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Original Contributions

Formation of Infectious Pancreatic Necrosis Virus–like Particles Following Expression of Segment A by Recombinant Semliki Forest Virus

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Abstract: Segment A of the Sp strain (a Norwegian field isolate) of infectious pancreatic necrosis virus (IPNV) was amplified by reverse transcriptase polymerase chain reaction in two stages from RNA isolated from infected cells, and cloned into the Semliki Forest virus (SFV) expression vector pSFV1. Expression and correct processing of IPNV proteins was confirmed by transfection of RNA transcribed from this plasmid into BHK cells. This clone was then used to produce recombinant replication-defective SFV particles (rSFV) expressing the IPNV segment A. Immunofluorescence studies with conformation-dependent monoclonal antibodies to IPNV confirmed that the recombinant proteins produced after infection of the salmonid cell line CHSE-214 with such rSFV retain their antigenicity. Infection of the CHSE cells with the rSFV resulted in the formation of IPNV-like particles, which were similar in size and morphology to IPNV.

Key words: infectious pancreatic necrosis virus, segment A, Semliki Forest virus, vaccine, expression vector.

Introduction

The rapid expansion of the aquaculture industry in recent years has led to an increase in disease outbreaks. Over the last few years bacterial disease problems have been brought under control, largely owing to the use of a new generation of oil-adjuvant vaccines. Development of vaccines for viral diseases such as infectious pancreatic necrosis, viral hemorrhagic septicemia, and infectious hematopoietic necrosis has proved to be more difficult. Reports have estimated that infectious pancreatic necrosis virus (IPNV) is responsible for the loss of approximately 5% of the Atlantic salmon smolts transferred to sea (Melby et al., 1994).

IPNV is a double-stranded RNA virus with a bisegmented genome (a birnavirus). Segment A of the virus encodes a polyprotein of 108 kDa that is posttranslationally cleaved to yield VP2, VP3, and VP4 (Hudson et al., 1986; Duncan and Dobos, 1986). The major component of the viral capsid is VP2, with VP3 forming the inner capsid. The nonstructural protein VP4 encodes a viral protease that is responsible for the processing of the segment A polyprotein (Azad et al., 1987; Duncan et al., 1987; Jagadish et al., 1988; Magyar and Dobos, 1997). Segment B of the virus encodes an RNA-dependent RNA polymerase. VP2 is the most con-

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served protein encoded by the genome, although the protein does contain a central variable region where major neutralization epitopes are known to be located (Frost et al., 1995; Tarrab et al., 1995). Vaccine research has focused on the use of inactivated virus, live-attenuated strains, or recombinant subunit vaccines. The traditional vaccines based on live IPNV have disadvantages related to safety, as well as environmental and economic concerns, so current methods of vaccine development have turned to recombinant DNA technology, in the hope that this technology may provide efficient and inexpensive vaccines (Vaughan et al., 1998). Recombinant IPNV proteins produced in *Escherichia coli* have been used successfully in vaccination experiments (Manning and Leong, 1990; Frost et al., 1995; Christie, 1997), and DNA vaccines have been constructed for infectious hematopoietic septicemia virus and viral hemorrhagic septicemia virus (Anderson et al., 1996; Heppell et al., 1998; Lorenzen et al., 1998). With this aim in mind, we examined the potential of the Semliki Forest virus (SFV) vector system to express IPNV genes; this vector system has already been used in the construction of prototype vaccines for a number of animal viruses (Berglund et al., 1998; Atkins et al., 1999).

SFV is a positive-stranded RNA virus, and the vector is a transient RNA expression vector based on the infectious clone pSP6-SFV4 (Liljeström and Garoff, 1991). The subgenomic 26S RNA is a gene amplification mechanism that codes for the viral structural proteins only. In the vector, the 26S coding region from the infectious clone has been deleted and a polylinker sequence and cassette of translational stop codons have been inserted downstream from the 26S promoter. The nonstructural coding region is retained, as are the short 5′ and 3′ regions required for RNA replication. Recombinant RNA encoding heterologous antigens may be used directly as a vaccine, or recombinant particles may be produced by an in vivo packaging mechanism. Efficient packaging of RNA replicons can be achieved by cotransfection of cells with RNAs transcribed from helper plasmids and recombinant plasmids (Berglund et al., 1993). The helper plasmid retains the 5′ and 3′ regions required for replication, and the complete structural coding region, but lacks the signal in the nonstructural *nsP2* gene required for packaging. Therefore, only recombinant RNA is packaged into particles. Recent modifications of the helper packaging system have further increased the biological safety of the SFV expression system; the capsid and spike genes can be split into two separate RNA molecules, thereby reducing the possibility of fully infectious virus being formed by recombination (Smerdou and Liljeström, 1999).

Expression of heterologous antigens using SFV expression technology offers many advantages over conventional vaccination strategies, including high levels of heterologous antigen expression in the cytoplasm, low-level vector protein expression, induction of apoptosis in infected cells (Glasgow et al., 1997, 1998), and a high level of biosafety. Expression of heterologous antigens results in the generation of strong humoral and cellular immune responses with prolonged memory. Antigens that have been shown to elicit protection when expressed by recombinant SFV particles (rSFV) include influenza nucleoprotein, simian immunodeficiency virus gp160, and louping ill virus prME and NS1 proteins (Mossman et al., 1996; Berglund et al., 1999; Fleeton et al., 1999, 2000).

The SFV expression system is able to function in mammalian cells at 37°C, producing recombinant proteins that are recognized by conformation-dependent monoclonal antibodies (MAbs). In a previous study we showed that the SFV expression system also functioned in fish cell lines, again producing a recombinant protein that is correctly folded. However, expression in fish cell lines is temperature dependent (Phenix et al., 2000). In this study we show that infection of a fish cell line with recombinant particles expressing the IPNV segment A resulted in the formation of IPNV virus-like particles (VLPs).

Materials and Methods

Cells and Virus

The Sp strain of IPNV (IMP-986) was supplied by Central Veterinary Laboratory, Oslo, Norway. The salmonid cell line, CHSE-214, was used for viral growth and RNA isolation. The cells were maintained at 25°C, 3% CO_2 in Eagle's minimum essential medium (MEM) supplemented with 20 mM glutamine, 1% nonessential amino acids, 10 mM Hepes, 10% fetal calf serum (FCS), and 100 IU/ml penicillin, 100 μg/ml streptomycin (GMEM). Maintenance medium (MMEM) consisting of GMEM with a reduced FCS concentration of 2% was used for virus growth.

For detection of expression from recombinant SFV constructs, BHK-21 cells were grown in 75-cm^2 flasks or on 13-mm glass coverslips in 6-well plates. Cells were cultured at 37°C, 5% CO_2 in BHK-21 medium (Gibco) supplemented with 5% (vol/vol) newborn calf serum, 5% (vol/vol) tryptose phosphate broth, 0.1% (vol/vol) penicillin-streptomycin solution, and 2 mM glutamine solution.

Table 1. Oligonucleotide Primers Used in PCR Amplification of the Sp Strain of IPNV (Norwegian field isolate)

Primer name	Primer sequence: 5′-3′*
SPF1	ATACCCGGGGGTCTATATCAATRCAAG
SPR1	TATCCCGGGGTRGCGSCCTCCKGCKGC
3′F1	ACGGTTAACTACTGTCCACGCTGTTTCC
3′R1	GCGGTTAACGAAAGAGAGTAGTCGTTAC

*R indicates A or G; K is G or T; and S is G or C.

Virus Growth and RNA Isolation

CHSE cells were grown to confluence in 75-cm^2 flasks and inoculated with IPNV. Following adsorption of the virus for 1 hour at 15°C, 15 ml of fresh MMEM was added and the cells were incubated at 15°C until a cytopathic effect was observed. At approximately 4 days after infection, total RNA was extracted from IPNV-infected CHSE cells using the Genosys RNA isolator according to the protocol recommended by the supplier.

Reverse Transcriptase Polymerase Chain Reaction

Segment A of IPNV was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) in two stages. Single-stranded complementary DNA was synthesized from 400 ng of total RNA from infected cells, using 150 ng of HPLC-purified primers and 15 U of avian myeloblastosis virus reverse transcriptase, according to the protocol recommended by the supplier (Promega). Double-stranded RNA was denatured at 95°C for 10 minutes prior to reverse transcription. Standard PCR reaction mixtures contained 1.5 mM $MgCl_2$, 1× Thermo DNA poly buffer, 200 μM each of dATP, dCTP, dGTP, and dTTP, and 100 ng each of forward and reverse primers along with 2 μl of first-strand cDNA mixture. The reaction was performed in an automated DNA thermal cycler (Hybaid Omnigene) programmed for 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and polymerization at 72°C for 2 minutes. The final cycle included a polymerization step at 72°C for 10 minutes. Hot-start PCR was performed in all cases. In this procedure 2.5 U *Taq* polymerase (Promega) was added after denaturation and preheating of the sample to 94°C for 10 minutes. Sequences of the primer pairs, with 5′-*Xma*I or *Hpa*I restriction ends, are shown in Table 1.

Construction of SFV Expression Plasmids

Following insertion of the two 1.5-kb fragments generated by RT-PCR into pPCR-Script using standard cloning procedures, a full-length segment A was constructed using a unique *Kpn*I restriction site. A primer was designed to incorporate the native SFV ribosome binding sequence in IPNV clones. The sequence of primers used in a proofreading PCR is as follows, with the SFV ribosome binding sequence underlined and the VP2 start codon in boldface in the forward primer: SFVF1, 5′-GCG GGA TCC TAT AGCACC **ATG** AAC ACA AAC AAG GC-3′; SFVR1, 5′-GAA AGA GAG TAG TCG TTA C-3′.

A standard proofreading PCR reaction contained 1× cloned *Pfu* polymerase buffer (Stratagene), 200 μM each of dATP, dCTP, dGTP, and dTTP, and 250 ng each of the forward and reverse primers, along with 100 to 200 ng of linearized DNA template. The reaction was performed in an automated DNA thermal cycler programmed for 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and polymerization at 72°C for 7 minutes. The final cycle included a polymerization step at 72°C for 10 minutes. Hot-start PCR was performed as described above using 2.5 U of cloned *Pfu* polymerase. The reamplified product was cloned into the *Sma*I site of the SFV expression vector pSFV1, and recombinant clones in the correct orientation were confirmed by restriction digest and sequence analysis.

Analysis of IPNV Protein Expression from rSFV

BHK-21 cells were transfected with in vitro transcribed RNA by electroporation, as described previously (Liljeström and Garoff, 1991). Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed using prestained protein molecular weight standards (NEB). Thirty-microliter cell lysates were stacked in 4.5% acrylamide and separated in 12% acrylamide at 50 mA. A standard protocol was followed for immunoblotting (Towbin et al., 1979). Proteins were transferred to Hybond-P membrane (Amersham) using the Bio-Rad semidry transfer apparatus in accordance with the manufacturers' instructions. Following electroblotting, membranes were immersed in a 10% (wt/vol) blocking solution consisting of dried milk powder (Marvel) in TS buffer (10 mM Tris-HCl, pH 7.4, 0.9% [wt/vol] NaCl) and incubated at room temperature for 2 hours with gentle shaking. A 1:1000 dilution of IPNV rabbit polyclonal antisera or a 1:2000 dilution of a MAb to IPNV-VP3 in 10% (wt/vol) blocking solution was added to blots that were incubated at 4°C overnight. Blots were then washed twice for 10 minutes each in TS buffer, twice in TS buffer with 1% (vol/vol) Tween-20, and twice again with TS buffer. Blots

were finally incubated in a 1:1000 dilution of HRP conjugated antirabbit IgG (Dako) for the polyclonal primary antibody blot, or a 1:1000 dilution horseradish-peroxidase-conjugated antimouse IgG (Dako) for the MAb blot, in 10% (wt/vol) blocking solution for 2 hours at room temperature with gentle shaking. Membranes were washed as above and developed using the ECL-Plus Western blotting kit (Amersham) according to manufacturer's instructions.

Production of rSFV Particles

Recombinant RNA produced in vitro was packaged into particles using a two-helper RNA system (Smerdou and Liljeström, 1999). Briefly, BHK cells were cotransfected with recombinant RNA and RNA from the split helper plasmids pSFV-S2 and pSFV-CS219A. After 24 hours of incubation, tissue culture supernatants were harvested and cell debris was removed by centrifugation. Aliquots of particles were frozen on dry ice and stored at −70°C until use. To determine the titer of stocks, BHK-21 cells were infected with serial dilutions of the recombinant particles and indirect immunofluorescence was performed using anti-IPNV antibody.

Antigenic Characterization of rSFV–Segment A Expression in Cell Culture

CHSE cells were propagated on 13-mm glass coverslips in 35-mm tissue culture dishes and incubated at 20°C in closed containers in a 3% CO_2 atmosphere. Serial dilutions of virus in infection medium (MEM, 0.2% BSA, 20 mM Hepes, 2 mM glutamine) were used to infect 80% confluent monolayers. Virus particles were allowed to adsorb for 1 hour at 20°C with shaking every 15 minutes. The inoculum was then aspirated, and cells were overlaid with growth medium that had a reduced FCS concentration of 2%, and incubated at 25°C, 3% CO_2 for 48 hours. Coverslips were washed twice with phosphate-buffered saline before fixing in acetone for 10 minutes. The coverslips were subjected to indirect immunofluorescence with IPNV polyclonal antiserum and a selection of IPNV MAbs. The panel of Mabs used in this study was obtained from Intervet Ltd. and is described by Frost et al. (1995).

Electron Microscopy

CHSE cells were propagated on 2-cm^2 Melinex film coverslips (Agar Scientific) in 35-mm tissue culture dishes and infected with 1.5×10^6 rSFV particles per well as described above. Cells were incubated until a cytopathic effect was visible (approximately 4 days). Cells were fixed in warm 2.5% glutaraldehyde in 0.09 M cacodylate buffer, pH 7.2, containing 3 mM $CaCl_2$. Cells were incubated in the fixative for 30 minutes, after which the fixative was aspirated and replaced with 0.1 M cacodylate buffer, pH 7.2, containing 3 mM $CaCl_2$. Uninfected CHSE cells were processed in the same manner. Fixed cultures were washed 6 times in 0.1 M cacodylate buffer before staining with 2% osmium tetroxide in 0.1 M cacodylate buffer for 30 minutes. Cells were dehydrated by successive incubations in 10%, 30%, 50%, 75%, and 95% ethanol solutions, each for 10 minutes. Coverslips were then incubated overnight in 100% ethanol. The ethanol was replaced with propylene oxide and incubated for 15 minutes followed by a second incubation step for 30 minutes. A solution of 50% agar 100 epoxy resin, containing DDSA hardener, MNA hardener, and BDMA accelerator, in propylene oxide was prepared and used to cover the cells for 2 hours. This was subsequently replaced with 100% resin for 2 hours. Resin was placed in an embedding mold onto which the Melinex coverslip was placed face down. The resin was allowed to polymerize at 60°C for 24 hours, after which the Melinex film was peeled from the polymerized resin. Another layer of resin was added to the monolayer of cells in the embedding mold and allowed to polymerize for 24 hours, thereby forming a sandwich of the monolayer in the resin. Ultrathin sections cut from the embedded monolayers were placed on copper grids and stained with 0.5% aqueous uranyl acetate and Reynolds lead citrate. Stained grids were studied using a Hitachi H7000 electron microscope.

Results

Expression of IPNV Antigens

The IPNV sequence encoding segment A was cloned into the SFV expression vector, pSFV1, at the *Sma*I site. SFV–segment A RNA was transfected into BHK cells, and the proteins expressed were examined by Western immunoblotting with IPNV polyclonal antiserum and an IPNV MAb to VP3. Expression of a number of IPNV proteins was detected in cells transfected with rSFV RNA (Figure 1, a). The proteins expressed were VP2 (50 kDa), VP3 (31.6 kDa), and VP4 (26.6 kDa). Cells electroporated with the SFV–segment A clone were also found to express proteins at 86.6 and 36.6 kDa. Labeling with the MAb directed against VP3

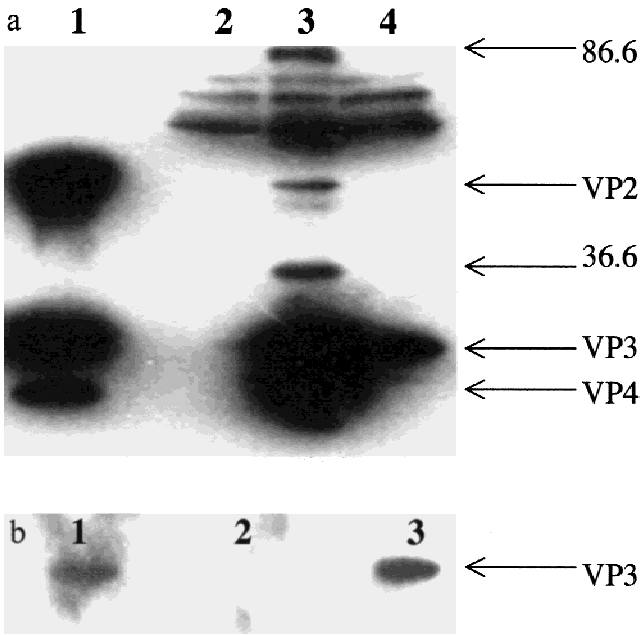

Figure 1. Western immunoblotting of SFV expression constructs. Cell lysates from BHK cells electroporated with SFV expression constructs were run on sodium dodecylsulfate polyacrylamide gels. Gels were subsequently blotted onto PVDF membrane, and bound proteins were detected using chemiluminescence with IPNV-specific antibodies and a horseradish-peroxidase-conjugated secondary antibody. In all panels IPNV proteins are indicated with arrows. **a:** Western immunoblot with IPNV polyclonal antisera. Lane 1, IPNV-infected CHSE cells; lane 2, pSFV1-VP2 at 24 hours after electroporation (nonexpressing clone); lane 3, pSFV1–segment A at 24 hours after electroporation; lane 4, untransfected cells (negative control). **b:** Western immunoblot with IPNV MAb to VP3. Lane 1, IPNV-infected CHSE cells; lane 2, untransfected cells; lane 3, pSFV1–segment A at 24 hours after electroporation.

resulted in the detection of one protein at 31 kDa with disrupted purified virus (not shown), IPNV-infected CHSE cells, and cells transfected by electroporation with rSFV RNA (Figure 1, b).

Antigenic Characterization of rSFV–Segment A Expression in Cell Culture

The antigenicity of the pSFV1 construct was tested in CHSE cells. IPNV polyclonal antiserum and IPNV-C12 (anti-VP3) gave rise to general cytoplasmic staining with dense nuclear staining (Figure 2, a and b). The cells infected with recombinant particles were also found to react with the conformation-dependent neutralizing MAbs B9, F2, and H8G2 (Figure 2, c–e).

Detection of IPNV Virus-like Particles

Having established that expression of IPNV segment A in mammalian and fish cells leads to the accumulation of the structural proteins VP2 and VP3, it was important to analyze whether they would assemble to form virus-like particles (VLPs). To test this hypothesis, CHSE-214 cells were infected with recombinant SFV particles expressing IPNV segment A and maintained for 5 days before fixing and processing for thin-section electron microscopy. As shown in Figure 3, the cytoplasm of infected cells contained membrane-bound structures with accumulations of small isometric particles. The particles have a morphology and size (diameter of 60 nm) that strongly resemble the appearance of particles found within the cytoplasm of IPNV-infected cells.

Discussion

In this study we have demonstrated the formation of virus-like particles by expression of the IPNV segment A using a vector. The cDNA encoding the entire segment A of IPNV was cloned into the SFV expression vector pSFV1. Expression in BHK-21 cells resulted in the correct processing of the polyprotein encoded by segment A. In addition to IPNV-specific proteins that comigrated with VP2, VP3, and VP4, proteins were produced with molecular weights of 86.6 and 37 kDa. In vitro translation of segment A RNA also resulted in the production of a similar protein (Duncan et al., 1987). This protein is believed to be an amino-truncated polyprotein produced by internal initiation, probably at Met-5. VP4-mediated cleavage of this protein results in the production of a 37-kDa protein, an amino-truncated VP2 polypeptide. Recombinant baculovirus expressing IPNV VP2 or segment A was also found to produce a protein of this size (Magyar and Dobos, 1994). Internal initiation of translation of IPNV Sp RNA, in vitro and in infected cells, is also responsible for the production of a 38-kDa protein. This protein is an amino-truncated VP4-VP3 polypeptide.

In infected cells, the polyprotein is rapidly cleaved into pVP2, VP4, and VP3. Further processing of pVP2 to VP2 and VP3 to VP3a is slow and most likely results from host cell preteases rather than the further proteolytic action of the VP4 protease. Although cloning of IPNV segment A into baculovirus expression vectors resulted in the correct processing of the polyprotein to pVP2, VP4, and VP3, neither VP2 nor VP3a was detected (Magyar and Dobos,

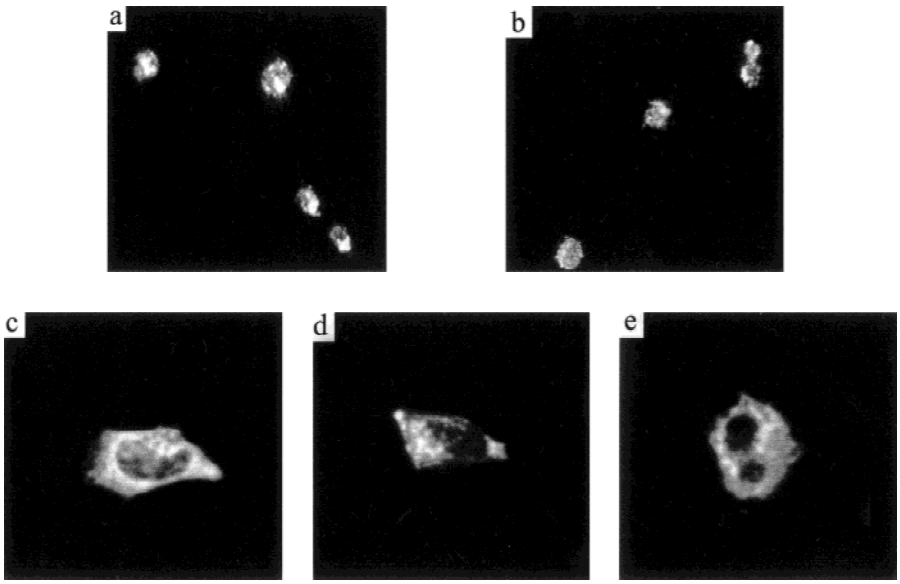

Figure 2. Immunofluorescent staining of CHSE cells infected with recombinant SFV particles expressing IPNV segment A. CHSE cells were grown to 80% confluence on 13-mm coverslips in 35-mm dishes and infected with recombinant SFV particles expressing the IPNV segment A; 72 hours after infection cells were fixed and incubated with IPNV polyclonal antiserum or IPNV MAbs. Samples were subsequently incubated with FITC-conjugated pig antirabbit Ig or FITC-conjugated pig antimouse Ig and viewed by epifluorescence. **a:** IPNV polyclonal antiserum. **b:** MAb C12–anti-VP3. **c:** MAb B9–anti-VP2. **d:** MAb F2–anti-VP2. **e:** MAb H8G2–anti-VP2.

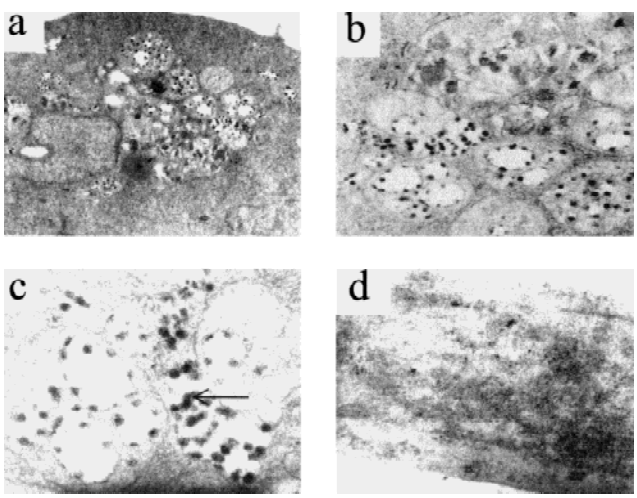

Figure 3. Electron microscopy of CHSE cells infected with recombinant SFV particles expressing the IPNV segment A. Ultrathin sections of CHSE cells were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy. The cytoplasm of infected cells shows vacuoles containing virus-like particles 60 nm in size (arrow). Original magnifications for infected cells, ×20,000 (**a**), ×40,000 (**b**), ×100,000 (**c**); for uninfected cells, ×30,000 (**d**).

1994). Expression of IPNV segment A by rSFV vectors appears to result in the correct processing of pVP2 to VP2. Processing of VP3 to VP3a also appears to occur efficiently.

Immunofluorescence studies with conformation-dependent Mabs to IPNV confirmed that the recombinant proteins produced after infection of CHSE cells with rSFV particles expressing IPNV segment A retain their antigenicity. Expression of the IPNV polyprotein by rSFV particles leads to the formation of IPNV VLPs. The VLPs accumulate in the cytoplasm of infected CHSE cells, with morphology and size similar to those reported for IPNV. As cells infected with rSFV particles expressing IPNV segment A react with conformation-dependent Mabs, it is likely that the VLPs produced elicit an immune response similar to that induced by purified virus. Purified VLPs may therefore be used as a candidate recombinant vaccine against IPNV. VLPs of purified infectious bursal disease virus (IBDV, a chicken birnavirus), from cells infected with a recombinant vaccinia virus vector expressing segment A, react strongly with anti-IBDV antiserum and are currently being assessed for vaccine potential (Fernández-Arias et al., 1998).

Our previous study indicated that the SFV expression

system functioned well in mammalian cells at 37°C and in fish cells at 25°C. The absence of expression in CHSE cells at lower temperatures indicated that the translation of SFV RNA or its replication is inhibited (Phenix et al., 2000). The system may, however, be suited for evaluation as a vaccine in warmwater fish, such as carp or catfish, which are economically important farmed species. Diseases such as grass carp hemorrhage disease (GCHD) or channel catfish virus disease (CCVD) normally occur at temperatures between 25° and 34°C. Grass carp are cultured extensively in China and are responsible for 20% of the country's freshwater fish production. Outbreaks of GCHD severely affect production and can result in production losses of up to 30%. Although inactivated and attenuated commercial vaccines for this virus are available in China, the disease remains a problem for this country's aquaculture industry. Channel catfish is the main aquaculture species in the United States. CCVD is caused by a herpesvirus and occurs mainly in fry and fingerlings. Attenuated virus vaccines for this disease have only had laboratory trials. There have been no reports of recombinant vaccine development for either of these economically important diseases (Dixon, 1997). Further studies on the ability of SFV expression systems to deliver recombinant antigens to fish under warmwater conditions may provide a solution to these problems.

Although the SFV system may not be suitable as a vaccine vector for salmonids in its present form because of its temperature-dependent expression, alphaviruses infecting salmonids have recently been isolated, and these may be suitable for development into vaccine vectors. One such vector undergoing development is based on salmon pancreas disease virus (SPDV). This is an atypical alphavirus that grows best at 15°C (Weston et al., 1999; Welsh et al., 2000). The similarity between the genomes of SPDV and SFV makes it likely that SPDV can be developed as an alphavirus vector similar to that based on SFV.

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