Characterization and expression of Aleutian mink disease and pseudorabies virus capsid proteins in insect cells using a baculovirus expression system

by

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EXPLANATION OF THESIS FORMAT

The following alternate format thesis consists of a general introduction, a review of the literature, two separate manuscripts (Sections I and II), summary and conclusions, literature cited, acknowledgements, and an appendix. The M.S. candidate Wai-Hong Wu, is the principal investigator and author of the work presented here. The references cited in each manuscript are listed in LITERATURE CITED at the end of each manuscript. References cited elsewhere in the thesis are listed in the LITERATURE CITED at the end of the thesis.

GENERAL INTRODUCTION

Previous work in our laboratory has demonstrated that pseudorabies virus (PRV) nucleocapsid proteins are suitable for use as companion diagnostic antigens with PRV subunit vaccines (Platt et al., 1986; McGinley et al., 1988). However, the large scale production of these proteins in highly purified form by traditional methods is too costly. Recombinant DNA technology may prove to be an alternative method. Consequently, the objectives of the studies described in this thesis were directed at ultimately expressing a truncated gene of the major 32 kD PRV nucleocapsid protein in the baculovirus expression system. This system was selected because of its ability to express large quantities of foreign viral proteins (Smith et al., 1983; Matsuura et al., 1987).

The work described in the following thesis consists of two phases. The first phase describes the establishment of the baculovirus system in our laboratory. This was done using a well characterized cloned cDNA representing the complete coding sequence of the 85 kD (VP1) and 75 kD (VP2) capsid proteins of Aleutian mink disease virus. The second phase describes the efforts to express a cDNA clone representing a truncated gene of the PRV 32 kD major nucleocapsid protein.

LITERATURE REVIEW

Aleutian Mink Disease Virus

Introduction

Aleutian mink disease virus (ADV) is an autonomous parvovirus (Bloom et al., 1980) that causes an economically significant disease of mink (Aasted, 1985; Hensen, 1989). The disease was first described in 1956 in mink with the Aleutian genotype (Hartsough and Gorham, 1956). The infection in adult mink is persistent and is characterized by hypergammaglobulinemia and plasmacytosis. Mink ultimately die as a result of chronic immune complex-mediated glomerulonephritis and arteritis (Alexandersen, 1986; Alexandersen et al 1988a; Alexandersen et al., 1989; Bloom, 1984; and Hadlow et al., 1983). Mink infected during the neonatal period develop an acute fulminant, fatal respiratory distress syndrome which is characterized by interstitial pneumonia and the formation of hyaline membranes (Alexandersen, 1986; Alexandersen and Bloom, 1987; Alexandersen et al., 1987; Alexandersen et al., 1989; Larsen et al., 1984).

The disease is most severe in mink that have the gene for the Aleutian coat color. The development of glomerulonephritis (Padgett et al., 1967), and arteritis (Porter et al., 1973) is slower and the overall mortality rate is lower in mink without the Aleutian genotype (Gorham et al., 1965). The severity of the

disease is also determined by virus strain (Hadlow et al., 1984). Low virulent strains like the Pullman ADV rarely cause disease in non-Aleutian mink (Eklund et al., 1968). Potential reservoirs of ADV are believed to be the cat, dog, and a variety of wild animals. The raccoon, dog, cat, ferret, fox, mouse and rabbit have been experimentally infected with the virus (Alexandersen et al., 1985). However, there is no evidence to show that these animals are capable of spreading the virus.

The Virus

The Aleutian mink disease virus has icosahedral symmetry and measures approximately 23-28 nm in diameter. The capsid consists of 2 major structural proteins, VP1 and VP2. The molecular weight of these proteins varies between strains and is approximately 85 and 75 kD respectively (Aasted, 1980; Bloom et al., 1980). Two nonstructural proteins, designated NS-1 and NS-2 have also been characterized. The molecular weight of these proteins are 71 (Alexandersen et al., 1986; Bloom et al., 1982) and 13.6 kD (Porter et al., 1990) respectively. Their function is not known.

Control of ADV

Vaccination increases the severity of Aleutian disease (Porter et al., 1972). Consequently the disease is controlled in mink populations by the serological identification and removal of infected mink (Cho and Greenfield,

1978). The most common serological tests used for this purpose include: counter current electrophoresis (CCE) (Cho and Ingram, 1972), indirect counter current electrophoresis (Aasted and Cohn, 1982), the enzyme-linked immunosorbent assay (Wright and Wilkie, 1982), the quantitative radio immunoassay (Aasted and Bloom, 1983) and the rocket line immunoelectrophoretic assay (RLIE) (Alexandersen and Hau, 1985). Quantitative radio immunoassay and the rocket line immunoelectrophoretic assay are the most sensitive (Aasted, 1985). Because the CCE is easy and quick to run it is considered to be the best method available for screening large numbers of serums (Aasted, 1985).

Most mink develop detectable levels of antibodies to VP1, VP2 and NS-1 within 10 to 15 days of infection (Porter, 1986). Virus specific IgM can be detected as early as 6 days post infection (p.i.) and persists in serum for at least 85 days (Porter et al., 1984). Virus specific IgG antibodies are usually not detected until 15 days p.i. (Porter, 1986) but will persist at relatively high levels for the life of the mink.

The time that ADV specific antigens can first be detected in serum can apparently be influenced by the source of the antigen used in the assay. For example, Clemens et al. (1992) conducted an experiment in which serums were collected from 4 ADV infected mink 10 and 60 days p.i.. Antibody was detected in all 4 mink on day 60 p.i. by CCE in which the antigen that was used was; recombinant ADV capsid proteins, ADV proteins derived from cell

culture and ADV proteins extracted from organs of infected mink. However, on day 10 p.i., no antibody was detected in these serum when ADV infected cell culture antigen was used in CCE. In contrast, antibody was detected on day 10 p.i. in 3 of 4 mink when organ extracted antigen was used, and in 2 of 4 mink when vaccinia derived recombinant antigen was used.

Pseudorabies Virus

Introduction

Pseudorabies or Aujeszky's disease virus is the etiological agent of pseudorabies which is an economically significant disease in swine. The virus is a member of the family Herpesviridae and subfamily alphaherpesvirinae (Gustatson, 1981; Roizman and Batterson, 1985). The disease was first described in cattle, dogs and cats in 1902 (Aujeszky, 1902). The most common clinical signs of infection in swine include, fever, abortion, pneumonia, excess salivation, convulsions, anorexia, paddling movements and death (Gustafson, 1986). Intense pruritus also occurs in infected mammals with the exception of the pig. Although pruritus is occasionally seen in the pig it is not generally severe. The disease is most severe in neonates and in pigs less than 6 months of age. Older pigs are often inapparently infected. The severity of the disease is also dependent on virus strain, the immune status, and the

presence of other pathogens in the animal. The infection can and usually does persist in latent form (Andrews, 1962; Beran et al., 1980; Roizman and Batterson, 1985; Gutekunst et al., 1980). These latent infections can be reactivated periodically, especially if the pig is subjected to stress (Davies and Beran, 1980).

The Virus

The pseudorabies virus consists of an envelope that surrounds an icosahedral protein shell that measures approximately 180 nm in diameter (Ben-Porat and Kaplan, 1985; Roizman and Batterson, 1985). Five major envelope glycoproteins; gI, gII, gIII, gp50, gp63 and 2 minor envelope glycoproteins, gIV and gV, have been identified. Glycoprotein gI has an apparent molecular weight of 120 to 130 kD (Hampl et al., 1984). Glycoprotein gII exists as a complex. The individual components of gII; gIIa, gIIb, and gIIc have molecular weights of 120-125, 74-67, and 58 kD respectively (Hampl et al., 1984; Kennedy et al., 1984; Lukacs et al., 1985). Glycoprotein gIII exists as an uncomplexed monomer (Hampl et al., 1984) and has a reported molecular weight that ranges between 82 and 98 kD (Hampl et al,1984; Lukacs et al., 1985; Robbins et al., 1986; Wittmann et al., 1983). The molecular weight of gp50 is reported to range between 50 to 60 kD (Petrovskis et al., 1986; Wathen and Wathen, 1984). The envelope glycoprotein gp63 has an apparent molecular weight of 63 kD. The minor glycoproteins gIV and gV have reported molecular weights of

98 and 62 kD respectively (Hampl et al., 1984). The capsid consists of 3 major proteins with molecular weights of 142, 62, and 32 kD respectively (Ben-porat and Kaplan, 1985; Stevely, 1975). There are 7 minor proteins with molecular weights of 112, 85, 41, 38, 35, 30, and 27 kD respectively (Stevely, 1975). Nonstructural PRV proteins include the 180 kD immediate early protein, DNA binding proteins, glycoprotein gX, and various PRV encoded enzymes.

Control

Two general approaches can be used to control pseudorabies: a) the use of vaccines to prevent losses due to disease, and b) the establishment and maintenance of PRV-free herds. The method of choice currently being employed by the swine industry today is the second. A critical requirement for the establishment of PRV - free herds is the availability of sensitive serological tests. The most commonly used of these tests are; the serum virus neutralization test, the enzyme labeled immunosorbent assay, and the latex bead agglutination test. In general, virus specific IgM can be detected within 4 to 6 days p.i. (Bitsch and Eskildsen, 1982; Mcferran and Dow, 1962; Wittmann and Rziha, 1989). The highest levels of this class of immunoglobulin usually occur at approximately 7 days p.i. (Wittmann et al., 1980). Virus specific IgM then declines and disappears between 14 and 21 days p.i. (Wittmann et al., 1980; Wittmann and Rziha, 1989). Virus specific IgG antibodies are generally detected at approximately 6 to 8 days p.i., reach peak levels between days 14

and 21 p.i. and then decline over time (Wittmann et al., 1980; Wittmann and Rziha, 1989). Generally pigs can remain antibody positive for at least 3 to 4 month p.i..

Vaccines have traditionally been an integral part of PRV control programs (McGinley and Platt, 1988). However because these vaccines do not prevent infection, it is necessary to have the means of differentiating vaccinated pigs that are infected with PRV from vaccinated pigs that are not infected. This is possible by the use of genetically engineered deletion mutant vaccines. The deletion mutant viruses that are used in these vaccines are engineered so that 1 or more non-essential genes are deleted. As a result, virus infection of vaccinated pigs can be detected by testing for the presence of antibody to the protein that would be encoded if the deleted genes were present.

The use of live deletion mutant vaccines is not without potential problems. First, false positive results may be obtained when pigs that are initially vaccinated with 1 deletion mutant vaccine are subsequently vaccinated with a different deletion mutant vaccine. The use of traditional vaccines that possess the proteins that are absent in deletion mutant viruses can also produce false positive reactions. Secondly, it is possible for recombination to occur between a deletion mutant vaccine virus and a virulent field virus (Henderson et al., 1990; Katz et al., 1990). As a result, virulent field viruses may be generated that also lack the gene representing specific proteins and their presence could go undetected in swine populations.

The potential problem of recombination can be avoided with the use of subunit vaccines which consist of 1 or more but not all of the proteins of the virus (McGinely and Platt, 1989). Such vaccines could consist of all of the envelope glycoproteins that are associated with virulence. Consequently viral nucleocapsid proteins could be used as antigen in companion differential diagnostic tests. Inactivated vaccines are not without their disadvantages. In general, multiple doses are required to stimulate good immunity and they do not elicit a good cellular immune response.

Expression of Recombinant Viral Proteins

The Expression of Aleutian Mink Disease Virus Proteins

Aleutian mink disease virus antigens were first expressed in *Eschericeria coli* by Mayer et al. (1983). Fragments of DNA from a cell culture-adapted virus, ADV-G, and a Danish strain, DK ADV, that represented map units from 54 to 88, were molecularly cloned into a pUC8 plasmid vector. Two recombinant plasmids were successfully constructed, pBM1 containing the ADV-G fragment and pBM2 containing the DK-ADV fragment. These plasmids were then used to transformed *E.coli* JM103. Mayer et al. demonstrated by Western immunoblot and immunoprecipitation that both recombinant plasmids,

pBM1 and pBM2, induced the expression of 3 polypeptides with approximate molecular weights of 55, 34, and 27 kD.

Subsequently, Bloom et al. (1987) expressed a 1.55 kbp DNA fragment encoded between map units 56 and 88 in *E.coli* using pUC8. They reported that 4 proteins with molecular weights of 67, 57, 47 and 36 kD were expressed. Concurrently, Lochelt et al. (1987) reported that proteins of 32 and 74 kD were encoded in a central region between map units 15 and 88 of the lymphotropic ADV strain, SL3.

The following year Bloom et al. (1988) expressed 2 proteins with molecular weights of 57 and 34 kD ± 2 kD from a 3.55 kbp genomic fragment of ADV-G, ADV-Utah-I and ADV-Pullman in *E.coli*. These fragments were located between map units 15 and 88.

More recently Clemens et al. (1992) described the expression of an 85 and 75 kD protein by a recombinant vaccinia virus which contained a 2.3 kbp ADV cDNA fragment that is located between map units 40 and 88. These 2 proteins self assembled in infected cells which lead the investigators to conclude that ADV proteins that were expressed in *E.coli* in the previous studies probably represented degradation forms of VP1 and VP2.

The Expression of Pseudorabies Virus Antigens

Several PRV glycoproteins have been expressed in the vaccinia virus expression system. These glycoproteins include gp50, gp63, gI, and

gX (Kost et al., 1989; Marchioli et al., 1987). All of these glycoproteins are encoded in whole or in part, within the BamHI fragment 7 of PRV genome (Kost et al., 1989). Recombinant PRV glycoproteins that have been expressed in vaccinia virus-infected cells, co-migrate with the authentic PRV glycoproteins in polyacrylamide gels (Kost et al., 1989; Marchioli et al., 1987). Recombinant gP50 expressed in this way has been evaluated for use as a PRV subunit vaccine (Marchioli et al., 1987), and has been shown to be efficacious in mice. (Marchioli et al., 1987).

Pseudorabies virus proteins have also been expressed in procaryotic cells. These include proteins gp-88 (Saman et al., 1986), gH (Klupp and Mettenleiter, 1991), ICP18.5 (Pederson and Enquist, 1991) a 74 kD glycoprotein and a 92 kD glycoprotein (Robbins et al., 1984). However glycoproteins expressed in *E.coli* are not as biologically active in the host as native glycoproteins or proteins expressed by recombinant viruses. The reason for this is that *E.coli* does not have glycosylation or splicing mechanisms to modify viral proteins after translation. Usually PRV antigens expressed in this way are used for the mapping of the PRV genome. Antibodies raised against expressed PRV proteins in *E.coli* will react with the native form of PRV proteins. As a result specific gene fragments that encode PRV proteins can be identified.

Baculovirus Expression Vector System

Introduction

The development of recombinant DNA technology makes it possible to manipulate and exchange different kinds of genes between different organisms. A major goal of the biologic industry is to use this technology to produce large quantities of purified recombinant viral proteins for use as vaccine antigens and diagnostic reagents. Many efforts have been undertaken to express viral proteins in yeast, insect, or mammalian cells. These eucaryotic cells are ideal for this purpose because they have the ability to splice mRNA and glycosylate recombinant viral proteins. Among the most efficient of the eucaryotic expression systems is the baculovirus / insect cell system. This system has distinct advantages over other eucaryotic expression systems. The invertebrate virus vectors such as the baculovirus, *Autographa californica nuclear polyhedrosis virus* (AcNPV) are nonpathogenic for vertebrates, they possess strong promoters and they are easy to manipulate.

The AcNPV is propagated in the Sf-9 continuous cell line. This cell line was cloned from the IPLB-Sf-21-AE cell line which was originally derived from immature ovaries of *Spodoptera frugiperda* (the fall army worm) pupae (Vaughn et al., 1977). This cell line was initially grown and maintained in media supplemented with hemolymph. Subsequently the hemolymph was substituted

by fetal bovine serum which proved to be more effective in supporting the cell cultures (Mitsuhashi, 1982).

The following review will described the general characteristics of the baculovirus, AcNPV. This review will also focus on the use of AcNPV as an expression system for foreign viral genes.

General Characteristics of Baculovirus AcNPV

The baculovirus AcNPV belongs to subgroup A of the family Baculoviridae (Matthews, 1982). More than 30 species of lepidopteran insects are known to be susceptible to infection by the virus (Gröner, 1986). The virus exists in 2 forms. The occluded or inactive form (OV) is found in dead insects or in the external environment. In this state the virus is embedded in a protective protein matrix referred to as a polyhedra. The non-occluded form (NOV) is found in living insects (Volkman et al., 1976).

The virus is enveloped and has a helical nucleocapsid with ds DNA. The nucleocapsid measures 40 by 300 nm (Faulkner and Carstens, 1986). It consists of a major 44 kD major polypeptide (Summers and Smith, 1987) and 8 minor proteins (Kelly, 1985). The envelope of the NOV contains 12 proteins, 8 of which are glycosylated (Stiles and Wood, 1983). The envelope of the OV contains 13 proteins none of which are glycosylated.

The genome of AcNPV is a circular, supercoiled ds DNA of 128 kilobase pairs (Smith and Summers, 1978a; Smith and Summers, 1978b). It comprises

approximately 13% of the virus particle by weight (Arif, 1986). Homologous repeating sequences (hr) have been identified within the baculovirus genome (Cochran and Faulkner, 1983). These homologous repeats (hr) are located at 5 specific locations: hr_1, EcoRI-B-EcoRI-I; hr_2, EcoRI-A-EcoRI-J; hr_3, EcoRI-C-EcoRI-G; hr_4, EcoRI-Q-EcoRI-L; and hr_5, EcoRI-S-EcoRI-X. Each homologous repeat consists of nucleotide sequences that range in size from 73 to 225 bp and are rich in EcoRI sites (Arif, 1986). These homologous regions may play a role in the replication and expression of the AcNPV genome (Arif, 1986).

The genome of AcNPV has a coding capacity for 70 to 100 viral proteins (Friesen and Miller, 1986). Clones of cDNA representing 18 early, 49 late and 45 very late mRNA transcripts have been identified in infected insect cells (Friesen and Miller, 1986).

Viral replication requires approximately 72 h. It is initiated by the ingestion of the OV and digestion of its protective polyhedrin protein by gut enzymes. Adsorption to epithelial cells lining the digestive tract is mediated by the 64 kD envelope protein (Volkman, 1986). Penetration into the cell is speculated to be an endocytotic process (Volkman and Goldsmith, 1985). Virus that is adsorbed to the surface of the insect cell is taken into a clathrin-coated pit which then forms a coated vessicle. Fusion of the viral envelope and the endosomal membrane results in the release of the viral nucleocapsid into the cell cytoplasm. The rod-shaped nucleocapsid either enters the nucleus or

discharges the AcNPV genome through a nuclear pore (Faulkner and Carstens, 1986).

Replication of AcNPV DNA begins in the nucleus within 6 h p.i.. The progeny vDNA is packaged as a complex in a rod-shaped nucleocapsid. Nucleocapsids that are produced early in the course of infection are transported through the cytoplasm and released from the cell by budding. Nucleocapsids that are produced late in the course of infection acquire their envelope from the nuclear membranes and are embedded in the polyhedrin protein which also accumulates in the nucleus of the cell.

Transcription and translation of the baculovirus genome can be divided into 3 temporal phases: early, late, and very late (Miller, 1988). Translated proteins from each phase are required before progression to the next phase. Early gene transcription occurs prior to 6 h p.i. (Miller, 1988). This period can be divided into an immediate early and a delayed early phase (Miller, 1988). A single transcript is thought to be transcribed during immediate-early transcription. This transcript encodes a nonstructural regulatory immediate-early protein (IE-1). The IE-1 protein functions as a transacting transcriptional activator of other early genes (Guarino and Summers, 1986).

Transcription of late genes extends from approximately 6 through 18 h p.i.. Most of the genes that encode the structural proteins which include the major capsid protein, the core protein and the envelope glycoproteins, are

transcribed during this phase (Miller, 1988). Replication of DNA and the formation of NOV also occur during this period.

The very late phase of gene expression, occurs approximately 20 to 72 h, p.i.. This phase is characterized by abundant polyhedrin protein synthesis and the formation of occlusion bodies within the nucleus. These occlusion bodies consist of a conglomerate of viral particles and polyhedrin protein. During this phase, the polyhedrin and the p10 protein genes are highly expressed (Faulkner and Miller, 1986).

Experimental findings suggested that cellular RNA polymerase is responsible for the transcription of the early phase genes (Fuchs et al., 1983) and a virus-induced α-amanitin-resistant RNA polymerase is responsible for the transcription of the late and the very late phase genes of AcNPV (Fuchs et al., 1983; Grula et al., 1981). The α-amanitin-resistant RNA polymerase recognizes a consensus sequence present in the late and very late phase genes and initiates their transcription (Rohrmann, 1986). Eight late RNAs of AcNPV were found to have similar consensus sequences containing a conserved region (ATAAG) at their 5' start site. This consensus sequence and the A + T rich leader region, 60 bases upstream from the ATG translation start site, are involved in the high level expression of the polyhedrin gene (Miller, 1988).

The Baculovirus as a Gene Expression Vector

The baculovirus expression vector system (BEVS) is a helper-independent eucaryotic expression system that was developed by Smith et al. (1983a; 1983b). It has been used for the high level expression of a wide variety of foreign genes (Doerfler, 1986; Luckow and Summers, 1988; Matsuura et al, 1987; Smith et al., 1983b; Smith et al., 1985). These genes were inserted into the polyhedrin gene of AcNPV which is not essential for virus replication (Fraser et al., 1982; Smith et al., 1983a).

The use of the polyhedrin gene as a site for the insertion of foreign genes is ideal for several reasons. It is controlled by a strong promoter which is responsible for high level expression of the foreign gene. As much as 1 to 500 mg of foreign protein per liter of cell culture have been reported (Luckow and Summers, 1988). The promoter of the polyhedrin gene functions late in the replication process when host cell protein synthesis is minimum to non existent. Consequently the expressed protein is relatively pure. Foreign genes as large as 15 kb can be inserted into AcNPV genome (Miller, 1989). Recombinant baculoviruses are occlusion negative which facilitates their identification and cloning. The AcNPV is not a vertebrate virus and therefore is inherently safe (Tjia et al., 1983). Most importantly, the expressed proteins are immunologically and functionally similar to their native counterparts because the insect host cell possesses the postranslational modification systems that are present in eucaryotic cells.

The factors affecting expression are complex and not well characterized. It has been shown that cell condition and the quality of media components used, directly affects the level of polyhedrin expression in wild type AcNPV infection (Summers and Smith, 1987). Healthy *Spodoptera frugiperda* cells with over 97% viability and a doubling time of 16-18 h are required for high level expression (Summers and Smith, 1987). Expression levels for different genes inserted into the same vector are often different and may be related to the length and nature of the leading sequence and protein encoded by the foreign genes. Each foreign gene may have its own codon preference so that nascent recombinant proteins are processed differently. In addition, the choice of the baculovirus transfer plasmid used in the transfection and their recombination events also affect the level of expression of the foreign gene (Luckow and Summers, 1988).

SECTION I: CHARACTERIZATION AND EXPRESSION OF ALEUTIAN MINK DISEASE VIRUS CAPSID PROTEINS IN INSECT CELLS USING A BACULOVIRUS EXPRESSION SYSTEM

SUMMARY

A 2.3 Kb cDNA clone encoding Aleutian mink disease virus (ADV) structural proteins VP1 and VP2 was inserted into the polyhedrin gene of *Autographa californica nuclear polyhedrosis virus* (AcNPV) downstream from its promoter. The cDNA was expressed by the recombinant virus, AcADV-1, in *Spodoptera frugiperda* 9 cells. The recombinant protein was found to be antigenic for mink as determined by Western immunoblot analysis. Serums which were collected from 16 mink that were not infected with ADV did not react with the recombinant proteins VP1 or VP2. Ten serums that were collected from mink which had antibody titers greater than 4 (log_2) as determined by counter current electrophoresis (CCE), reacted with VP1 and VP2. Two of 20 serums from mink with CCE titers equal to or less than 4 reacted with VP1 and VP2. Fluorescence microscopic examination of AcADV-1 infected *Spodoptera frugiperda* 9 cells indicated that the recombinant protein was present within the nucleus of the cells. Electron microscopic examination of positive-stained thin sections of these cells revealed the presence of small particles that measured 23-25 nm in diameter. These particles resembled native ADV core particles. This observation suggests that recombinant baculovirus-expressed VP1 and VP2 self assemble to form ADV capsids.

INTRODUCTION

Aleutian disease of mink is caused by an autonomous parvovirus (11). It was first described in 1956 (20) and is responsible for significant economic loss in commercial mink ranching operations (3,19,30). The infection in adult mink is persistant and is characterized by plasmacytosis, hypergammaglobulinemia and ultimately immune complex mediated glomerulonephritis and arteritis (17,26,28,29). Affected mink usually die within 3-4 months after infection. Mink, without maternal antibodies that are infected during the neonatal period usually develop an acute fulminant, fatal respiratory distress syndrome which is characterized by interstitial pneumonia and the formation of hyaline membranes (4,6,7,9,21). The disease is most severe in mink that have the gene for the Aleutian coat color. However, the severity of disease is also influenced by the virus strain (15,18,25).

Vaccination increases the severity of Aleutian disease (27). Consequently the disease is controlled in mink populations by serologically identifying and subsequently removing infected mink (3,30). Experimental studies have demonstrated that detectable levels of IgM appear within 6 days after infection and can persist for at least 85 days (29). Detectable levels of IgG usually appear within 10 to 15 days of infection and will persist for the life of the mink (30). These immunoglobulins are primarily specific for the capsid proteins, p85 (VP1) and p75 (VP2), and the nonstructural protein, p71.

The most commonly used serological tests for detection of Aleutian mink disease virus (ADV) antibody are counter current electrophoresis (CCE) (14) and indirect counter current electrophoresis (1). These assays require a highly purified ADV antigen preparation. Traditionally, antigen for these assays has been produced in cell culture (2,11) or extracted from tissues of infected mink (13). More recently, ADV capsid protein has been expressed by recombinant vaccinia virus containing a 2.3 Kb cDNA insert which represents the complete coding sequence for VP1 and VP2 (14). This recombinant capsid protein was found to be slightly more immunoreactive than cell culture derived ADV antigen (14). The purpose of the present study is to determine if VP1 and VP2 can be expressed by the baculovirus expression system (22,23,24,32) in a form that is also highly antigenic for mink (31). This expression system was chosen because of its ability to produce large amount of recombinant protein in either insect cell cultures or Lepidopteran larvae (22).

MATERIALS AND METHODS

Cells and virus

The wild-type *Autographa californica nuclear polyhedrosis virus* (AcNPV) (Invitrogen, San Diego, CA) and recombinant baculovirus stocks were propagated in *Spodoptera frugiperda cell* (Sf-9) cultures which were maintained at 28^0 C in TNM-FH medium, which was prepared by supplementing Grace medium (GIBCO BRL/Life Technologies, Gaithersburg, MD) with 0.3% yeastolate, 0.3% lactalbumin hydrolysate (Difco Laboratories, Detroit, MI) gentamycin (50 ug/ml), amphotericin B (2.5 ug/ml) and 10% fetal calf serum.

Constructions of recombinant plasmid

A 2.3 kb cDNA representing ADV structural proteins VP1 and VP2 was provided in the plasmid vector, pGEM3, by Dr. M. E. Bloom of the National institution of Health, Rocky Mountain Laboratory, Hamilton, MN. The cDNA insert was excised from pGEM3 by Bgl II digestion and ligated into the dephosphorylated Bgl II site of the baculovirus transfer plasmid, pVL1392. This recombinant transfer plasmid was then transformed into *Escherichia coli* NM522. Clones containing the ADV cDNA were identified by colony hybridization with the ^{32}P-labelled 2.3 Kb ADV cDNA (specific activity $7x10^7$ dpm/ug). A recombinant plasmid containing the insert in the correct transcriptional orientation was identified by restriction enzyme analysis.

This transfer plasmid was designated pVLADVS and was used to produce recombinant baculovirus.

Production and identification of recombinant baculovirus

One ug of purified wild type AcNPV and 2 ug of the recombinant plasmid, pVLADVS DNA were mixed in 0.75 ml of transfection buffer pH 7.1 (25 Mm Hepes, 140 Mm NaCl, 125 Mm $CaCl_2$). This mixture was then added to a 25 cm^2 flask containing $2x10^6$ Sf-9 cells that were seeded 1 h previously and subsequently maintained at 28° C for 4 h. The cells were then rinsed with TNM-FH and incubated in fresh TNM-FH for 5 days at 28° C. At the end of this period, culture media was collected and screened for recombinant baculovirus by a dot-blot hybridization procedure originally described by Fung, et al (16).

Spodoptera frugiperda 9 cells were seeded into 96-well microplates at a density of $2x10^4$ cells/well in a volume of 100 ul of TNM-FH. Individual wells were then inoculated with 50 ul of 10 fold dilutions of media containing potential recombinant baculovirus and incubated at 28° C. Six days later all media was removed from each well, transferred to a duplicate microtiter plate, and stored at 4° C for future use. The cell monolayers in each well were then lysed with 200 ul of 0.5 N NaOH. Cell lysates were transferred to Hybond N+ filters (Amersham International, Amersham, U.K.) using a dot blot microfiltration apparatus (Bio-Rad Laboratories, Richmond, CA), all filters were previously rinsed with 0.5 N NaOH. Individual filters were subsequently washed with

200 ul of 0.5 N NaOH, removed from the apparatus and treated for 2 h at 65° C in a 3 fold concentration of sodium phosphate EDTA buffer (SSPE) pH 7.4, containing 5X Denhardt's solution, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA. Normal (1X) SSPE consists of 0.18 M NaCl, 10mM $NaH_2PO_4 \cdot H_2O$ and 1mM EDTA. Filters were subsequently exposed to the ^{32}P-labelled 2.3 Kb ADV cDNA (specific activity $7x10^7$ dpm/ug) overnight in the same buffer. Filters were then washed at 65° C for 30 min one time in 2X SSPE and once in 0.1X SSPE which contained 0.1% SDS. Washed filters were exposed to Kodak X-Omat XAR-5 film at -70° C for 2 h which permitted the identification of wells that contained recombinant baculovirus.

Individual recombinant viruses were cloned from mixed populations of baculovirus by 4 subsequent rounds of limiting dilutions, and identified as previously described. The homogeneity of the cloned populations was verified by thoroughly examining virus-infected Sf-9 monolayers for the presence of nuclear occlusions. The presence of occlusions would indicate contamination with non-recombinant virus.

Expression and identification of ADV recombinant protein

Spodoptera frugiperda 9 cells in 25 cm^2 flask were singly infected with wild type, and recombinant baculovirus at an m.o.i. of 10, and incubated at 28° C for 72 h. Virus infected cells and non-infected control cells were then harvested and solubilized by adding 100 ul of Laemmli sample buffer

(50 Mm Tris-Hcl, pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 0.1% bromophenol blue) to each 1 ml volume of original cell suspension that contained approximately $6x10^5$ cells. The treated cell preparations were boiled for 10 min and electrophoresed through a 10% sodium dodecyl sulfate polyacrylamide gel using a Mini-protean II dual slab system (Bio-Rad, Richmond, CA). The electrophoretically separated proteins were transferred from the gel to nitrocellulose membranes using a Mini trans-blot apparatus (Bio-Rad, Richmond, CA). The membranes were blocked by immersion for 2 to 4 h in TBS (Tris base 25mM, NaCl 500mM, Ph 7.5) containing 3% gelatin. The membranes were stained with an optimal dilution of pooled serums that were collected from ADV infected and noninfected mink. The stained membranes were washed twice with TBS containing 0.05% Tween 20. The Immunoglobulin stained ADV proteins were visualized by treating the membranes with an optimal dilution of horseradish peroxidase conjugated to protein A (Kirkegaard & Perry Laboratories Inc, Gaithersburg, Maryland) and subsequently with the chromogen, 4-chloro-1-naphthol. Both mink serum and the conjugate were diluted in TBS containing 1% gelatin.

Direct immunofluroresence

The location of recombinant ADV protein within infected cells was demonstrated by immunofluroresence. Cell monolayers were infected with the recombinant baculovirus at an m.o.i. of 1 and incubated for 72 h. Infected cells

were collected and resuspended in TNM-FH with uninfected cells at a ratio of 1:3 respectively. Eight-well teflon coated glass slides (ICN Biomedicals, Costa Mesa, CA) were spotted with 5 ul of the cell suspensions. The cells were permitted to settle onto and adhere to the glass slides for 30 min. Cell monolayers were then fixed with acetone for 5 minutes at 4^0 C. The fixed cell preparations were overlaid with mink anti-ADV specific IgG conjugated to FITC and incubated for 1 h at 37^0 C in a humidified atmosphere. Slides were then washed 3 times by immersion in distilled water for 5 minutes each, air dried and examined with a Leitz fluorescence microscope.

Electron microscopy

The structure of recombinant ADV protein was visualized in a cell associated and a cell free state by electron microscopy. The cell free state was visualized by infecting a Sf-9 cell monolayer contained in a 150 cm^2 flask with recombinant baculovirus at an m.o.i. of 10. The infected cells were harvested 72 h later and resuspended in 1 ml of PBS. The cells in the suspension were disrupted first by 4 cycles of freezing at -70^0 C and thawing at 37^0 C and subsequently by sonication. The cell lysate was clarified by centrifugation at 13,600 X g for 30 minutes at 4^0 C. The clarified suspension was then added to an equal volume of Freon 113. This mixture was vortexed for 3 min and centrifuged again at 13,600 X g at 4^0 C for 10 min. The upper phase containing virus and recombinant protein was collected and adsorbed for

30 min onto a parlodion coated 300-mesh copper grid. The grid was then inverted and placed onto a drop of distilled water for 5 min and subsequntly onto a drop of an aqueous solution of 0.5% uranyl acetate for 20 seconds. Excess stain was removed by blotting and the grid was examined with an Hitachi HS-9 electron microscope (Hitachi, Tokyo, Japan) at 75 kV.

The cell associated state was visualized by fixing infected cells with 2% glutaraldehyde prepared in phosphate buffer. The fixed cells were washed twice in phosphate buffer and then stained with 1% osmium tetroxide in 0.1 M phosphate buffer, rinsed in distilled water, dehydrated with alcohol, infiltrated with propylene oxide and embedded in epoxy resin. Sections with a thickness of 600-900 Å were collected onto bare 3 mm copper grids and stained with a solution containing 2% uranyl acetate diluted in methanol and Reynolds' lead acetate (31) and examined by electron microscopy.

Evaluation of the antigenicity of the recombinant protein

The antigenicity of ADV recombinant protein for mink was evaluated further by Western immunoblot analysis as previously described using a panel of mink serums that represented pre and post infection sequential bleedings from 16 adult mink. These serums were provided by Dr. M. E. Bloom of the National institution of Health, Rocky Mountain Laboratory, Hamilton, MN. The antibody titers of these serums was previously determined by CCE and ranged from less than 4 (log_2) to about 14 (log_2).

RESULTS

Construction and isolation of a recombinant baculovirus

A recombinant baculovirus transfer vector containing cDNA representing the complete coding sequences of the ADV VP1 and VP2 gene in the correct orientation was constructed as described in Fig. 1. Recombinant baculovirus was produced by the co-transfection of Sf-9 insect cells with the DNA of the wild-type baculovirus, AcNPV and the transfer vector pVL1392. Recombinant baculoviruses were identified by dot-blot hybridization (Fig. 2) and cloned by 4 rounds of limiting dilution. The procedure was effective based on the absence of viral inclusions in cells infected with the cloned populations of recombinant baculovirus. A single clone was designated AcADV-1 and used for the expression of recombinant VP1 and VP2.

Expression of ADV capsid protein in *Spodoptera frugiperda* cells

Spodoptera frugiperda 9 cells were singly infected at a m.o.i. of 10 with wild-type baculovirus AcNPV and the recombinant baculovirus AcADV-1. Infected cells were harvested at 72 h, p.i. and analyzed by SDS-PAGE and Western immunoblotting. A representative coomassie blue-stained gel is shown in Fig. 3. The 29 kD polyhedrin protein which is present in solubilized wild-type AcNPV infected cells as indicated in lane 3, is not present in the lysate of recombinant baculovirus infected Sf-9 cells as indicated by the absence of a

Figure 1. Construction of the recombinant baculovirus transfer plasmid pVL-ADVS

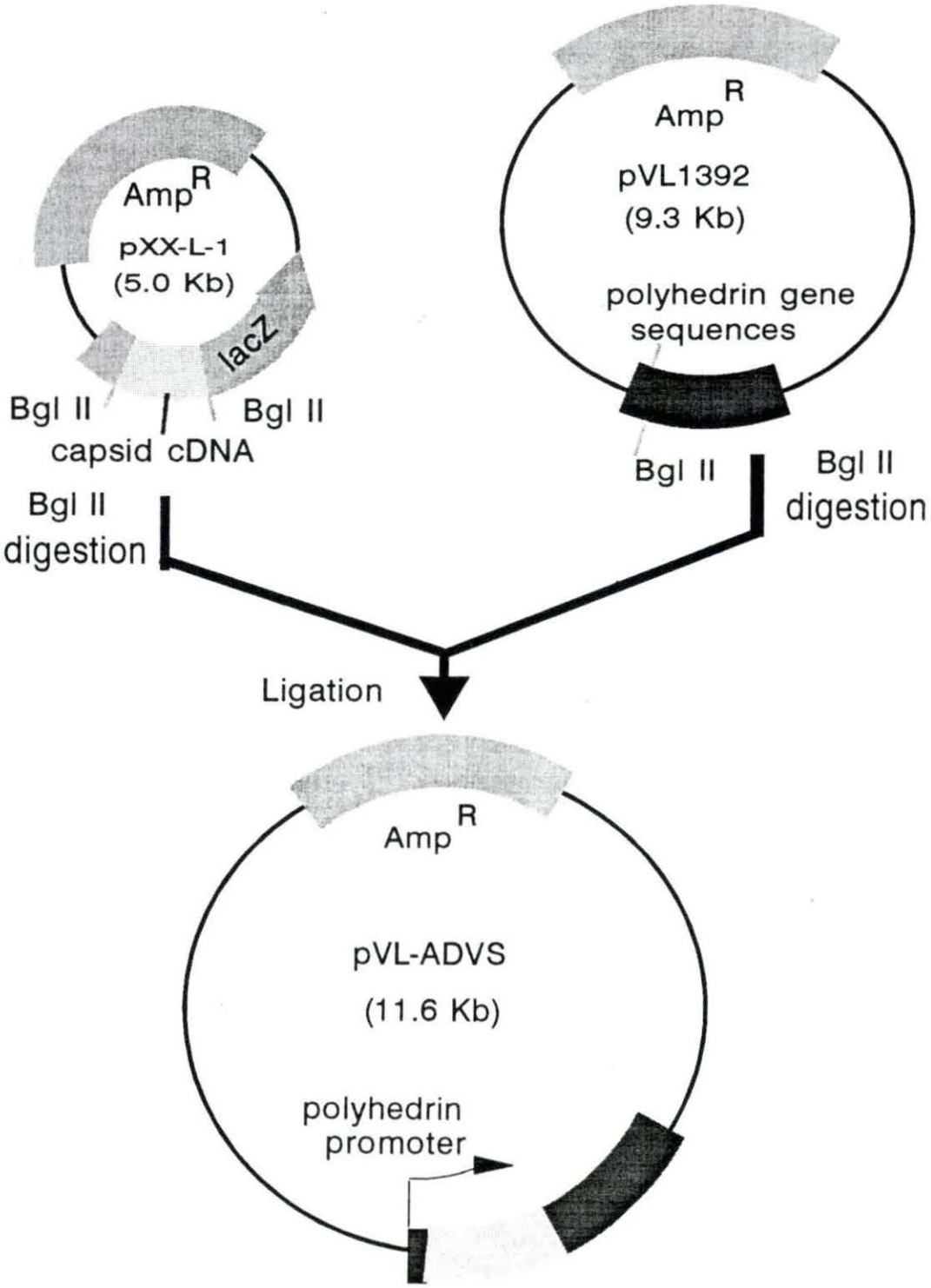
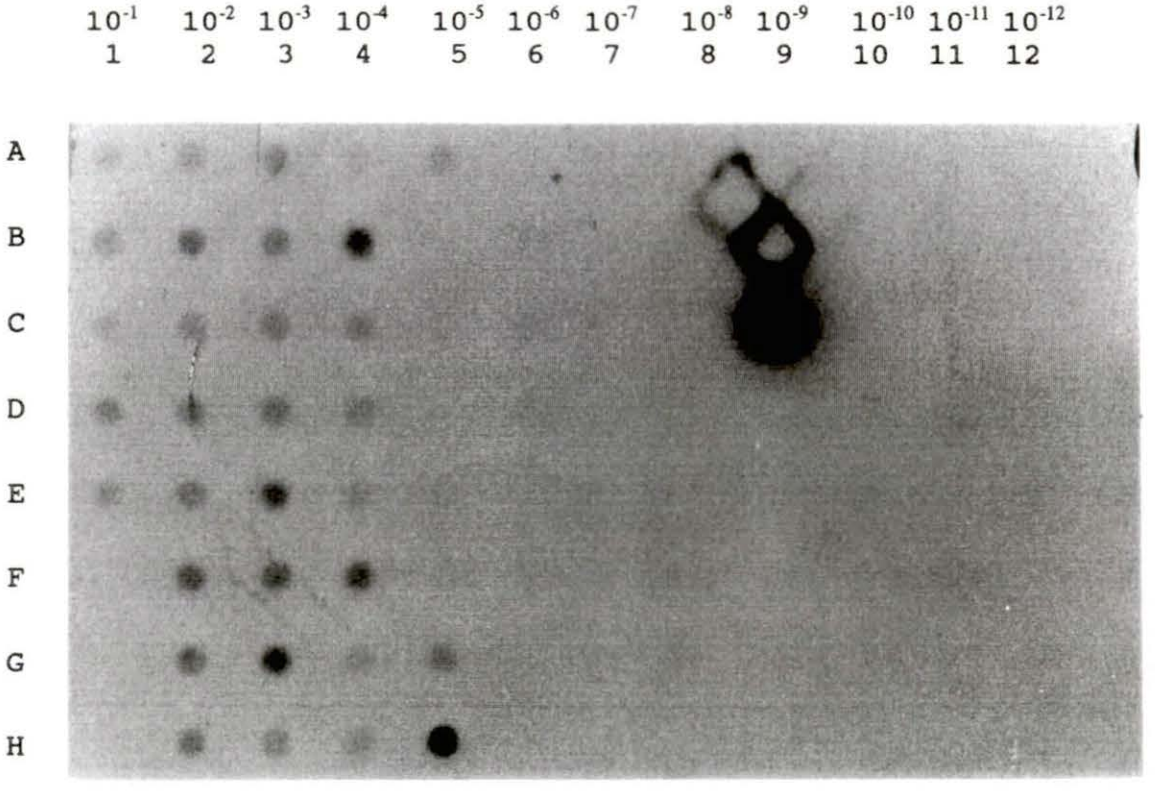

Amp R
pXX-L-1
(5.0 Kb)
lacZ
Bgl II
Bgl II
capsid cDNA
Bgl II
digestion
Amp R
pVL1392
(9.3 Kb)
polyhedrin gene
sequences
Bgl II
Bgl II
digestion
Ligation
Amp R
pVL-ADVS
(11.6 Kb)
polyhedrin
promoter

Figure 2. Identification of recombinant baculovirus by dot blot hybridization. *Spodoptera frugiperda 9* cells were seeded in 96 well plates and infected with serial dilutions of a virus population containing potential recombinant baculoviruses. After 6 days, cells were lysed, their DNA transferred to a Hybond N+ membrane and hybridized with a ^{32}P labelled ADV cDNA probe. Well C9 = positive control containing the ADV cDNA, wells F1, G1 and H1 = negative controls containing lysates of uninfected cells


10^{-1} 1
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10^{-10} 10
10^{-11} 11
10^{-12} 12
A
B
C
D
E
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G
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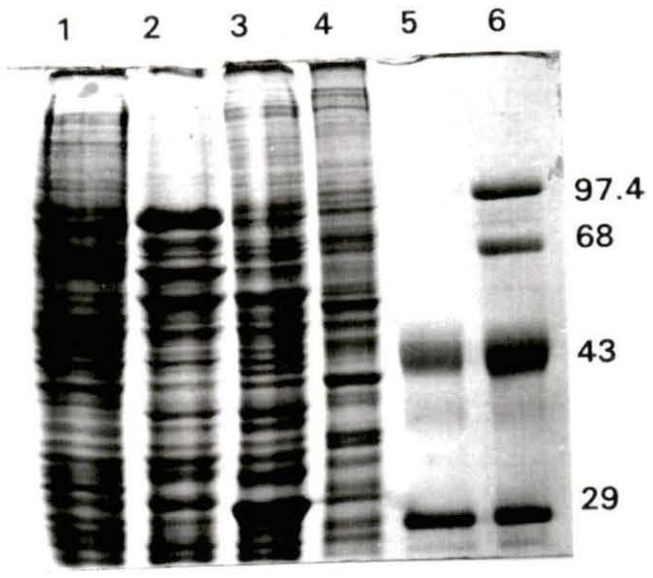
Figure 3. SDS-PAGE analysis of wild-type baculovirus, AcNPV and recombinant baculovirus AcADV-1. Lane 1, mock-infected cells; lane 2, AcADV infected cells; lane 3, wild-type AcNPV infected cells; lane 4 ADV infected CRFK cells; lane 5, low molecular weight markers; lane 6, high molecular weight markers

Figure 4. Western immunoblot analysis of ADV capsid proteins expressed in insect cells. Lane 1, wild-type ADV infected CRFK cells; lane 2 and 3, recombinant baculovirus infected cells; lane 4, wild-type baculovirus infected cells; lane 5, mock-infected cells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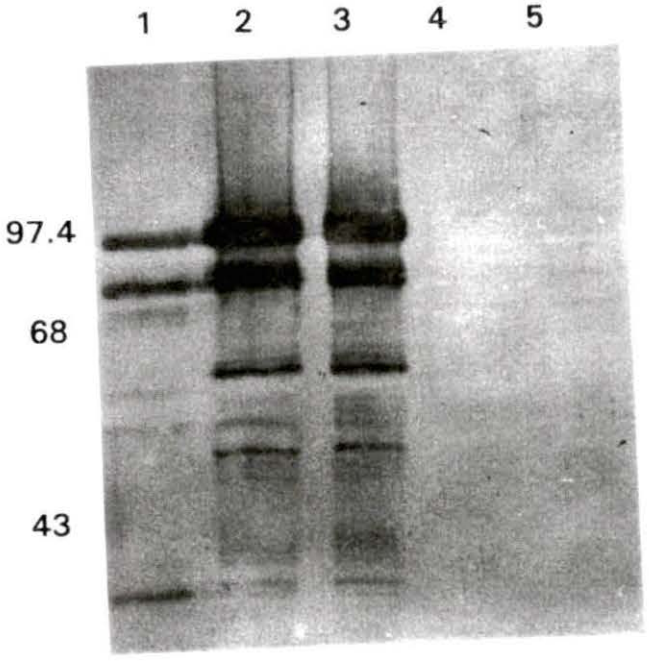
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representative band in lane 2. The polyhedrin protein has been replaced by a protein with a molecular weight of approximately 85 kD, which is similar to the molecular weight of native VP1 (85 kD).

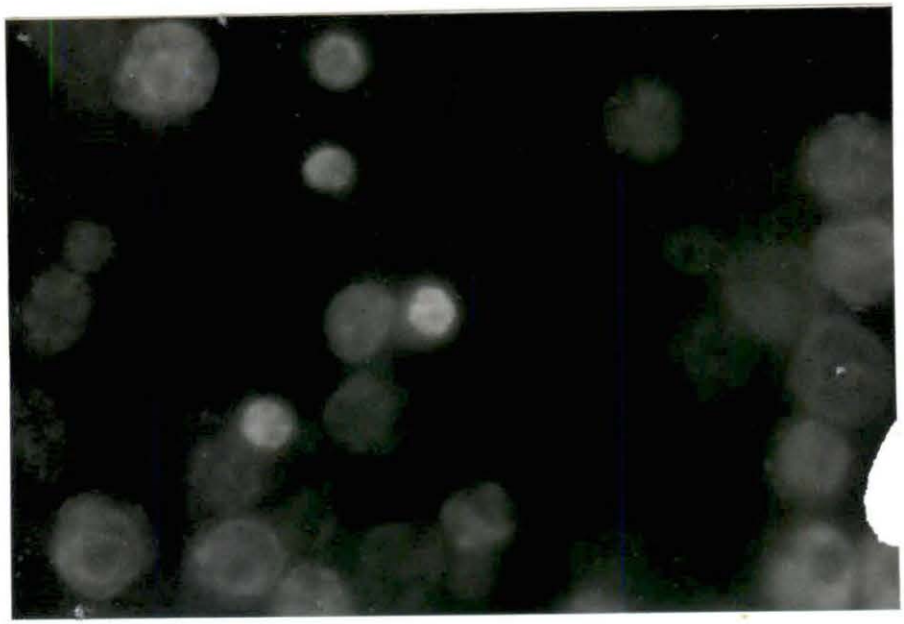
Fluorescent microscopic examination revealed that recombinant ADV capsid proteins localized in the nucleus of infected cells. See Fig. 5, panel A. There is not any fluorescence in the nucleus of uninfected cells (Fig. 5, panel B). Examination of intact and lysed AcADV-1 infected cells by electron microscopy revealed the presence of small particles with icosahedral symmetry. See Fig. 7. These particles are 23 to 25 nm in diameter (Fig. 6 and 7) and resemble natural ADV core particles which range in size from 23 to 28 nm in diameter (3).

Evaluation of the antigenicity of baculovirus-expressed VP1 and VP2

The antigenicity of baculovirus expressed ADV recombinant protein for mink was demonstrated by Western immunoblot analysis as illustrated in Fig. 4. Capsid proteins VP1 and VP2 derived from ADV infected cells (lane 1) and the recombinant baculovirus AcADV-1 (lane 2 and 3) are clearly visible. The lower molecular weight proteins seen in lanes 2 and 3 most likely represent degradation products of VP1 and VP2. These proteins were not present in lysates of cells infected with the wild type virus or in lysates of uninfected cells as illustrated in lanes 4 and 5 respectively.

Figure 5. Immunofluorescence of recombinant baculovirus-infected *Spodoptera frugiperda* cells 72 h postinfection. Panel (A): insect cells infected with recombinant baculovirus. Panel (B): uninfected cells

A



B

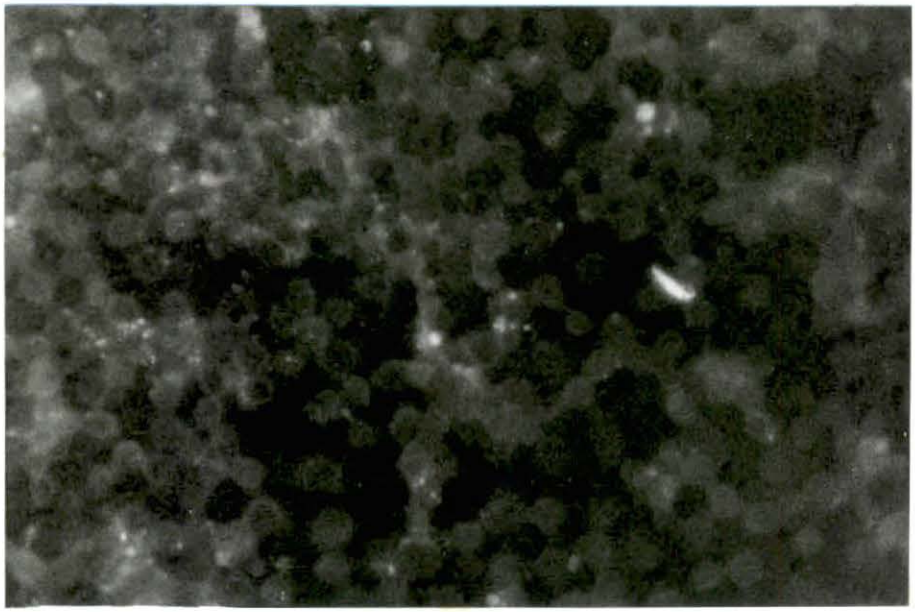


Figure 6. Electron microscopy of of a sectioned *Spodoptera frugiperda 9* cell 72 h after infection with recombinant baculovirus AcADV. Arrow indicates capsid-like structures approximately 23-25 nm in diameter (magnification, X 28,080). Bar = 200 nm

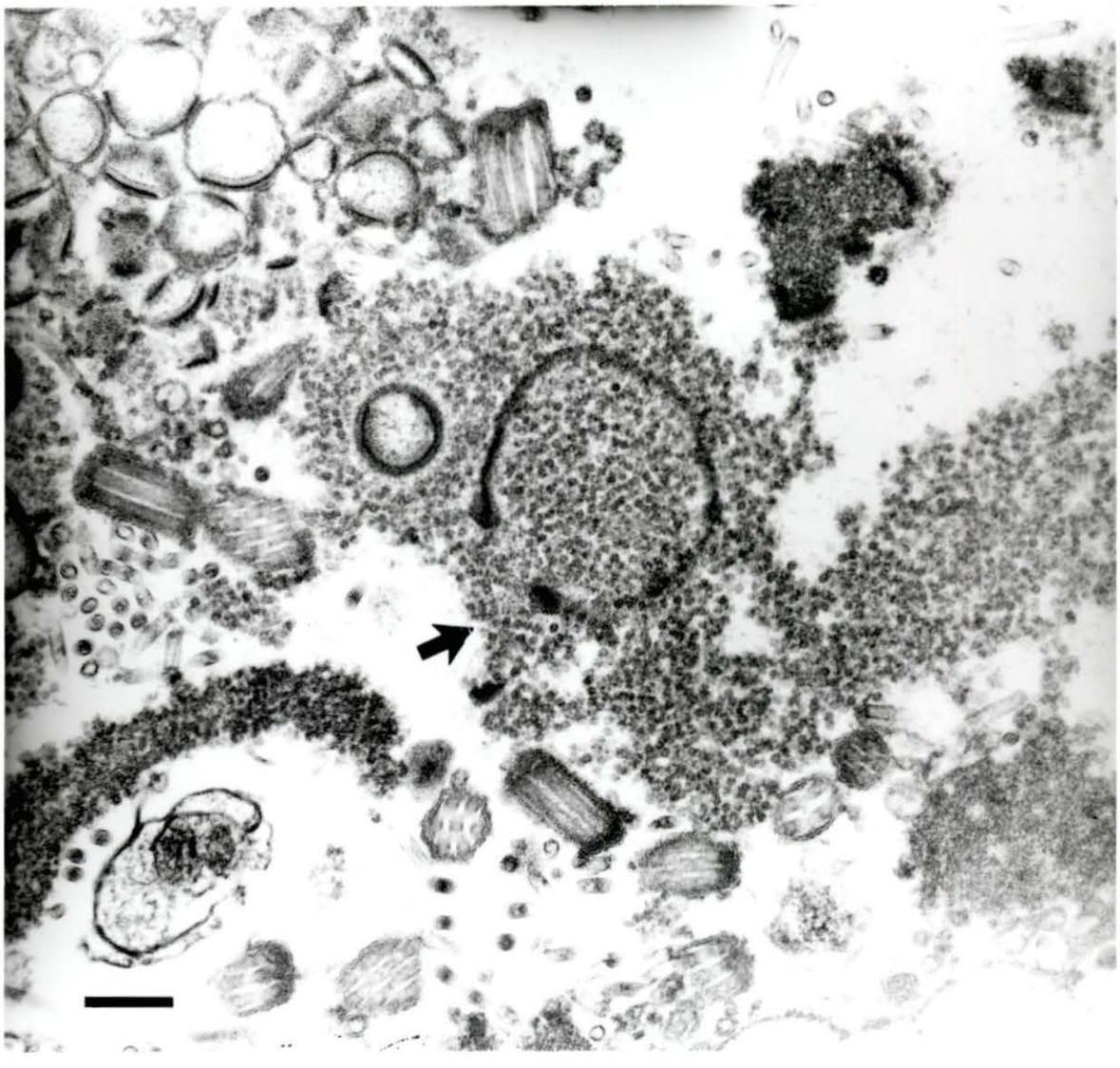
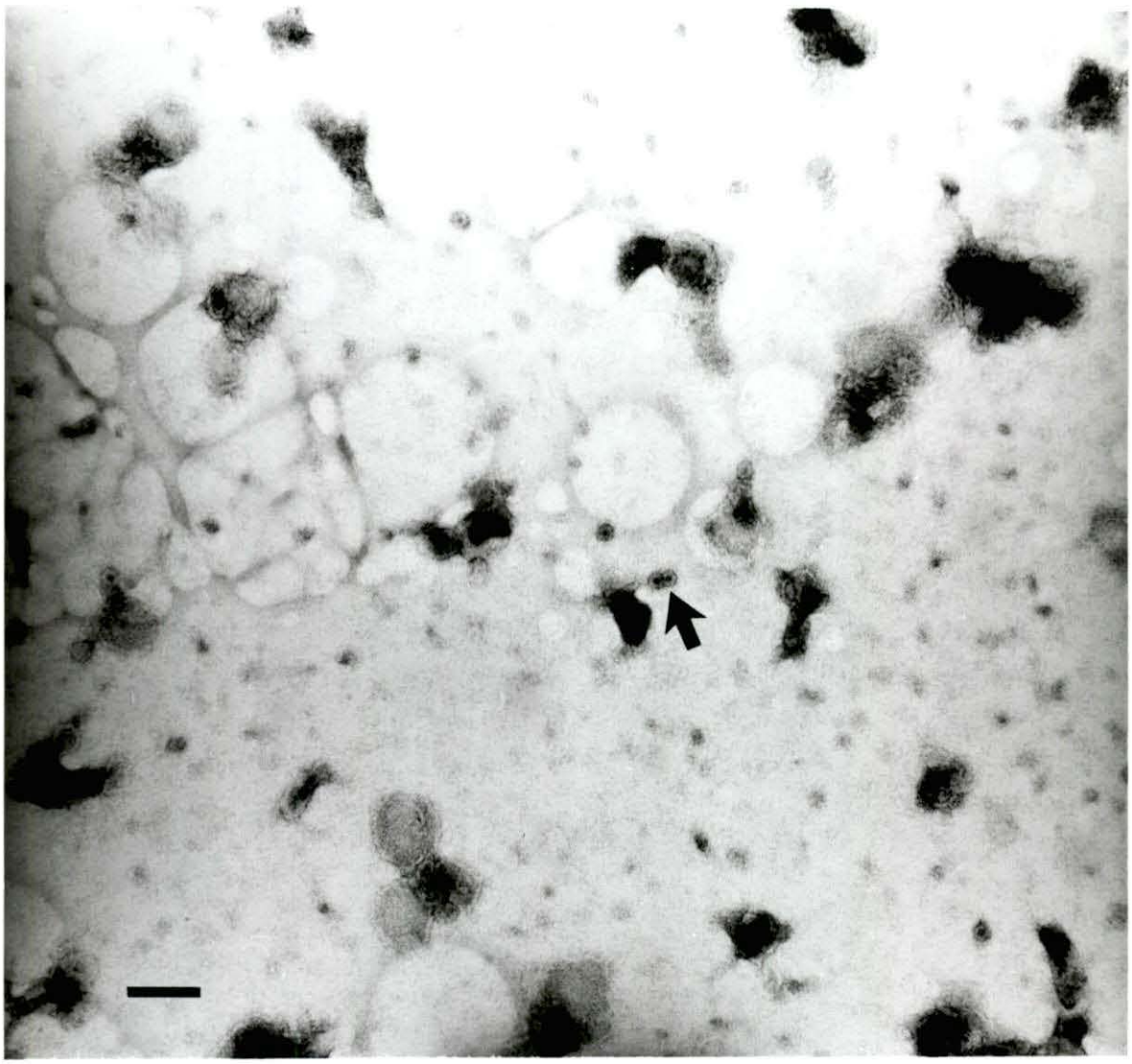


Figure 7. Electron microscopy of a postive stained AcADV-1 infected cell lysate. Arrow indicates ADV corelike particles approximately 23-25 nm in diameter (magnification X 47,500). Bar = 100 nm



Further evaluation of the antigenicity of the recombinant ADV protein was made by Western immunoblot assay of a panel of mink serums that were collected before and after infection with ADV. The ADV antibody titers of these serums were previously determined by CCE and expressed as $\log_2$. The results of this experiment are summarized in Table 1.

No antibody specific for recombinant VP1 and VP2 was detected by Western immunoblot analysis in 16 serums that were collected from uninfected mink. Antibody specific for these recombinant proteins was detected by Western immunoblot analysis in all 10 serums with CCE titers greater than 4. Western immunoblot analysis only detected antibody to the recombinant proteins in 2 of 6 serums that had CCE titers equal to or less than 4.

Table 1. Evaluation of the antigenicity of baculovirus-derived recombinant Aleutian mink disease virus (ADV) capsid protein by Western immunoblot assay of serums collected from mink experimentally infected with ADV

		Presence / titer of ADV specific antibody					
		pre-infection		Early post-infection[a]		Late post-infection[b]	
Mink #	Experiment	CCE[c]	WB[d]	CCE	WB	CCE	WB
4493	1	-	-	<4	-	<4	-
4494		-	-	<4	-	<4	-
4498		-	-	<4	-	<4	-
4499		-	-	<4	-	<4	+
4495		-	-	>4	+	>4	+
4496		-	-	>4	+	>4	+
4497		-	-	>4	+	NA[e]	
4500		-	-	>4	+	>4	+
4501		-	-	>4	+	>4	+
4502		-	-	>4	+	NA	
4508	2	-	-	<4	-	<4	-
4518		-	-	<4	-	4	-
4519		-	-	<4	-	4	-
4523		-	-	<4	-	4	-
4515		-	-	4	+	<4	-
4526		-	-	4	+	4	-

[a] Early post-infection = day 28, experiment 1 and day 32, experiment 2.

[b] Late post-infection = day 68, experiment 1 and day 93, experiment 2.

[c] CCE = Counter current electrophoresis titers (log_2) determined by using CRFK cell culture derived ADV antigen.

[d] WB = Western immunoblot assay. Presence (+) or absence (-) of antibody was determined using recombinant VP1 and VP2 as antigen.

[e] NA = Not available.

DISCUSSION

The objective of the above study was to determine if a 2.3 kb cDNA representing VP1 and VP2 of ADV could be expressed in a highly antigenic form by the baculovirus expression system. This cDNA was originally cloned from mRNA by Alexandersen et al. (8) and was recently expressed by a recombinant vaccinia virus (14). In the present study we succeeded in producing a recombinant baculovirus by inserting the same cDNA clone into the polyhedrin gene adjacent to its promoter. Evidence that supports this conclusion was the absence of the 29 kD polyhedrin protein in lysates of Sf-9 cells that were infected with the recombinant virus as indicated by SDS-PAGE analysis and the presence of polyhedrin protein in lysates of Sf-9 cells infected with wild type virus. See Fig. 3, lanes 2 and 3 respectively.

Further evidence that indicated the successful construction of the recombinant baculovirus containing ADV cDNA, was provided by Western immunoblot analysis. See Fig. 4. Lysates of cells infected with the recombinant virus contained 2 proteins with molecular weights of 85 and 75 kD. The pooled ADV specific mink serum reacted with both of these proteins (lane 2 and 3). The molecular weights of these 2 proteins are similar both to the molecular weights of native VP1 and VP2 (11) and to the molecular weights of the recombinant forms of these proteins produced by vaccinia

virus (14). Lysates of mock-infected and wild-type infected cells did not contain any proteins that were immunologically recognized by ADV positive mink serum. See Fig. 4, lane 4 and 5.

Additional evidence of the antigenicity of these recombinant proteins for mink is presented in Table 1. Ten serums with CCE titers greater than 4 all tested positive for ADV antibody by Western immunoblot analysis in which the recombinant proteins were used as antigens. However, only 2 of 20 serums with CCE titers equal to or less than 4 tested positive by Western immunoblot analysis. The failure to detect antibody in 18 of these 20 serums by Western immunoblot analysis could have been due to a difference in sensitivity between the Western immunoblot and CCE assays. No false positive reactions were observed by Western immunoblot analysis in 16 serums that were collected from mink prior to ADV infection.

Analysis by SDS-PAGE of lysates of cells infected with recombinant baculovirus also revealed that VP1 was produced in larger quantities than VP2 as indicated by the relative densities of the 2 bands representing these proteins (Fig. 3, lane 2). Clemens et al. (14) also reported that vaccinia recombinant VP1 was produced in greater quantities than VP2. These are interesting observations because VP2 is produced in greater quantities than VP1 in mammalian cells infected with wild type ADV (5,10,12). This difference could be due to the relative affinity of the ribosomes of Sf-9 and Green monkey cells for the start codons within the mRNA transcript of the cDNA.

Fluorescent microscopy (Fig. 5) and electron microscopic studies (Fig. 6) suggest that the recombinant protein is assembled within the nucleus of Sf-9 cells in a form that resembles the size and appearance of the natural ADV capsid. These observations suggest that the signal sequences of recombinant proteins function the same in the baculovirus system as they do in the mammalian cell and most importantly that the 3 dimensional configuration of antigenic determinants of the recombinant protein may be identical to that of the native protein.

The fact that the expressed protein is immunologically recognized by mink justifies additional studies in which native, vaccinia-expressed and the baculovirus-expressed ADV capsid proteins can be compared for efficacy by the same serological test. In a recent study recombinant VP1 and VP2 was produced by vaccinia virus and compared to native ADV proteins obtained from infected cell cultures (14). The vaccinia recombinant protein was recognized by ADV specific antibodies on day 10 p.i. in 2 of 4 mink. However antibodies were not detected in these serums when cell culture derived antigen was used in the assay. These results suggest that vaccinia recombinant protein may be superior to cell culture protein as diagnostic antigen. Comparative studies will confirm whether or not baculovirus recombinant ADV capsid protein is a better diagnostic antigen than cell culture derived antigen and vaccinia recombinant ADV capsid proteins. These studies will also determine if baculovirus recombinant protein can be produced more economically.

ACKNOWLEDGEMENTS

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SECTION II: CONSTRUCTION OF A RECOMBINANT BACULOVIRUS CONTAINING A TRUNCATED PSEUDORABIES VIRUS GENE REPRESENTING THE 32 kD MAJOR NUCLEOCAPSID PROTEIN.

SUMMARY

A recombinant baculovirus was constructed containing an 800 bp cDNA that represented a truncated gene of the major 32 kD nucleocapsid protein. Attempts to express this gene in *Spodoptera frugiperda 9* cells were unsuccessful.

INTRODUCTION

Pseudorabies or Aujeszky's disease is the source of significant economic lost to the swine industry. Annual losses are estimated at $107 to $117 million (2). The disease is caused by a Herpesvirus which can persist in a latent state in swine. Consequently, pigs that recover from acute infection or which are inapparently infected can periodically shed virus over their lifetime (1).

Vaccines can prevent clinical disease but do not prevent infection or the establishment of latency. Consequently vaccinated pigs must be considered infected unless they are immunized with either a live deletion mutant vaccine or a subunit vaccine. These vaccines permit the identification of pigs infected with wild type virus by testing for antibodies to PRV proteins that are not represented in the vaccines (6).

The use of live deletion-mutant vaccines is not without some risks. *In-vitro* and *in-vivo* recombination has been demonstrated (3,4,5). Consequently, recombination between vaccine strains and field strains may result in the emergence of virulent PRV strains that would not be detectable by companion differential diagnostic tests. This possibility can be avoided with the use of subunit vaccines (15). The ideal subunit vaccine would contain all of the glycoproteins that are associated with virulence and protective immunity.

Recent work in our laboratory has demonstrated that the non-glycosylated nucleocapsid proteins (NCP) are suitable diagnostic antigens

(10,11). Their utilization would permit the use of all PRV glycoproteins which have been shown to be associated with virulence, including gpI (7,9,14,16).

The production of NCP by traditional methods would not be economical. However recombinant methods may prove to be economically feasible. The following study describes the construction of a recombinant baculovirus (12,13,17) containing a truncated gene representing the 32 kD major NCP. Efforts to express the recombinant protein are also described.

MATERIALS AND METHODS

Cells and virus

The wild-type *Autographa californica nuclear polyhedrosis* (AcNPV) (Invitrogen, San Diego, CA) and recombinant baculovirus stocks were propagated in *Spodoptera frugiperda cell* (Sf-9) cultures which were maintained at 28^0 C in TNM-FH medium, which was prepared by supplementing Grace medium (GIBCO BRL/Life Technologies, Gaithersburg, MD) with 0.3% yeastolate, 0.3% lactalbumin hydrolysate (Difco Laboratories, Detroit, MI) gentamycin (50 ug/ml), amphotericin B (2.5 ug/ml) and 10% fetal calf serum.

Constructions of the recombinant plasmid

An 800 bp cDNA designated 462A that represented the truncated gene of the PRV 32 kD major nucleocapsid protein was provided in the transfer vector, pcDNA II, by M. McGinley of Iowa State University. The cDNA insert was excised from pCDNA II by digestion with Xba I and Spe I and ligated into the dephosphorylated Nhe I site of the baculovirus transfer plasmid pBlueBac. A recombinant baculovirus transfer plasmid containing the insert in the correct transcriptional orientation was identified and designated pBlue462A.

Production and identification of recombinant baculovirus

One ug of purified wild-type baculovirus DNA and 2 ug of the recombinant baculovirus transfer plasmid, pBlue462A were mixed in 0.75 ml of transfection buffer pH 7.1 (25 Mm Hepes, 140 Mm NaCl, 125 Mm $CaCl_2$). This mixture was then added to a 25 cm^2 flask containing 2 x 10^6 Sf-9 cells that were seeded 1 h previously and subsequently maintained at 28^0 C for 4 h. The cells were then rinsed with TNM-FH and incubated in fresh TNM-FH for 5 days at 28^0 C. At the end of this period, culture media was collected and screened for the presence of recombinant baculovirus by plaque assay.

Isolation of recombinant baculovirus by plaque assay

Spodoptera frugiperda 9 cells were seeded into 60 mm^2 dishes (Lux Contour Dishes, ICN Biomedicals, Costa Mesa, CA) at a density of 1.75 X 10^6 cells/dish in a volume of 3 ml of TNM-FH. After 1 h the cells were inoculated with 10 fold serial dilutions of supernatants that contained potential recombinant virus. Inoculum was removed after 1 h. The cell monolayer was overlaid with a 1.5% solution of agarose [seaplaque, FMC corp., Rockland, Maine] prepared in TNM-FH medium containing 100 ug/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (GIBCO BRL/Life Technologies, Gaithersburg, MD). The dishes were incubated for 5 days at 28^0 C in a humid environment. Blue plaques with occlusion (-) morphology were picked with Pasteur pipettes and placed in 1 ml aliquotes of TNM-FH which were maintained overnight at 4^0 C to

permit elution of the virus from the agarose. This procedure was repeated 3 times. The presence of PRV cDNA in the recombinants was confirmed by dot-blot hybridization.

Dot-blot hybridization

Essentially Sf-9 cells were seeded into 96-well microplates at a density of 2.0 X 10^4 cells/well in a volume of 100 ul of TNM-FH. Individual wells were then inoculated with 50 ul of 10 fold dilutions of media containing potential recombinant baculovirus and incubated at 28^0 C. Six days later all media was removed from each well, transferred to a duplicate microtiter plate, and stored at 4^0 C for future use. The cell monolayers in each well were then lysed with 200 ul of 0.5 N NaOH. The cell lysates were transferred to a dot blot microfiltration apparatus (Bio-Rad Laboratories, Richmond, CA.) which contained a Hybond N+ filter (Amersham International, Amersham, U.K.), that was previously rinsed with 0.5 N NaOH. The filter was subsequently washed with 200 ul of 0.5 N NaOH, removed from the apparatus and treated for 2 h at 65^0 C in a 3 fold concentration of sodium phosphate EDTA buffer (SSPE), pH 7.4, containing 5X Denhardt's solution, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA. Normal (1 X) SSPE consists of 0.18 M NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$ and 1 mM EDTA. The filter was subsequently exposed to the ^{32}P-labelled 800 bp PRV cDNA fragment (specific activity 8 X 10^7 dpm/ug) overnight in the same buffer. The filter was washed by immersion once in 2 X SSPE and then

in 0.1 X SSPE containing 0.1% SDS at 65° C for 30 min each. Washed filters were exposed to Kodak X-Omat XAR-5 film at -70° C for 2 h which permitted the confirmation of the presence of PRV cDNA in the cloned virus population.

Expression and identification of PRV recombinant protein

Spodoptera frugiperda 9 cells contained in 25 cm^2 flasks were singly infected with either wild-type, or recombinant baculovirus at an m.o.i. of 10, and incubated at 28° C for 72 h. Virus infected cells and non-infected control cells were then harvested and solubilized by adding 100 ul of Laemmli sample buffer (50 Mm Tris-Hcl pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 0.1% bromophenol blue) to each 1 ml volume of original cell suspension that contained approximately 6 X 10^5 cells. The treated cell preparations were boiled for 10 min and electrophoresed through a 10% sodium dodecyl sulfate polyacrylamide gel using a Mini-protean II dual slab system (Bio-Rad, Richmond, CA). The electrophoretically separated proteins were transferred from the gel to nitrocellulose membranes using a Mini trans-blot apparatus (Bio-Rad, Richmond, CA). The membranes were blocked by immersion for 2 to 4 h in TBS (Tris base 25 mM, NaCl 500 mM, pH 7.5) containing 3% gelatin. The membranes were stained with an optimal dilution of rabbit anti-PRV-nucleocapsid antibody. The stained membranes were washed twice with TBS containing 0.05% Tween 20 and subsequently stained with goat

anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories Inc, Gaithersburg, Maryland). Excess conjugate was removed by washing with TBS containing 0.05% Tween 20. Substrate degredation was revealed with the chromogen 4-chloro-1-naphthol.

RESULTS

Construction and isolation of a recombinant baculovirus

A recombinant baculovirus transfer vector containing cDNA that represented the truncated nuclocapsid gene in the correct orientation was constructed. Orientation was confirmed by analysis of restriction enzyme digests (Fig. 1). Recombinant baculovirus was produced by co-transfection of Sf-9 cells with wild-type AcNPV DNA and the recombinant transfer plasmid pBlue462A. Recombinant baculoviruses were cloned by plaque purification. The presence of PRV cDNA in these clones was confirmed by dot blot hybridization as illustrated in Fig. 2. A cloned recombinant baculovirus containing the PRV cDNA was designated Ac462A.

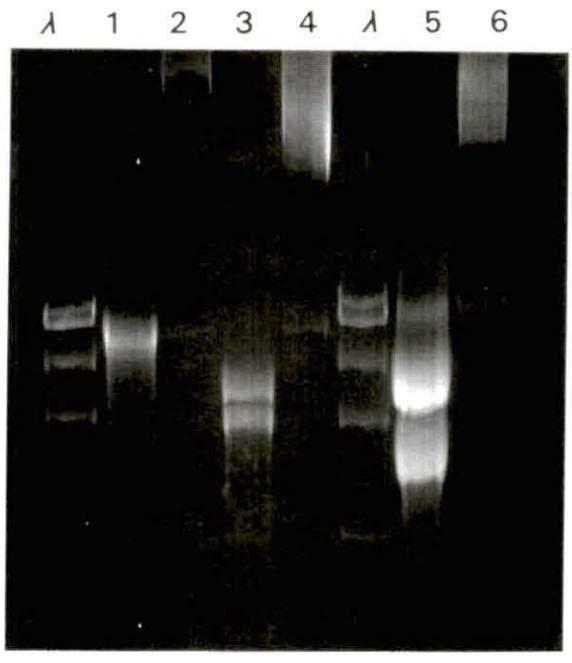
Expression of the truncated PRV nucleocapsid protein in Sf-9 cells

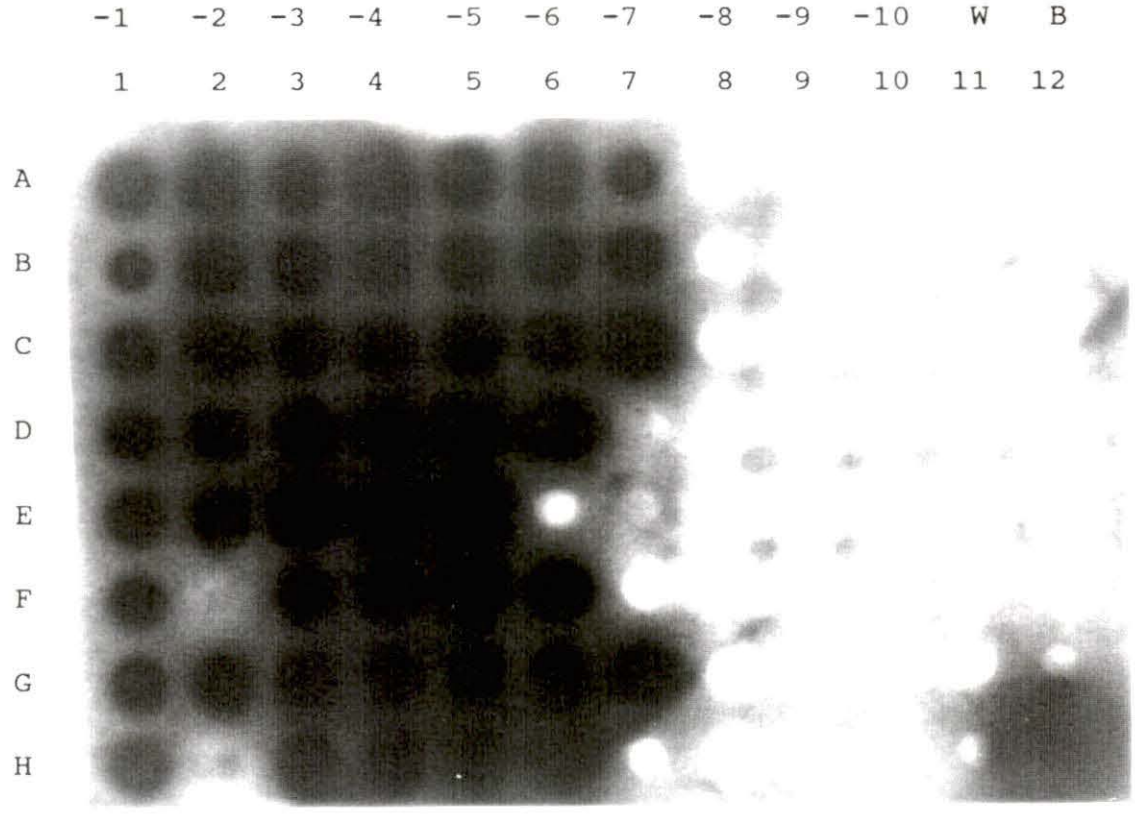
Spodoptera frugiperda 9 cells were singly infected at a m.o.i. of 10 with wild-type AcNPV and recombinant baculovirus Ac462A. Infected cells were harvested at 72 h post-infection. Lysates of mock, wild-type AcNPV and recombinant virus infected cells were analyzed by SDS-PAGE and Western immunoblot. The normal 29 kD polyhedrin protein was not produced by the

recombinant baculovirus as indicated by SDS PAGE analysis. However there was no indication of the presence of a band representing an additional low molecular weight protein in the lysate. Rabbit anti-PRV nucleocapsid protein serum also failed to demonstrate the presence of recombinant PRV protein in Western immunoblots.

Figure 1. Etidium bromide stained agarose gel demonstrating the correct orientation of the cDNA insert 462A in transfer plasmid pBlueBac. Lanes 1 and 2, XhoI digested and undigested pBlueBac plasmid DNA; lanes 3 and 4, XhoI digested and undigested recombinant plasmid DNA in the correct orientation; lanes 5 and 6, XhoI digested and undigested recombinant plasmid DNA in the incorrect orientation; lane λ, HindIII fragments of Lambda bacteriophage DNA

Figure 2. Dot blot analysis of DNA produced in Sf-9 cells infected with virus from blue recombinant plaques. Cells infected with dilutions of viral stock ranging from 10^{-1} to 10^{-10} were lysed with 200 ul of 0.5 N NaOH per well, transferred to a Hybond N+ filter and hybridized with the ^{32}P labelled 462A cDNA. Well H12 contains 462A cDNA and represents a positive control. Negative controls are represented by wells: E11, E12, F11, F12, G11, G12 and H11 which contain lysates of uninfected cells; A11, B11, C11 and D11 which contain lysates of wild type AcNPV infected cells; and A12, B12, C12 and D12 which contain lysates of cells infected with recombinant virus containing only the β-galactosidase foreign gene 462A


λ 1 2 3 4 λ 5 6


-1 -2 -3 -4 -5 -6 -7 -8 -9 -10 W B
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

DISCUSSION

Failure of the recombinant baculovirus Ac462A to express the PRV cDNA can be attributed to several reasons. First the cDNA represented a truncated gene which may not have contained an ATG start site. This is a likely possibility because nucleotide sequence analysis of 144 and 108 bp from the Sp6 and T7 primers respectively did not reveal any ATG-met translation start site (M. J. McGinley, personal communication). Secondly, the polyhedrin leader is very AT-rich. The insertion of a GC-rich PRV cDNA immediately downstream from the polyhedrin leader may affect the level of expression (8). Third, good expression of nonfusion foreign proteins with pBlueBac may require the presence of a short gene sequence before the real ATG translation start site (8).

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SUMMARY AND CONCLUSIONS

The objective of this study was to establish the baculovirus expression system for the production of recombinant pseudorabies virus (PRV) nucleocapsid proteins. The system was initially optimized by using a well characterized cDNA that represented the complete coding sequence of the 85 kD (VP1) and 75 kD (VP2) capsid proteins of Aleutian mink disease virus. Subsequently, attempts were made to express an 800 bp cDNA that represented a portion of the gene for the 32 kD major nucleocapsid protein of PRV.

The cDNA representing the ADV capsid proteins, VP1 and VP2 was cloned into the transfer vector, pVL1392 and inserted into the polyhedrin gene of *Autographa californica nuclear polyhedrosis* virus (AcNPV) downstream from its promoter. A recombinant virus, AcADV-1, was identified by dot blot hybridization and cloned by limiting dilutions. The cDNA was expressed by the recombinant virus in *Spodoptera frugiperda 9* cells. Fluorescence microscopy revealed that recombinant protein localized in the nucleus of the infected cell. These proteins were also shown by electron microscopy to self assemble in the form of empty ADV capsids. Western immunoblot analysis demonstrated that these proteins were immunologically recognized by AD infected mink.

Recombinant Ac462A containing the truncated gene of the 32 kD major nucleocapsid protein of PRV was constructed by first cloning the 800 bp cDNA

into the transfer vector pBlueBac. This vector was successfully used to produce recombinant Ac462A containing the cDNA in the correct orientation. The use of the blue gene made it possible to identify and clone recombinants with less effort and time than that required by the dot blot hybridization technique. However, the size of the plasmid containing the cDNA reduced transformation efficiency of *E.coli* (Hanahan, 1983).

Efforts to express the PRV cDNA were not successful. It is speculated that this failure was probably due to the absence of a start codon within the cDNA itself or its presence at a location not controlled by the promoter of the polyhedrin gene.

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APPENDIX. METHODOLOGIES USED IN THE BACULOVIRUS EXPRESSION SYSTEM

Preparation of insect cell culture medium

The following method is used for the preparation of Complete TNM-FH medium for the insect cell line: *Spodoptera frugiperda* clone no.9 (Sf-9).

Required Equipment:

- Sterile container e.g. 500 ml bottles
- 0.2 u desktop filter
- Vaccum pump

Required Reagents:

- Grace insect medium (Invitrogen)
- Supplement: Yeastolate and Lactalbumin hydrolysate (Difco)
- Fetal calf serum (Sigma)

Protocol:

1. Add 1.67 g of yeastolate, 1.67 g lactalbumin hyrolysate and 50 ml of fetal calf serum to 450 ml of Grace insect cell medium.
2. Filter sterilze with the 0.2 u desktop filter.
3. Store at 4^{0} C.

General Handling techniques of Sf-9 cell monolayers

The following techniques are used for the handling of the *Spodoptera frugiperda* 9 (Sf-9) cell line.

- Cell culture medium should be store at room temp before use.
- The viability of the cells can determined by adding 100 ul of a 0.4% solution of trypan blue to 1 ml of the cell suspensions. Stained cell suspension are examined microscopically. Dead cells will be stained blue. A viability of 98% is required.
- Avoid adding medium directly onto the cell monolayer. This may dislodge the cells.
- Sf-9 cells are sensitive to centrifugal force. Low speed centrifuge (1000 X g) for less than 10 min is recommended.
- 50 ug/ml of gentamycin and 2.5 ug/ml of amphotericin B are recommended for use in the insect cell cultures.

The subculture of Sf-9 cell monolayer culture

The following protocol is for the subculture of Sf-9 cells. *Spodoptera frugiperda* cells are seeded at an initial density of approximately 0.5 X 10^6 cells/ml and subcultured before the cell density reaches 4 X 10^6 cells/ml.

Protocol:

1. Remove the medium in the 25 cm^2 flask and add 5 ml of the fresh TNM-FH medium.
2. Dislodge the cell monolayer by gently pipeting medium across its surface.
3. Each 1 ml of the cell suspension (2-2.5 X 10^6 cells) can be transferred to a new 25 cm^2 flask containing 4 ml of fresh TNM-FH medium.
4. Cells are maintained at 28^0 C.
5. Healthy Sf-9 cells will have a doubling time of 18-22 h. Therefore they should be subcultured about 3 times a week.

Freezing and Storage of Sf-9 cell lines

The folowing procedure is for the storage of Sf-9 cells in liquid nitrogen. Logarithmic grow phase cells (1.0-2.5 X 10^6 cells/ml) with a viability of 98% and a double time of 18-22 h are required.

Required Equipment:

- 1.0 ml cryogenic tubes
- Styrofoam container
- -20° C freezer
- -70° C freezer
- Liquid nitrogen tank

Required Reagents:

- Fetal calf serum (Sigma)
- Dimethylsulfoxide (DMSO) (Sigma)

Protocol:

1. Dislodge the cell monolayer by gently pipeting medium across its surface. Transfer the cell suspension to a 15 ml centrifuge tube.
2. Centrifuged at 1,000 X g for 10 min.
3. Remove the supernatant and resuspend cells (1 X 10^7 cells) in 1 ml of medium containing 90% FCS and 10% DMSO.

4. Transfer the cell suspension into a cryogenic tube and place it into a stryrofoam container.
5. Store the container at at -20^0 C for 1 h, then at -80^0 C overnight.
6. Next day transfer the cryogenic tube into liquid nitrogen for storage.

Thawing of *Spodoptera frugiperda* (Sf-9) Cells

The following procedure describes the thawing of Sf-9 cells. Each vial of 1 ml aliquote containing approximately 10^7 cells (Invitrogen).

Required Equipment:

- 37° C water bath
- Ice bath
- 25 cm^2 tissue culture flask

Required Reagents:

- TNM-FH medium
- 70% ethanol
- 27° C incubator

Protocol:

1. Sf-9 cells are removed from liquid nitrogen and thawed rapidly by immersion in a 37° C water bath.
2. After the contents are thawed, clean the outside of the vial with 70% ethanol and transfer into an ice bath.
3. Add the cell suspension to a 25 cm^2 tissue culture flask and put 5 ml of cold (4° C) TNM-FH into the flask.
4. Let the cells adhere to the flask for 1 h at room temperature and then transfer to a 28° C incubator.
5. Two to 3 h later remove the medium from the flask and add fresh TNM-FH medium. Maintain the cells at 28° C incubator.

Transfection of Sf-9 cells

Recombinant baculovirus transfer plasmid and wild-type AcNPV DNA are transfected into Sf-9 cells by a modified calcium phosphate precipitation method (Burand et al., 1980). The recombination event may happen between the recombinant plasmid and the AcNPV genome within the insect cell. As a result a recombinant baculovirus containing the foreign gene will be produced.

Required Reagents:

- Transfection buffer (25 mM Hepes, pH 7.1, 140 mM NaCl, 125 mM $CaCl_2$)
- TNM-FH medium
- Grace medium with 10% fetal calf serum
- Antibiotics (50 ug/ml gentamycin and 2.5 ug/ml amphotericin B)

Protocol:

1. One ug of purified wild-type baculovirus (AcNPV) (Invitrogen) and 2 ug of the recombinant baculovirus transfer plasmid DNA are added to 0.75 ml of transfection buffer by very gently vortexing the solution.
2. Seed 2 X 10^6 Sf-9 cells into a 25 cm^2 flask and allow 30 min for cell attachment.

3. Add 0.75 ml of Grace medium containing 10% FCS and antibiotics to 2 X 10^6 of *S.frugiperda* cells, that have been seeded in a 25 cm^2 flask.
4. Add the DNA mixture to the flask and incubate at 28° C for 4 hours.
5. The cells are then rinsed with TNM-FH and incubated in fresh TNM-FH for 5 days at 28° C.
6. Use an inverted phase microscope to confirm the result of transfection. Positive signs include the presence of occlusions inside the nucleus, an increase in the size of the nucleus and cell lysis.
7. Five days later the transfection solution is collected and used either in the plaque assay or dot blot hybridization.

Plaque purification of recombinant baculovirus

Required Equipment:

- 60 X 15 mm culture dishes with grid (Lux Contour Dishes,ICN)
- Inverted microscope
- 50^0 C water bath
- Sterile Pasteur pipet

Required Reagents:

- Low melting point agarose (SeaPlaque, FMC corp.)
- Sterile distilled water
- Complete 1 X TNM-FH medium
- Fetal calf serum
- 200 X stock solution of 5-bromo-4-chlore-3-indolyl-B-D-galoactoside (X-gal) (20 mg/ml, in DMSO)
- Dimethylsulfoxide (DMSO)
- Gentamycin
- Amphotericin B

Protocol:

1. Sf-9 cells are seeded into 60 mm^2 dish at a density of 1.75 X 10^6 cells/dish in 3 ml of TNM-FH and allowed to settle for 30 min at 28^0 C.
2. Cell monolayers are inoculated with 1 ml aliquotes of TNM-FH containing virus and maintained at 28^0 C for 1 h.

3. During the incubation period, prepare the agarose overlay (5 ml/dish) as follows:

   - Add 3 g agarose per 100 ml of deionized distill water and autoclave for 15 min.

   - Place 1 X TNM-FH medium into a 50^0 C water bath.

   - Mix equal volumes of the 3% low melting point agarose solution and 1 X TNM-FH in a steriled container.

   - Add gentamycin (50 ug/ml), amphotericin B (2.5 ug/ml) and X-gal (100 ug/ml), to the melted agarose and maintain at 50^0 C water bath until used.

4. Remove inoculum from cell monolayer after the 1 hour incubation period and replace with 5 ml of agarose overlay. Maintain cultures for 5 days at 28^0 C in a humid environment.

5. Examine each dish at the end of 5 days for the presence of blue plaques. Blue plaques in the dishes are further examined for the presence of occlusions with an inverted phase microscope at 250-400 magnification.

6. Blue plaques without occlusions are picked with Pasteur pipettes and placed in 1 ml of TNM-FH medium overnight.

7. The following day the agarose plugs containing medium are gently emulsified and serially diluted 10 fold.

8. All recombinant clones are plaque purified at least 3 times.

Identification of recombinant baculovirus by dot-blot hybridization

Required Equipment:

- 96 well microtiter plate
- Hybond N + filter (Amersham)
- dot blot apparatus (Bio-Rad)
- Vaccum pump
- Sealable plastic bags
- Plastic bag sealer
- 65°C shaking water bath
- Autoradiography cassettes with intensifying screens
- Kodak X-Omat XAR-5 film

Required Reagents:

- 0.5 N NaOH.
- ^{32}P-labelled RNA or DNA probe
- 2 X SSPE, 0.1% SDS (1 X SSPE = 0.18 M NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA)
- 0.1 X SSPE, 0.1% SDS
- DNA hybridization buffer (3 X SSPE pH 7.4, 5 X Denhardts solution, 0.1% SDS, 0.1 mg/ml salmon sperm DNA).

Protocol:

1. Seed Sf-9 cells into a 96 well microtiter plate at a density of 2.0 X 10^4 cells/well in a volume of 100 ul TNM-FH. Allow cells to settle for 30 min before proceeding to step 2.
2. Supernates collected from the transfection flasks are serialy diluted 10 fold. The dilutions are used to infect Sf-9 cells.
3. Six days later the medium is removed from each well, transfered to a duplicate plate, and stored at 4°C to serve as future virus stock.
4. Add 200 ul of 0.5 N NaOH to each well to lyse the infected cell monolayer.
5. The cell lysates are transferred to the dot blot apparatus, containing a 0.5 N NaOH rinsed Hybond N+ filter.
6. After adding the lysates to the wells on the dot blot apparatus, vacuum is applied until all the lysates run down through the filter.
7. Wash each well with 200 ul of 0.5 N NaOH.
8. The filter is removed from the apparatus, and prehybridized as follows.
9. Rinse a plastic bag with 2 X SSPE and put the filter to be probed into the bag.
10. Add 50 ml of DNA prehybridization buffer into the bag, express excess air bubbles from the bag and seal the bag.
11. Prehybridize the filter at 65°C in a shaking water bath for 2 hours.

12. Boil the double-stranded DNA probe for 10 minutes. Then place it in an ice bath for 2 minutes.
13. After prehybridization remove the buffer from the bag.
14. Add 10 ml of the buffer that was first removed to a 15 ml polypropylene tube.
15. Add 5 ng of probe/ml containing at least 10^5-10^6 cpm into the above tube and pipette the contents back into the bag.
16. Hybridize at 65^0 C overnight.
17. After hybridization, remove the buffer to a 15 ml tube.
18. Rinse the filter in the bag at 65^{0}C, 2 times with 2X SSPE and then 2 times with 0.1X SSPE. Each wash lasts 30 min.
19. Discard the first rinse directly into a liquid radioactive waste container. Discard subsequent rinses into the sink.
20. Remove the filter from the wash bag and transfer it into a new plastic bag wetted with 2 X SSPE, 0.1% SDS.
21. Expose the filters at -70°C for 2 hours to X-ray film using a film cassette equipped with intensifying screens.
22. According to the result on the X-ray film, a supernate from one of high dilution postive wells in the duplicate plate can be selected for another round of dot-blot hybridization screening as described above.

23. After 3-4 rounds of screening, the supernate from one of the hybridization positive high dilution wells is used to infect Sf-9 cells.
24. The recombinant clone is then tested for its ability to express the foreign cDNA.