regulation of a promoter at map unit 36 and encodes two capsid proteins, VP1 and VP2. The amino acid sequence of VP2 is entirely contained within VP1, and transcriptional studies suggest that both proteins are derived from the same spliced mRNA transcript through alternate initiation events (2). cDNA clones that contain the coding sequences for both VP1 and VP2 have also been prepared, and thus, DNA segments corresponding to the spliced mRNA for VP1 and VP2 are available (2).

One expression system in which a number of proteins have been expressed is vaccinia virus and other poxviruses (for reviews, see references 23 and 45). Recombinant poxviruses containing foreign viral genes have successfully been used in studies involving the host immune response, and in some cases, vaccination with such viruses has resulted in protection from viral infection (36, 45).

In this study, we have expressed the ADV structural proteins in recombinant vaccinia viruses, VV:ADSP. Cells infected with VV:ADSP were specifically stained by ADV antiserum when immunohistochemical examination was performed. Characterization of protein from VV:ADSP-infected cells revealed that both the 85-kDa VP1 and the 75-kDa VP2 structural proteins of ADV were expressed and comigrated with proteins expressed by ADV-G when analyzed by immunoblot or radioimmunoprecipitation. These proteins were shown to be transported to the nucleus and assembled into ADV empty capsids. Mice inoculated with VV:ADSP produced ADV-specific antibodies, and some of these animals produced detectable levels of neutralizing antibodies to ADV.

* Corresponding author.
† Present address: Veterans Administration Medical Center, Omaha, NE 68105.

Aleutian disease, in addition to being an interesting model for the pathogenesis of parvovirus infections and persistent viral infections, is also a significant economic problem in the mink industry. Therefore, diagnosis and control of ADV infections are also of importance. ADV infection can be eradicated by culling ADV-seropositive animals from infected herds (17). Counterimmunoelectrophoresis (CIE) is currently the standard diagnostic test used for the detection of ADV. This test is currently performed by using antigen prepared from ADV-infected cell culture and is highly effective in identifying seropositive animals. VV:ADSP-infected cell lysates were, therefore, used as a source of antigen in a CIE assay and compared favorably with commercially available test antigens.

MATERIALS AND METHODS

Cells and virus. Green monkey kidney cells (CV-1) and Crandell feline kidney (CRFK) cells were grown in Dulbecco's modified Eagle medium with 4.5 mg of glucose per ml and 2 mM L-glutamine, supplemented with 10% fetal bovine serum and 50 μg of gentamicin per ml (DMEM-HG). TK-143 cells were grown in DMEM-HG in the presence of 25 μg of 5-bromodeoxyuridine per ml. ADV-G was propagated as previously described (10).

Plasmid constructions. The vaccinia virus recombination vector pSC11 (a gift from Bernard Moss) contains two vaccinia virus early transcriptional promoters, p11 and p7.5, flanked by portions of the vaccinia virus thymidine kinase (TK) gene (16). The p11 promoter directs expression of the *Escherichia coli lacZ* gene, which allows for selection of recombinant viruses by screening for β-galactosidase activity. Transcription from the p7.5 promoter occurs in the opposite direction. Located immediately downstream from p7.5 is a unique *Sma*I site into which foreign genes can be inserted (for these experiments, we used a pSC11 in which the *Sma*I site had been changed to a unique *Bgl*II site). The flanking TK sequences allow for homologous recombination of pSC11 derivatives into the thymidine kinase gene of wild-type vaccinia virus, and such recombinants can be selected by growth in thymidine kinase-negative cells in the presence of 5-bromodeoxyuridine.

A 2.3-kb ADV DNA segment corresponding to the mRNA coding sequences for the ADV-G capsid proteins, VP1 and VP2, was derived by digesting cDNA clone pIB21 (2) with *Xho*I and *Xba*I (Fig. 1). *Bgl*II linkers were added to this fragment following repair of the 3′ ends with Klenow fragment. The ADV fragment was ligated into pSC11, and the products were transformed into *E. coli* JM109. Colonies containing the ADV insert were identified, and the proper orientation of the ADV sequences was verified by restriction enzyme digestions. The clone selected for recombination into vaccinia virus was denoted pXVIII-B-6 (Fig. 1).

Another construction, denoted pXX-R-14, was developed by replacing the *Eco*RI-*Hin*dIII segment of the ADV-G insert in pXVIII-B-6 with the corresponding segment from a molecular clone of ADV-Utah 1. Plasmids pXVIII-B-6 and pXX-R-14 were propagated and used in the creation of the recombinant vaccinia viruses VV:IL-1 and VV:XXU-1, respectively. These two viruses behaved identically in all assays and, therefore, will collectively be referred to as VV:ADSP throughout this report.

Production and isolation of recombinant vaccinia viruses. Recombinant vaccinia viruses were produced by established procedures (16, 32). Briefly, 2×10^6 CV-1 cells were seeded in 60-mm^2 dishes and grown overnight. The following day, the monolayers were infected with vaccinia virus (strain WR) at a multiplicity of infection (MOI) of 0.02 PFU per cell and incubated at 37°C for 1 h. Following the incubation, each plate was transfected with 40 μg of plasmid DNA by the $CaPO_4$ method (21), incubated at 37°C for 4 h, and shocked for 1 min with 15% glycerol. The cultures were then incubated at 37°C for 2 days. Cells were scraped from the plates, pelleted by centrifugation, and lysed by three cycles of freeze-thawing, followed by digestion with trypsin (162 μg/ml, final concentration) for 30 min and sonication.

Recombinant thymidine kinase-negative, β-galactosidase-positive vaccinia viruses were selected by growth in TK-143 cells in the presence of 25 μg of 5-bromodeoxyuridine per ml overlaid with agarose containing 0.02 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) per ml. Following two more rounds of plaque purification, large-scale preparations of the recombinant viruses were made and used throughout these experiments.

Immunoprecipitation of proteins metabolically labeled with [^{35}S]methionine. ADV-G-infected CRFK cells (60 h postinfection [p.i.]) and VV:ADSP-infected CV-1 cells (16 h p.i.) were labeled with [^{35}S]methionine as previously described (10, 11). Following preclearing, the aliquots from each sample were incubated with mink anti-ADV or with normal mink serum for 4 h at 4°C while being gently inverted. Fifty microliters of hydrated Sepharose CL4B beads (Pharmacia) (100 mg/ml) was added to each sample, and the incubation was continued for 30 min. The beads were washed five times in lysis buffer, resuspended in 50 μl of Laemmli sample buffer (30), and boiled for 2 min. The beads were again pelleted by centrifugation, and the proteins within the sample buffer were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels. Following electrophoresis, the gels were fixed, dried, and fluorographed by using Entensify (Du Pont). The gels were finally exposed to X-ray film until appropriate exposures were obtained.

Immunohistochemistry. Cells were grown on Lab Tek slides (Nunc), infected at an MOI of 1 PFU per cell, and at 24 h p.i. fixed in cold acetone for 10 min. Immunohistochemistry of the samples was essentially performed as described by Mori et al. (35).

Buoyant density centrifugation. CV-1 cells were seeded into 150-cm^2 flasks at a density of 4×10^6 per flask and infected the following day with 1 PFU per cell of VV:ADSP. After 60 h of incubation at 37°C, the cells were scraped into the media, pelleted by centrifugation, and resuspended in 1 ml of Dulbecco's phosphate-buffered saline (DPBS) per flask. Following lysis by four cycles of freeze-thawing and sonication, 1/2 volume of Freon 113 was added, and the preparation was vortexed 3 min prior to centrifugation at $12{,}000 \times g$ for 10 min at 4°C. The aqueous phase was layered over 2 ml of 40% sucrose in DPBS and centrifuged at $100{,}000 \times g$ overnight at 4°C in an SW 50.1 rotor. The pellets were dissolved in 0.5 M Tris–0.005 M EDTA containing 0.1% Sarkosyl, and solid CsCl was added to achieve a final density of approximately 1.33 g/ml. Gradients were allowed to form by centrifugation in an SW 41 rotor at $100{,}000 \times g$ for 35 h at 18°C. Eleven fractions were collected, and the densities were determined with a refractometer.

Immunoblot analysis of the fractions was performed following precipitation of the fractions with 10 volumes of methanol. The precipitates were resuspended in 50 μl of Laemmli sample buffer. The proteins were separated by SDS-PAGE on 10% gels, blotted onto nitrocellulose mem-

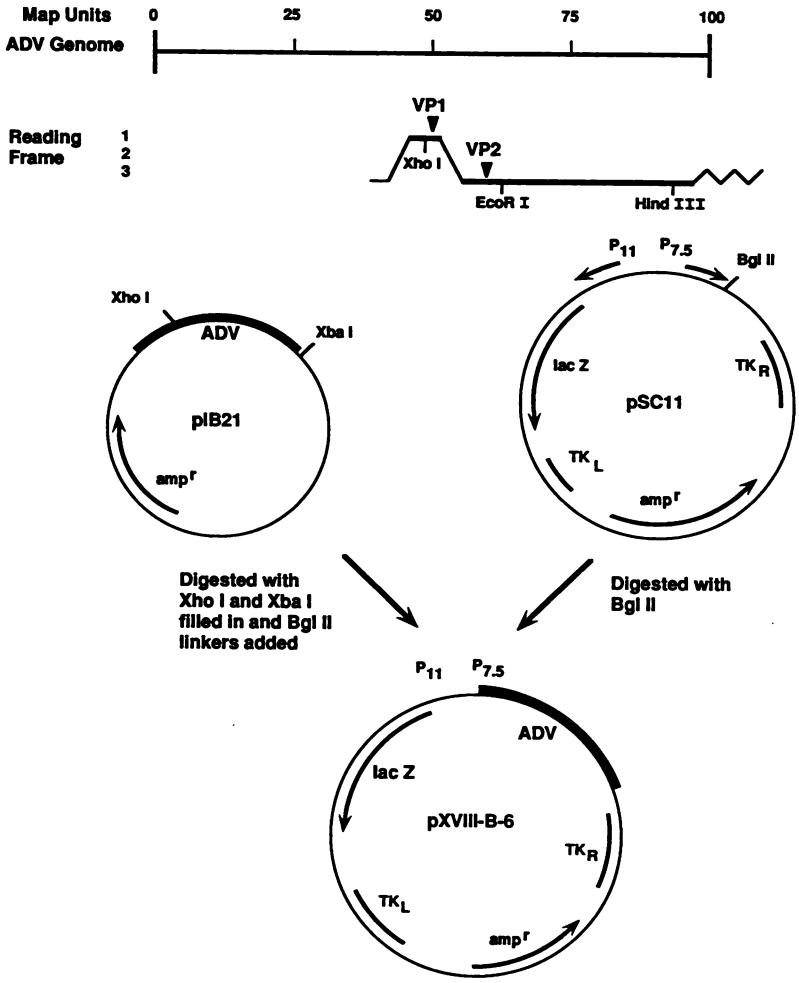

FIG. 1. Construction of plasmid pXVIII-B-6. Plasmid pXVIII-B-6 was constructed by digesting the cDNA containing plasmid pIB21 with *Xho*I and *Xba*I and isolating the 2.3-kb fragment containing the ADV structural protein gene. This fragment, corresponding to the R3 mRNA of ADV-G, was then inserted at the unique *Bgl*II site directly downstream of the vaccinia virus promoter p7.5 in the vaccinia virus expression vector pSC11 following addition of *Bgl*II linkers.

branes as previously described (7, 33), and reacted with pooled serum from ADV-infected mink.

Samples to be analyzed by electron microscopy were placed as droplets on clean Parafilm. An inverted parlodion-coated 300-mesh copper grid was placed on top of each droplet for 10 min to allow the sample to adsorb. The supernatants were absorbed from the grids with Whatman filter paper. The grids were then washed twice by inverting them on a drop of water and allowing them to wash by diffusion. Samples were stained with 0.5% aqueous uranyl acetate, pH 3.9, for 30 s and examined by using a Hitachi Hu11E1 electron microscope at 75 kV. The images were captured on Kodak SO163 electron film.

CIE. CIE was performed, as previously described (9), with commercial ADV-G antigens (kindly provided by United Vaccines, Madison, Wis., and the Danish Fur Breeders Cooperative) or an ADV-Utah 1 antigen prepared from ADV-Utah 1-infected mink (42). When VV:ADSP-infected cells were used as the source of antigen, CV-1 cells were seeded in 150-cm^2 flasks as described above and infected at an MOI of 0.5 PFU. At 3 days p.i., the cell lysates were prepared by scraping the cells into the media and pelleting. The supernatants were discarded, and the cell pellets were resuspended in 1 ml of DPBS, freeze-thawed three times, and sonicated. Trypsin was added to the lysate to a final concentration of 162 μg/ml, and the lysates were incubated at 37°C for 1 h. PMSF (phenylmethylsulfonyl fluoride) was added to a final concentration of 1 mM prior to analysis of the samples.

Inoculation of mice with VV:ADSP. RML (Swiss-Webster) mice were inoculated intradermally by tail scratch; eight received 10^8 PFU of VV:ADSP, and four received 10^8 PFU of vaccinia virus strain WR (VV:WR). Three weeks later, they were boosted with a second inoculation. After 2 weeks following the second inoculation, sera were collected and analyzed for ADV-specific antibodies by indirect immunofluorescence (IFA), CIE, or immunoblot. These sera were also analyzed for the ability to neutralize ADV.

The neutralization assay was performed by incubating dilutions of the mouse sera for 1 h at 37°C with ADV-G that had been diluted 1:100 in DPBS. The serum samples containing virus were then added to CRFK cells in 96-well tissue culture plates. Three days p.i., virus infectivity was determined by IFA as previously described (8). Serum samples

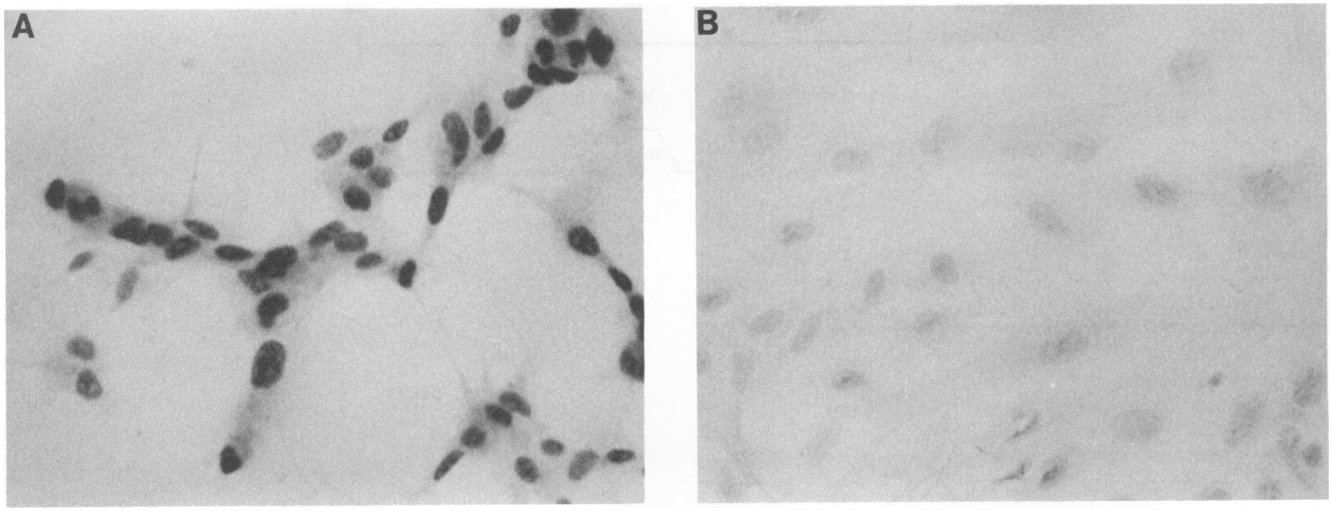

FIG. 2. Immunohistochemistry of VV:ADSP-infected cells. CV-1 cells were grown on slides and infected at an MOI of 1 PFU per cell. At 24 h p.i., the cells were fixed in cold acetone for 10 min. The slides were incubated with either rabbit antiserum specific for the ADV structural proteins (A) or normal rabbit serum (B) and developed as previously described (35). The specimens were counterstained with methyl green prior to microscopic examination. The cells stained with the ADV-specific serum showed dark staining primarily localized in the nuclei of infected cells (panel A). The cells stained with the normal rabbit serum exhibited no such staining (panel B).

were considered to have neutralizing activity when they reduced the number of fluorescence-forming units by 70% compared with the number in cells infected with virus treated with normal mouse serum.

RESULTS

Expression of ADV structural proteins in VV:ADSP-infected cells. Immunohistochemistry was performed to determine whether VV:ADSP-infected cells expressed ADV antigen. CV-1 cells infected with VV:ADSP or VV:WR were incubated with either normal rabbit serum or a polyclonal rabbit serum specific for ADV capsid proteins. The VV:ADSP-infected cells stained strongly with the ADV antiserum (Fig. 2A), suggesting that high levels of ADV capsid proteins were expressed. The majority of the antigen appeared to be localized in the nucleus, indicating that the antigen was being transported to the nucleus, the site of normal ADV replication (19). Cells incubated with the normal serum did not react (Fig. 2B), indicating that the staining was ADV specific. Furthermore, neither uninfected CV-1 cells nor cells infected with VV:WR stained with the anti-ADV serum (data not shown).

In order to define the ADV antigen expressed in VV:ADSP-infected CV-1 cells, radioimmunoprecipitation was performed. Radiolabeled cell lysates were incubated with mink anti-ADV or normal mink serum and separated on SDS-polyacrylamide gels. Autoradiography revealed that VV:ADSP-infected cells contained two ADV-specific proteins that comigrated with VP1 and VP2 (Fig. 3). Although VV:ADSP-infected cells expressed both structural proteins, VP1 was consistently more abundant than VP2. In contrast, ADV-infected CRFK cells expressed VP1 and VP2 in approximately equal quantities (Fig. 3). Identical results were also observed when lysates of VV:ADSP-infected cells were analyzed by immunoblot (data not shown).

No ADV-specific proteins were detected in CV-1 cells infected with recombinant vaccinia virus VV:IR-1 (a virus which contains an irrelevant insert) or in uninfected CV-1 cells. As expected, the nonstructural protein p71 that is normally produced in an ADV infection (11) was also not detected in the VV:ADSP-infected cells. In addition, when the same samples were reacted with normal mink sera, no bands were observed (Fig. 3). Because vaccinia virus replicates in the cytoplasm of infected cells and does not splice mRNA molecules (43), the fact that both VP1 and VP2 were

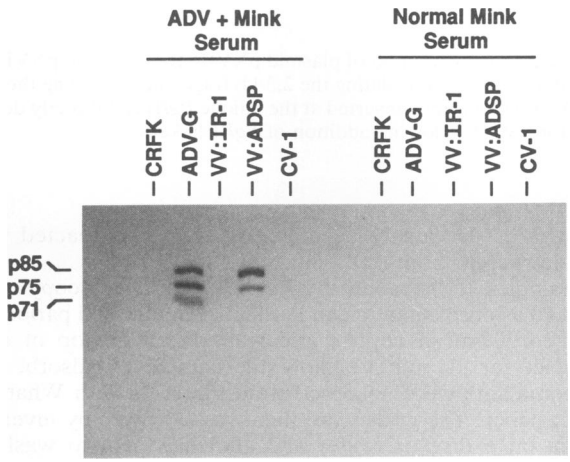

FIG. 3. Radioimmunoprecipitation of proteins expressed in VV:ADSP-infected cells. CV-1 cells were infected at an MOI of 1 with recombinant vaccinia virus VV:ADSP or VV:IR-1 (a recombinant virus that contains an irrelevant insert). At 24 h p.i., the cells were labeled with [^{35}S]methionine for 2 h. For comparison, ADV-infected (60 h p.i.) or uninfected CRFK cells were similarly labeled. Cell lysates were prepared and incubated with either ADV-positive or normal mink serum. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Proteins observed in the VV:ADSP-infected cell lysates reacted with the ADV-positive serum and comigrated with VP1 and VP2 in the ADV-G-infected CRFK sample. The major nonstructural protein was observed only in ADV-G-infected CRFK cells. No ADV-specific proteins were observed in the control samples.

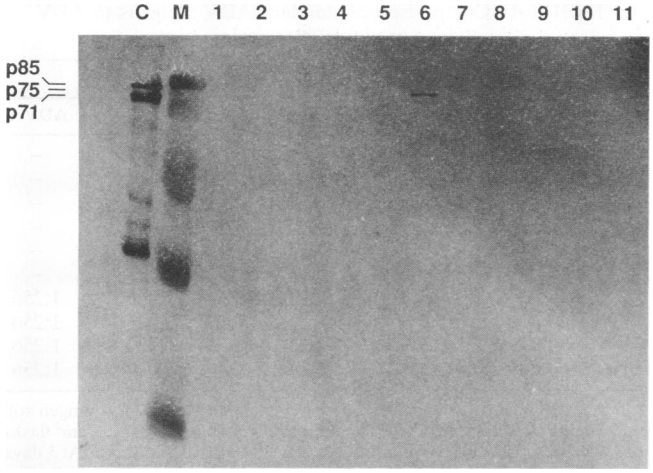

FIG. 4. Immunoblot analysis of isopycnic density centrifugation fractions. Eleven fractions ranging in density from 1.46 to 1.24 g/ml were collected following isopycnic density centrifugation of VV:ADSP-infected cell lysates. The fractions were precipitated with methanol and analyzed by immunoblot. The blot was incubated in the presence of ADV-positive mink serum. Lanes: C, lysate of ADV-G-infected CRFK cells; M, prestained markers; 1 through 11, fractions 1 through 11, respectively. A band comigrating with the ADV structural protein VP1 was detected in lane 6; this fraction had a density of approximately 1.32 g/ml.

produced implied that both proteins can be expressed from the same ADV mRNA.

Self-assembly of ADV proteins into particles. It has been reported that when the structural proteins of parvoviruses are expressed in either bovine papillomavirus vectors (29, 37) or baculoviruses (12, 25), these proteins spontaneously assemble into empty particles with physical characteristics of empty virions. To determine whether the ADV proteins expressed by the recombinant vaccinia virus VV:ADSP also assembled into empty particles, VV:ADSP-infected cell lysates were prepared and purified by isopycnic CsCl density gradient centrifugation. Eleven fractions ranging in density from 1.24 to 1.46 g/ml were collected and analyzed by immunoblot and electron microscopy.

Immunoblot analysis of the fractions revealed that the bulk of the ADV proteins were located at a density of approximately 1.32 g/ml (Fig. 4, fraction 6), which corresponds well to the reported density of 1.33 g/ml for ADV empty particles (10). VP1 was readily observed, and VP2 could be detected in this fraction after longer incubation (data not shown). VP1 was also detected in fraction 5 and after longer incubation could also be observed in fraction 7 (data not shown).

Electron microscopic analysis of the fractions confirmed that large numbers of icosahedral structures, approximately 27 nm in diameter, were also present in fraction 6 (Fig. 5). Thus, it was highly likely that the ADV structural proteins expressed in VV:ADSP-infected cells self-assembled into ADV-like particles with a density similar to that of empty ADV particles. Because VP1 was the predominant ADV protein in this fraction, this result also suggested that the particles contained more VP1 than VP2. Furthermore, we have stored these preparations for several months in CsCl with no noticeable changes in the number of particles present, implying that these particles are quite stable (data not shown).

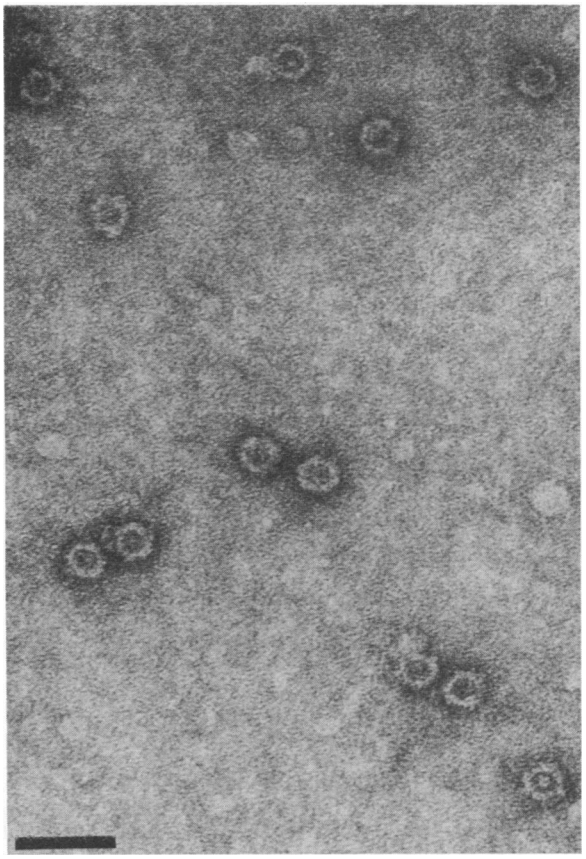

FIG. 5. Electron micrograph of particles purified from VV:ADSP-infected cells. Cell lysates from VV:ADSP-infected cells were partially purified by buoyant density gradient centrifugation. The samples were stained with 0.5% uranyl acetate. Parvovirus-like particles, approximately 27 nm in diameter, were observed in the fraction with a corresponding density of approximately 1.32 g/ml. The bar represents 50 nm.

CIE. We wished to determine the usefulness of the ADV antigen expressed by VV:ADSP as a diagnostic antigen. CIE is the standard immunological test for diagnosis of Aleutian disease and was carried out as previously described (9). We first determined the reactivity of the VV:ADSP antigen to an ADV-positive serum and compared this reactivity with those of other CIE antigens. For these assays, cell lysates from VV:ADSP- or VV:WR-infected cells, as well as two commercial ADV-G antigens, ADV-G DK antigen (a Danish antigen kindly provided by the Danish Fur Breeders Cooperative) and ADV-G USA antigen (kindly provided by United Vaccines), were used. The VV:ADSP antigen was also compared with antigen prepared in our laboratory from ADV-Utah 1-infected mink tissue.

Using a standard ADV-positive serum, fourfold dilutions of these five antigen preparations were tested (Fig. 6). The VV:ADSP antigen was specifically recognized by the ADV-positive serum in a pattern that was similar to those of the two ADV-G antigens. None of the antigens reacted to the normal mink serum, nor did the ADV-positive serum react with the VV:WR-infected cell lysate. These results indicated that the serum specifically recognized ADV antigens expressed by VV:ADSP in the CIE assay and that the reactivity of the VV:ADSP antigen was similar to that observed with standard ADV antigens.

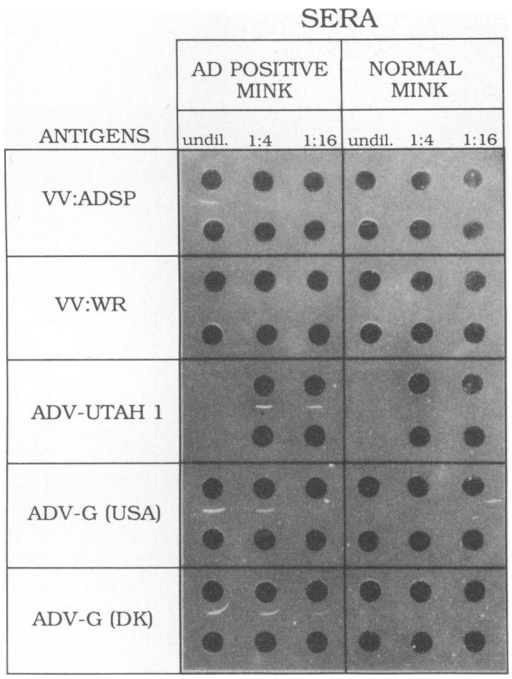

FIG. 6. Comparison of VV:ADSP antigen to standard ADV CIE antigens. Cell lysates from VV:ADSP- and VV:WR-infected CV-1 cells were compared with standard ADV test antigens in a CIE assay. A standard ADV-positive mink serum sample diluted 1:100 was used as the source of antibody in all cases. The antigens were assayed as a series of 1:4 dilutions. The VV:ADSP antigen showed the same pattern of reactivity as both the ADV-G USA and the ADV-G DK antigens reacting at all three concentrations assayed. The ADV-Utah 1 antigen was more sensitive than the ADV-G antigens. The ADV-positive mink serum showed no reaction to a lysate of VV:WR-infected CV-1 cells lysate. There was also no reaction with any of the antigens when normal mink serum was used.

We next tested the VV:ADSP antigen against a panel of mink sera and again compared its reactivity with those of other antigens (Table 1). In these assays, serial fourfold dilutions of sera from eight experimentally infected mink and two uninfected animals were used. Four of the infected sera were collected at 10 days p.i. (early in infection) and four were collected at 60 days p.i. (in the later stages of the disease). Because the ADV-specific antibody levels are much lower in 10-day p.i. sera than in 60-day p.i. sera (9), use of these sera would give an indication of the sensitivity of this antigen against both low and high titers in sera. Two of the 10-day sera (mink no. 5 and no. 6) reacted to the VV:ADSP, whereas three of the sera reacted with the ADV-Utah 1 preparation. None of these sera were positive with the standard ADV-G antigen (Table 1). These results suggested that the VV:ADSP antigen might be superior to the standard ADV-G USA antigen in detecting antibody early in the course of the disease.

All of the antigens reacted with the antibody in mink sera collected at 60 days p.i. However, the VV:ADSP antigen appeared to be more sensitive than the commercially available ADV-G USA antigen in all samples tested, yielding higher titers in all cases (Table 1). Again, none of the sera reacted with a control lysate from VV:WR-infected cells. The results from this limited comparison suggested that the ADV antigen produced in VV:ADSP-infected cells might be superior to the commercially available test antigen.

TABLE 1. Comparison of standard ADV antigens to ADV antigens produced by vaccinia virus

Mink no.	Serum	Reactivity of antigen[a]			
		VV:WR	VV:ADSP	ADV-Utah 1	ADV-G
1	Normal	–[b]	–	–	–
2	Normal	–	–	–	–
3	10 day p.i.[c]	–	–	1:4	–
4	10 day p.i.	–	–	–	–
5	10 day p.i.	–	1:4	1:4	–
6	10 day p.i.	–	1:16	1:16	–
7	60 day p.i.	–	1:1,024	1:1,024	1:256
8	60 day p.i.	–	1:1,024	1:1,024	1:256
9	60 day p.i.	–	1:1,024	1:1,024	1:256
10	60 day p.i.	–	1:1,024	1:1,024	1:256

[a] Results indicate the highest dilution at which reactivity of the antigen still occurred. VV:WR and VV:ADSP antigens were prepared from 150-cm^2 flasks of CV-1 cells. The cells were infected at an MOI of 1 PFU per cell. At 3 days p.i., they were harvested, pelleted, and resuspended in 1 ml of PBS. The samples were freeze-thawed three times and sonicated. Trypsin was added to a final concentration of 162 g/ml, and the lysates were digested for 1 h at 37°C. Following trypsinization, PMSF was added to a final concentration of 1 mM. The antigen (10 μl) was loaded into the cathodal well, while 10 μl of each serum dilution was loaded into the anodal well. The assays were performed as previously described (9). ADV-Utah 1 antigen used in these assays was prepared as previously described (9). ADV-G antigen used in these assay was produced by United Vaccines.

[b] – signifies that these samples were negative at a 1:4 dilution, the lowest dilution assayed.

[c] All infected animals were inoculated with ADV-Utah 1.

Inoculation of mice with VV:ADSP. An ultimate goal of the expression of these proteins in vaccinia virus is to determine the ability of these proteins to protect mink from ADV challenge. As a first step in determining the immunogenicity of VV:ADSP-expressed ADV antigens, we investigated the ability of VV:ADSP to induce ADV-specific antibodies in mice. Twelve RML (Swiss-Webster) mice were inoculated intradermally with 10^8 PFU of either VV:ADSP (eight animals) or VV:WR (four animals) and were boosted 3 weeks later with a second inoculation. Two weeks following the second inoculation, sera were collected and analyzed for ADV-specific antibodies. When tested against ADV-G-infected CRFK cells by indirect IFA, all eight of the serum samples from VV:ADSP-inoculated animals were positive. None of the sera from animals receiving VV:WR reacted (Table 2).

These sera were further analyzed by CIE and tested for neutralizing activity. Three of the eight positive mouse serum samples were positive in the CIE assay. Two of these serum samples had neutralizing activity (Table 2). These results indicated that the ADV proteins expressed in VV:ADSP-infected cells were immunogenic in mice. In addition, because some of these sera were positive by CIE and were able to neutralize ADV, these results implied that some of the antibodies recognized epitopes present on native ADV particles.

The sera were also analyzed by immunoblot to determine the specific proteins that they recognized. All of the sera were negative at the 1:50 dilution used (data not shown).

DISCUSSION

ADV structural proteins were expressed in recombinant vaccinia viruses. These recombinant viruses directed the

TABLE 2. Immunological response of mice to recombinant vaccinia virus VV:ADSP[a]

Mouse no.	Inoculum	Immunological response by:		
		IFA[b]	CIE[c]	Neutralization
1	VV:WR	–	–	–
2	VV:WR	–	–	–
3	VV:WR	–	–	–
4	VV:WR	–	–	–
5	VV:ADSP	+	ND[d]	ND
6	VV:ADSP	+	–	–
7	VV:ADSP	+	–	–
8	VV:ADSP	+	+	1:10[e]
9	VV:ADSP	+	+	1:10
10	VV:ADSP	+	–	–
11	VV:ADSP	+	+	–
12	VV:ADSP	+	–	–

[a] Twelve RML Swiss-Webster mice were inoculated intradermally with 10^8 PFU of VV:ADSP and boosted 3 weeks later with a similar inoculum. At 2 weeks subsequent to the second inoculation, sera were collected for analysis.
[b] IFAs were performed with ADV-infected CRFK cells by using a 1:20 dilution of the sera.
[c] CIE assays were performed by using a standard ADV antigen (United Vaccines) at a 1:4 dilution of the sera.
[d] ND, these assays were not done.
[e] Highest dilution at which the serum was able to neutralize ADV.

synthesis of both ADV structural proteins, and the proteins self-assembled into particles.

Immunohistochemical examination of cells infected with VV:ADSP revealed that ADV-specific protein was primarily localized in the nuclei of infected cells. Because vaccinia virus replicates in the cytoplasm of infected cells (36), the presence of the antigen in the nucleus indicated that the proteins were transported to the nucleus, as is normally observed for parvovirus infections (19). Thus, the appropriate nuclear localization signals must have been present and functional. The amino-terminal end of ADV VP1 contains the amino acid sequence PGKKRSA, which corresponds well with the simian virus 40 T-antigen nuclear localization signal sequence PKKKRKV (18, 27, 31). Analysis of the VP2-specific sequence revealed the presence of no easily recognizable nuclear transport consensus sequence. Nevertheless, the fact that parvoviruses replicate and assemble in the nucleus suggests that there must also be signal sequences located in the VP2-coding region. Use of this cytoplasmic expression system might be useful in defining the parvovirus sequences required for nuclear transport and localization.

Analysis of VV:ADSP-infected cell lysates by either radioimmunoprecipitation or immunoblot revealed that both the 85-kDa VP1 and the 75-kDa VP2 were expressed in infected cells. The fact that vaccinia virus replicates in the cytoplasm and, therefore, is incapable of splicing mRNA transcripts (43) led us to speculate that the single cDNA molecule inserted into the vaccinia virus expression vector can direct synthesis of both VP1 and VP2, as previously proposed (2). This speculation supports previous work and is in contrast to the reported transcriptional organization of other autonomous parvoviruses in which VP1 and VP2 appear to be synthesized from separate transcripts (13, 24, 34).

Infection of CV-1 cells with VV:ADSP consistently produced more VP1 than VP2. This finding differs from the results normally obtained from in vitro (11) infection of CRFK cells with ADV-G or in vivo infection of mink kits with ADV-Utah 1 (3), as well as expression of parvovirus proteins in other systems (15, 26, 29, 37). In all of these cases, VP2 is usually the more abundant species. In the native ADV R3 mRNA transcript, which codes for the capsid proteins, the initiation codon for VP1 is in a poorer translational initiation context (28) than that for VP2, and this initiation constraint was speculated to be the cause for the preferential synthesis of VP2. However, because VV:ADSP-infected cells produce more VP1 than VP2, other factors may also be operative in controlling which AUG is used.

The native ADV R3 mRNA contains 380 residues 5′ to the VP1 initiation site. Included in this leader sequence are two minicistrons of 8 and 100 amino acids containing five in-frame AUGs. However, the truncated cDNA used to construct VV:ADSP contains no ATGs upstream of the VP1 initiation site. Perhaps, the presence of these small upstream open reading frames influences the recognition of the start sites for the VP1- and VP2-coding sequences as has previously been suggested to occur in a feline parvovirus, feline panleukopenia virus (14). The possibility also exists that initiation constraints for protein synthesis in vaccinia virus-infected cells differ from those in ADV-infected cells and, therefore, may be involved (4).

The results from immunoblot analysis of fractions collected following partial purification of VV:ADSP-infected cells revealed that the majority of the ADV antigen in these cells was located at a density of 1.32 g/ml and consisted primarily of VP1. These results mirrored those obtained by analysis of whole-cell lysates of VV:ADSP-infected cells, in which VP1 was consistently more abundant than VP2.

Electron microscopic analysis of these fractions confirmed the presence of apparently empty particles with a buoyant density near 1.33 g/ml, the reported density of empty ADV particles (10). Because the immunoblot of these fractions showed a greater abundance of VP1, the assembled particles produced in VV:ADSP-infected cells likely contained more VP1 than VP2. It is interesting to note that although the ratio between the two structural proteins differed from standard ADV infections, the ability of these proteins to assemble into particles was apparently unaffected. These data coupled with those from other systems suggest that the requirements for parvovirus capsid assembly may not be very stringent (12, 25). It will be interesting to develop recombinant vaccinia viruses that express only VP1 or VP2 to investigate the requirements for ADV particle assembly.

Examination of the VP1 to VP2 ratios of parvovirus B19 expressed by using a baculovirus eukaryotic expression system revealed that the VP1-to-VP2 ratio in the cell lysates of coinfected cells and the particles produced (12) were the same as that normally observed for viral infections. This is in contrast to what we have observed for VV:ADSP-expressed particles. This difference may be due to our use of a cDNA clone or the use of vaccinia virus as the vehicle for expression. Nevertheless, these results imply that the ratio of parvovirus proteins expressed in eukaryotic cells is reflected in the particles produced.

An outbreak of ADV on a mink ranch can be economically devastating. The current diagnostic antigens for ADV infections utilize live virus, and their use could conceivably lead to inadvertent contamination of an ADV-free mink herd. Therefore, the production of recombinant, noninfectious ADV antigens would be advantageous. We have shown that ADV antigens can be expressed and used as a diagnostic reagent that compares favorably with commercially available live-virus reagents. Thus, use of VV:ADSP may repre-

sent a significant practical improvement in serodiagnosis of ADV infections.

The ability to express specific proteins in vitro can be a valuable tool in the study of disease. Aleutian disease is characterized by an aberrant immune response, leading to immune complex deposition in the kidneys and subsequent kidney failure. It has been suggested that uncontrolled B-cell proliferation is caused by viral infection of macrophages and follicular dendritic cells (35), cells intimately involved in the regulation of the immune response. This abnormal B-cell response may lead to the hypergammaglobulinemia and ultimately to the immune-complex deposition in the kidneys. It is possible that the infection of these cells affects the normal production of cytokines, causing this aberrant B-cell response. Alternatively, it is also possible that the ADV proteins are mitogenic and themselves directly stimulate B-cell proliferation. Vaccination of mink with recombinant proteins may allow a more detailed investigation of the role that the individual proteins and the immune response to them have in this disease.

It will be of interest to see whether mink inoculated with VV:ADSP develop ADV antibodies and what effect preexisting antibodies have on subsequent challenge with ADV. The result that inoculation of mice with VV:ADSP can induce detectable titers of neutralizing antibodies suggests that epitopes accessible on the native virion were also present on the particles produced in VV:ADSP-infected cells. The fact that only two of the eight mice inoculated with VV:ADSP developed detectable levels of neutralizing antibodies suggests that perhaps the aberrant VP1-to-VP2 ratio may mask or limit the neutralizing epitopes. Again, development of recombinant vaccinia viruses that express only VP1 or VP2 will be useful in defining these epitopes and determining whether they are available. Nevertheless, this finding is encouraging and suggests that recombinant vaccines may have some prophylactic use in the control of Aleutian disease.

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REFERENCES

1. **Alexandersen, S.** 1986. Acute interstitial pneumonia in mink kits: experimental reproduction of the disease. Vet. Pathol. **23:**579–588.
2. **Alexandersen, S., M. E. Bloom, and S. Perryman.** 1988. Detailed transcription map of Aleutian mink disease parvovirus. J. Virol. **62:**3684–3694.
3. **Alexandersen, S., A. Uttenthal-Jensen, M. Hansen, and B. Aasted.** 1986. Demonstration of non-degraded Aleutian disease virus (ADV) proteins in lung tissue from experimentally infected mink kits. Arch. Virol. **87:**127–133.
4. **Bablanian, R., S. K. Goswami, M. Esteban, A. K. Banerjee, and W. C. Merrick.** 1991. Mechanism of selective translation of vaccinia virus mRNAs: differential role of poly(A) and initiation factors in the translation of viral and cellular mRNAs. J. Virol. **65:**4449–4460.
5. **Bloom, M. E., S. Alexandersen, C. F. Garon, S. Mori, W. Wei, S. Perryman, and J. B. Wolfinbarger.** 1990. Nucleotide sequence of the 5′-terminal palindrome of Aleutian mink disease parvovirus and the construction of an infectious molecular clone. J. Virol. **64:**3551–3556.
6. **Bloom, M. E., S. Alexandersen, S. Perryman, D. Lechner, and J. B. Wolfinbarger.** 1988. Nucleotide sequence and genomic organization of Aleutian mink disease parvovirus (ADV): sequence comparisons between a nonpathogenic and a pathogenic strain of ADV. J. Virol. **62:**2903–2915.
7. **Bloom, M. E., O. R. Kaaden, E. Huggans, A. Cohn, and J. B. Wolfinbarger.** 1988. Molecular comparison of in vivo and in vitro derived strains of Aleutian disease of mink parvovirus. J. Virol. **62:**132–138.
8. **Bloom, M. E., R. E. Race, B. Aasted, and J. B. Wolfinbarger.** 1985. Analysis of Aleutian disease virus infection in vivo and in vitro: demonstration of Aleutian disease virus DNA in tissues of infected mink. J. Virol. **55:**696–703.
9. **Bloom, M. E., R. E. Race, W. J. Hadlow, and B. Chesebro.** 1975. Aleutian disease of mink: the antibody response of sapphire and pastel mink to Aleutian disease virus. J. Immunol. **115:**1034–1037.
10. **Bloom, M. E., R. E. Race, and J. B. Wolfinbarger.** 1980. Characterization of Aleutian disease virus as a parvovirus. J. Virol. **35:**836–843.
11. **Bloom, M. E., R. E. Race, and J. B. Wolfinbarger.** 1982. Identification of a nonvirion protein of Aleutian disease virus: mink with Aleutian disease have antibody to both virion and nonvirion proteins. J. Virol. **43:**606–616.
12. **Brown, C. S., J. W. M. Van Lent, J. M. Vlak, and W. J. M. Spaan.** 1991. Assembly of empty capsids by using baculovirus recombinants expressing human parvovirus B19 structural proteins. J. Virol. **65:**2702–2706.
13. **Carlson, J., K. Rushlow, I. Maxwell, F. Maxwell, S. Winston, and W. Hahn.** 1985. Cloning and sequence of DNA encoding structural proteins of the autonomous parvovirus feline panleukopenia virus. J. Virol. **55:**574–582.
14. **Clemens, D. L., and J. O. Carlson.** 1989. Regulated expression of the feline panleukopenia virus P38 promoter on extrachromosomal FPV/EBV chimeric plasmids. J. Virol. **63:**2737–2745.
15. **Clemens, K. E., D. R. Cerutis, L. R. Burger, C. Q. Yang, and D. J. Pintel.** 1990. Cloning of minute virus of mice cDNAs and preliminary analysis of individual viral proteins expressed in murine cells. J. Virol. **64:**3967–3973.
16. **Chakrabarti, S., K. Brechling, and B. Moss.** 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. **5:**3403–3409.
17. **Cho, H. J., and J. Greenfield.** 1978. Eradication of Aleutian disease of mink by eliminating positive counterimmunoelectrophoresis test reactors. J. Clin. Microbiol. **7:**18–22.
18. **Colledge, W. H., W. D. Richardson, M. D. Edge, and A. E. Smith.** 1986. Extensive mutagenesis of the nuclear location signal of simian virus 40 large T antigen. Mol. Cell. Biol. **6:**4136–4139.
19. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. **33:**91–174.
20. **Eklund, C. M., W. J. Hadlow, R. C. Kennedy, C. C. Boyle, and T. A. Jackson.** 1968. Aleutian disease of mink: properties of the etiological agent and the host response. J. Infect. Dis. **118:**510–526.
21. **Graham, F. L., and A. J. Van Der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
22. **Hadlow, W. J., R. E. Race, and R. C. Kennedy.** 1983. Comparative pathogenicity of four strains of Aleutian disease virus for pastel and sapphire mink. Infect. Immun. **41:**1016–1023.
23. **Hruby, D. E.** 1990. Vaccinia virus vectors: new strategies for producing recombinants vaccines. Clin. Microbiol. Rev. **3:**153–170.
24. **Jongenell, C. V., R. Sahli, G. K. McMaster, and B. Hirt.** 1986. A precise map of splice junctions in the mRNAs of minute virus of mice, an autonomous parvovirus. J. Virol. **59:**564–573.
25. **Kajigaya, S., A. Fujii, A. M. Field, S. Anderson, T. Shimada, and N. S. Young.** 1991. B19 parvovirus capsids produced in a baculovirus system are antigenically and immunologically similar to native virions. Proc. Natl. Acad. Sci. USA **88:**4646–4650.
26. **Kajigaya, S., T. Shimada, S. Fujita, and N. S. Young.** 1989. A

genetically engineered cell line that produces empty capsids of B19 (human) parvovirus. Proc. Natl. Acad. Sci. USA **86**:7601–7605.

27. **Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith.** 1984. A short amino acid sequence able to specify nuclear location. Cell **39**:499–509.
28. **Kozak, M.** 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNA. Nucleic Acids Res. **12**:857–872.
29. **Labieniec-Pintel, L., and D. Pintel.** 1986. The minute virus of mice P_{39} transcription unit can encode both capsid proteins. J. Virol. **57**:1163–1167.
30. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
31. **Landford, R. E., and J. S. Butel.** 1984. Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. Cell **37**:801–813.
32. **Mackett, M., and G. L. Smith.** 1986. Vaccinia virus expression vectors. J. Gen. Virol. **67**:2067–2082.
33. **Mayer, L. W., B. Aasted, C. F. Garon, and M. E. Bloom.** 1983. Molecular cloning of the Aleutian disease virus genome: expression of Aleutian disease virus antigens by a recombinant plasmid. J. Virol. **48**:573–579.
34. **Morgan, W. R., and D. C. Ward.** 1986. Three splicing patterns are used to excise the small intron common to all minute virus of mice RNAs. J. Virol. **60**:1170–1174.
35. **Mori, S., J. B. Wolfinbarger, M. Miyazawa, and M. E. Bloom.** 1991. Replication of Aleutian mink disease parvovirus in lymphoid tissues of adult mink: involvement of follicular dendritic cells and macrophages. J. Virol. **65**:952–956.
36. **Moss, B.** 1991. Vaccinia virus: a tool for research and vaccine development. Science **252**:1662–1667.
37. **Pintel, D., M. J. Merchlinsky, and D. C. Ward.** 1984. Expression of minute virus of mice structural proteins in murine cell lines transformed by bovine papillomavirus-minute virus of mice plasmid chimera. J. Virol. **52**:320–327.
38. **Porter, D., A. E. Larsen, and H. Porter.** 1969. The pathogenesis of Aleutian disease of mink. I. In vitro viral replication and the host antibody response to viral antigen. J. Exp. Med. **130**:575–589.
39. **Porter, D., A. E. Larsen, and H. Porter.** 1972. The pathogenesis of Aleutian disease of mink. II. Enhancement of tissue lesions following the administration of a killed virus vaccine or passive antibody. J. Immun. **109**:1–7.
40. **Porter, D., A. E. Larsen, and H. Porter.** 1973. The pathogenesis of Aleutian disease of mink. III. Immune complex arteritis. Am. J. Pathol. **71**:331–344.
41. **Porter, D., A. E. Larsen, and H. Porter.** 1980. Aleutian disease of mink. Adv. Immunol. **29**:261–286.
42. **Race, R. E., B. Chesebro, M. E. Bloom, B. Aasted, and J. B. Wolfinbarger.** 1986. Monoclonal antibodies against Aleutian disease virus distinguish virus strains and differentiate sites of virus replication from sites of sequestration. J. Virol. **57**:285–293.
43. **Stamatos, N. M., S. Chakrabarti, B. Moss, and J. D. Hare.** 1987. Expression of polyomavirus virion proteins by a vaccinia virus vector: association of VP1 and VP2 with the nuclear framework. J. Virol. **61**:516–525.
44. **Stolze, G., and O. R. Kaaden.** 1987. Apparent lack of neutralizing antibodies to Aleutian disease is due to masking of antigenic sites by phospholipids. Virology **158**:174–180.
45. **Tartaglia, J., S. Pincus, and E. Paoletti.** 1990. Pox-based vectors as vaccine candidates. Crit. Rev. Immunol. **10**:13–29.