Mar. Biotechnol. 2, 27–37, 2000
DOI: 10.1007/s101269900005

MARINE BIOTECHNOLOGY

Cell Culture Evaluation of the Semliki Forest Virus Expression System As a Novel Approach for Antigen Delivery and Expression in Fish

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Abstract: Heterologous gene expression by Semliki Forest virus (SFV) expression vectors was investigated in fish cell culture. Experiments performed using an infectious strain of SFV, replication-defective SFV particles, and recombinant SFV RNA constructs encoding the *Escherchia coli* LacZ or firefly luciferase reporter genes indicated that levels of SFV-mediated expression in fish cells were dependent on cell type and temperature. Maximal expression levels were observed in the two salmonid-derived cell lines CHSE-214 and F95/9 at 25°C and 20°C. As the temperature was lowered to 15°C or below, levels of reporter gene expression were reduced up to 1000-fold, indicating that the SFV replication complex functioned inefficiently at low temperatures. The ability of SFV expression systems to function in fish cells was further investigated by analyzing the expression of the protective VP2 antigen of infectious pancreatic necrosis virus (IPNV) from the various constructs, including a novel DNA-based SFV plasmid. The VP2 protein produced in CHSE-214 and F95/9 cells transfected or infected with the recombinant SFV-IPNV VP2 constructs appeared to be synthesized in an antigenically correct form, as evidenced by the ability to react with several conformation-dependent IPNV-specific monoclonal antibodies. Whether the temperature-restricted replication and expression displayed by SFV-based constructs in fish cell culture also occurs in vivo remains to be determined.

Key words: IPNV, VP2, expression, recombinant DNA, vaccine

INTRODUCTION

There is a critical need for the production of safe, cost-effective fish vaccines for use in acquaculture species. The suitability of methodologies based on recombinant DNA (rDNA) for the development of vaccines against viral diseases of farmed fish is currently being investigated (Leong and Fryer, 1993; Munn, 1994; Leong, 1997; Vaughan et al., 1998). For example, overexpression of the protective virus protein in *Escherichia coli* has been used to produce a commercially available infectious pancreatic necrosis (IPN) vaccine (Norvax Protect-IPN, Intervet Norbio, Bergen, Norway; Frost and Ness, 1997). A more recent approach, and one that has received much interest in the mammalian vaccine field, is that of DNA vaccines. DNA vaccines behave like live viral vaccines but preclude the risk of inadvertent

Received January 29; accepted June 29, 1999.

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infection, reactivation, or conversion to increased virulence. The potential use of DNA vaccines in aquaculture was recently demonstrated by intramuscular inoculation of trout with experimental DNA vaccines (Anderson et al., 1996; Heppell et al., 1998; Lorenzen et al., 1998). Plasmid DNA encoding the G and N proteins of infectious hematopoietic necrosis virus (IHNV) or viral hemorrhagic septicemia virus (VHSV) under the control of the cytomegalovirus (CMV) promoter were used to immunize rainbow trout. In both cases, fish inoculated with the plasmid encoding the G protein, either alone or in combination with the plasmid encoding the N protein, developed virus-neutralizing antibody responses and a strong protective immunity against severe disease challenge.

The development of a rDNA vaccine depends on correct identification of the viral protein inducing protective immunity and on cloning and expression of the gene encoding the protective antigen. Virus-derived eukaryotic expression vectors (e.g., for adenovirus, vaccinia virus and alphavirus) are available for investigating the processing and antigenic properties of mammalian and avian virus proteins synthesized within appropriate cell cultures. Similar expression systems to study the proteins specified by salmonid fish viruses within fish cells at temperatures supportive of virus growth are lacking. Use of the baculovirus expression system has been described for expression of the G glycoprotein of VHSV in insect cells (Lecocq-Xhonneux et al., 1994; Lorenzen et al., 1994); however, problems such as incorrect protein processing and folding at elevated temperatures were encountered.

The eukaryotic expression system based on the alphavirus Semliki Forest virus (SFV) has been widely used to study the processing, antigenicity, and biological properties of proteins and, more recently, as a basis for vaccine development (Mossman et al., 1996; Berglund et al., 1997, 1998, 1999; Tubulekas et al., 1997). The antigen is synthesised in vivo and processed similarly to an antigen synthesized by an infectious virus. Correct presentation to the immune system and induction of long-lasting immunologic memory protect against subsequent antigenic challenge. Alphaviruses such as SFV display a broad host range, infecting mammalian, avian, insect, reptilian, and fish cell cultures (Griffin, 1986; Clarke et al., 1973; Leake et al., 1977; L'Heritier, 1997). The objective of this study was to investigate the suitability of SFV expression for use in fish. Recombinant SFV RNA produced in vitro can be used directly for transfection of cells and the production of replication-defective particles when transfected with a helper virus construct. Such particles comprise the structural proteins of SFV encapsidating RNA that encodes the SFV replicase and heterologous proteins only (Liljestrom and Garoff, 1991; Berglund et al., 1993). The broad host range and high expression levels of the SFV expression system provide vector-associated replication and expression events in the cytoplasm without a DNA intermediate, obviating many of the problems encountered by conventional nuclear DNA-based expression such as shortage of transcription factors, messenger RNA splicing, capping, and mRNA transport (Liljestrom and Garoff, 1991). More recently, SFV vector design has extended to a DNA-based vector, hereafter referred to as the layered SFV DNA construct, in which the SFV replicon has been placed under the control of the CMV promoter (Berglund et al., 1998). Unlike conventional DNA vectors, the CMV promoter drives the synthesis of a recombinant SFV RNA transcript rather than the expression of the antigen-encoding genes. Transfection of cells with this DNA results in transcription of SFV RNA and high-level expression of the cloned heterologous gene.

The aim of this work was to investigate the suitability of SFV vectors to function as eukaryotic expression systems in fish cells at temperatures supportive of fish virus growth with a view to examining their vaccine potential in fish.

Materials and Methods

Expression Constructs

SFV Reporter Gene Constructs

Plasmid pSFV-lacZ, which was used for the production of SFV-lacZ RNA, has previously been described (Liljestrom and Garoff, 1991). The layered SFV DNA expression vector pBKTSFV-lacZ was constructed by encompassing the sequences encoding the SFV replicon under the transcriptional control of the CMV immediate early promoter as described by Berglund et al. (1998). A recombinant SFV plasmid expressing the firefly luciferase (*luc*) reporter gene was constructed by amplifying the luciferase coding sequence from pGl2 (Promega, Dublin, Ireland) using primers 5′-GATATAGGATCCTATTATAGCACCATGGAAGACGCCAAAAACATA-3′ and 5′-GATATAGGATCCTTACAATTTGGACTTTCCGCC-3′ and subcloning into the *Bam*H1 site of pSFV1.

SFV-IPNV VP2 Expression Constructs

A full-length (1.5-kb) complementary DNA fragment encoding the protective VP2 antigen of a Norwegian field

isolate (Sp-like serotype) of IPNV was produced by reverse transcription–polymerase chain reaction (RT-PCR) and inserted into pBluescript (Stratagene, Cambridge, U.K.). A set of primers based on published Sp VP2 sequences (Sp F1, 5′-GTACCCGGGGGTCTATATCAAT(AG)CAAG-3′; and SpR1, 5′-ATTCCCGGGGT(AG)GCG(GC)CCTCC(GT)GC(GT)GC-3′) were used to amplify the 1.5-kb fragment by RT-PCR using total RNA extracted from IPNV-infected CHSE-214 cells as template, prepared as described by Heppell et al. (1992). The 1.5-kb PCR fragment was inserted into pBluescript using the T-vector cloning procedure (Stratagene) and sequenced, and the VP2 gene was reamplified from clone pBLVP26 with the proofreading enzyme Pfu (Stratagene) using primers SFV F1, 5′-ATACCCGGGTATTATAGCACCATGAACACAAACAAGGCA-3′, and SpR1. The SFV forward primer was designed to incorporate the native SFV ribosome binding sequence. Reamplified products were cloned into the *Sma*1 site of the SFV expression vector SFV1, and recombinant clones in the correct orientation were confirmed by restriction digest and sequence analysis (pSFV-VP2 clones). In addition, the 1.5-kb fragment was subcloned into the *Bam*H1 site of the layered SFV DNA vector pBKSFV5 using standard procedures to generate pSFVL-VP2 clones.

Naked Nucleic Acid Constructs

The DNA plasmid pCMV-β (Clontech) encoding the *Escherichia coli lacZ* gene was used as a control for DNA transfections.

CMV-IPNV VP2 DNA Constructs

The VP2 gene was reamplified from pBLVP26 using Pfu polymerase (Stratagene) and the primers 5′-GATCAAGCTTGCCGCCACCATGAACACAAACAAGGCAACCGCA-3′ and 5′-GCTATCTAGATTAGTGGTACCTTCCGCCTGCTGCGTTGGTCTTGGTGAG-3′ and cloned into the *Hin*dIII and *Xba*I sites of the eukaryotic expression vector pcDNA3 (Invitrogen, The Netherlands) under the control of the CMV promoter. Recombinant clones were confirmed by restriction and sequence analysis.

All plasmids were prepared by ion-exchange chromatography using Qiagen DNA-prep columns as described by the manufacturer (Qiagen, Crawley, U.K.).

Cells and Virus

The salmonid cell lines, chinook salmon embryo (CHSE-214; ATCC:CRL-168), Atlantic salmon (AS: Ref 02-776; ICN Pharmaceuticals Inc., Oxfordshire, U.K.), and F95/9 (derived from a diagnostic salmon obtained from the Fish Unit, Veterinary Sciences Division, Department of Agriculture for Northern Ireland), and the nonsalmonid cell lines, bluegill fry (BF-2; ATCC:CCL-91) and common carp epithelioma (EPC; EATCC:93120820), were maintained in Eagle's minimal essential medium (MEM) supplemented with 20 mM glutamine, 1% nonessential amino acid, 10 mM Hepes, 10% fetal calf serum, (FCS) and, 100 IU penicillin ml^{-1}, 100 μg ml^{-1} streptomycin (GMEM). Cells were propagated at 20°C for CHSE-214 and F95/9 cells and at 25°C for AS, BF-2, and EPC cells in 3% CO_2 atmosphere. Maintenance medium (MMEM) consisting of GMEM with a reduced FCS concentration of 2% was used during virus growth. Fish cells (approx. 90% confluent) were inoculated with an infectious strain of SFV (SFV4) in 25-cm^2 flasks or on glass coverslips. Following adsorption of the virus for 1 hour at a range of temperatures (25°C, 20°C, 15°C, and 10°C), the inoculum was removed and the cells were washed three times with phosphate-buffered saline (PBS) before replacement with MMEM. Cells were incubated at the above range of temperatures for up to 10 days postinoculation. Flasks were harvested at several timepoints postinoculation, and the virus titer was determined by 50% end-point titration in BHK cells. Inoculated coverslips were harvested at similar timepoints and subjected to immunocytochemical analysis for the detection of SFV antigens.

Titration of SFV4 and transfection with nucleic acid and SFV-based constructs was carried out in baby hamster kidney cells (BHK-21; ATCC:CCL-10), a cell line routinely used for propagation of SFV. Cells were maintained in BHK medium (Glasgow minimal essential medium [GMEM]; Life Technologies, Paisley, Scotland) supplemented with 10% tryptose phosphate broth, 10% FCS, 2 mM glutamine, 10 mM Hepes, and 100 IU penicillin ml^{-1}, 100 μg ml^{-1} streptomycin.

Immunocytochemical Detection of SFV Antigens in Fish Cells

Immunocytochemical detection of SFV-specific antigens was performed according to a standard streptavidin-biotin peroxidase method (Zymed, Cambridge, U.K.) using SFV polyclonal antiserum made available in the laboratory.

Preparation of Recombinant SFV RNA and Replication-Defective SFV Particles

RNA for transfection was prepared by in vitro transcription of *Spe*1-linearized preparations of pSFV-lacZ and pSFV-

IPNV VP2 DNA according to a standard protocol described elsewhere (Liljestrom and Garoff, 1991). Replication-defective SFV-VP2 and SFV-luc particles were made by co-transfecting BHK cells with equal amounts (approx. 25 μg) of SFV-VP2 or SFV-luc RNA and SFV-Helper 2 RNA using the method described by Berglund et al. (1993). Clarified recombinant virus was activated with chymotrypsin and titrated in BHK cells by indirect immunofluorescence (IIF) using the IPNV polyclonal antiserum, essentially as described by Berglund et al. (1993).

Transfection of Animal and Fish Cell Lines with Recombinant SFV Nucleic Acid Constructs

CHSE-214 and F95/9 cells were transfected with recombinant SFV RNA by electroporation essentially as described for transfection of BHK cells (Liljestrom and Garoff, 1991), except that cells were pulsed twice at 0.65 Kv, 25 μF (1.625 V/cm). Cells were incubated at the required temperatures (25°C, 20°C, 15°C, and 10°C) in a sealed container with 3% CO_2 and harvested at various times posttransfection.

BHK cells were transfected with pSFVL-VP2 DNA using Lipofectamine (Life Technologies) according to the method described by Berglund et al. (1998). Transfection of CHSE-214 and F95/9 cells was similarly performed except that the cells were incubated with the DNA-lipid complexes for 4 hours at 20°C with gentle agitation every 30 minutes. Cells were harvested at 24 to 96 hours posttransfection and tested for the expression of IPNV VP2 by IIF. Cells transfected with the SFV-based reporter DNA plasmid pBKTSFV-lacZ and mock-transfected cells were included as positive and negative controls, respectively.

Infection of Animal and Fish Cells with Recombinant, Replication-Defective SFV Particles

Replication-defective SFV-VP2 and SFV-luc particles were activated with chymotrypsin and used at a multiplicity of infection of 1. Virus was diluted in MEM/0.1% BSA and adsorbed onto coverslips of BHK cells for 1 hour at 37°C and onto CHSE-214 and F95/9 cells for 1 hour at 20°C. Following removal of the inoculum, cells were fed again with GMEM and incubated at the required temperature for 24 to 72 hours before being harvested and tested for the expression of IPNV VP2 by IIF.

Detection of LacZ Expression in Fish Cells Transfected with SFV-LacZ Reporter Constructs

Expression of lacZ in transfected cells, as determined by β-galactosidase (β-gal) activity, was assayed in situ using a commercial β-gal staining kit (Roche Diagnostics Ltd, Lewes, U.K.) according to the manufacturer's instructions. Transfection efficiencies, measured as the percentage of cells expressing active β-gal, were calculated by counting the average number of stained cells from 10 fields in triplicate wells and expressing this as a percentage of the total number of cells.

Detection of IPNV VP2 in Animal and Fish Cells Transfected or Infected with SFV-VP2 Constructs

Coverslips of transfected or infected cells were harvested at a given time point by fixing in acetone for 10 minutes. Cells were reacted with IPNV polyclonal and the IPNV VP2 neutralizing monoclonal antibodies H8G2, F2, and B9, made available in the laboratory, for 1 hour at 37°C, washed in PBS for 10 minutes, and then incubated for a further 1 hour at 37°C with the appropriate fluorescein isothyacyanate–conjugated antispecies antibody (Sigma). Positive control coverslips of IPNV-infected CHSE-214 cells reacted with the above antisera were also included.

SDS-PAGE and Western Blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Separated proteins were transferred to nitrocellulose, and immunoblotting was performed by standard procedures using the IPNV polyclonal antiserum at 1:250 dilution.

Metabolic Labeling and Immunoprecipitation of Proteins

Metabolic labeling of BHK cells transfected with recombinant SFV RNA was performed 14 hours postelectroporation with SFV-VP2 RNA, essentially as described by Liljestrom and Garoff (1994). Immunoprecipitation of the labeled proteins with IPNV polyclonal antiserum was performed as described elsewhere (Liljestrom and Garoff, 1994). Samples were analyzed on a 12.5% SDS-PAGE gel. BHK cells transfected with SFV-lacZ RNA were similarly processed and included as a negative control.

Luciferase Assays

Luciferase activity was measured in extracts prepared from adhered cells and cells in culture supernatant. Adhered cells were rinsed twice with PBS before addition of 200 μl of

luciferase lysis buffer (Roche Diagnostics Ltd). Cells were scraped off the plate, transferred to a microfuge tube, and incubated at room temperature for 15 minutes. Cellular debris was removed by spinning at maximum speed for 30 seconds. Supernatants were collected, and 20-μl aliquots were mixed with 100 μl of luciferase assay reagent (Roche Diagnostics Ltd) as described by the manufacturer. Light emission was measured for 20 seconds with a luminometer (Turner Designs, Dublin, Ireland) and expressed as relative light units (RLU) per second. Cells in the culture supernatant were harvested by centrifugation at 1500 *g* for 10 minutes, lysed in 200 μl of lysis buffer, and analyzed for luciferase activity as described above.

Results

Growth of Infectious SFV4 in Fish Cell Culture

Replication and expression of SFV was restricted by fish cell type and temperature (Table 1). SFV4 grew best in the salmonid-derived cell line F95/9 at 25°C. This was demonstrated by the development of an extensive cytopathic effect, an increase in virus titer, and expression of SFV-specific antigen. There was no evidence of productive virus replication in this cell line when the temperature was lowered to 20°C or below, although SFV antigen expression was detected to reduced levels. SFV replication and antigen expression were also detected in CHSE-214 cells at 25°C but not at 20°C and below. Low levels of SFV antigen expression were observed in AS cells at 25°C, and no virus growth or antigen expression was detected in the two nonsalmonid cell lines BF2 and EPC.

Table 1. Growth of SFV4 in Fish Cell Culture at Different Temperatures*

Cell type	Incubation temperature (°C)	CPE†	Infectivity ($TCID_{50}$/0.5 ml) in BHK cells‡	SFV antigen expression§
F95/9	25	+++	10^7	++++
	20	−	−	+++
	15	−	−	+
	10	−	−	−
CHSE-214	25	+++	10^7	++++
	20	−	−	−
	15	−	−	−
	10	−	−	−
AS	25	−	−	++
	20	−	−	−
	15	−	−	−
	10	−	−	−
BF-2	25	−	−	−
	20	−	−	−
EPC	25	−	−	−
	20	−	−	−

*Salmonid (CHSE-214, AS, and F95/9) and non salmonid (BF2 and EPC) cell lines were infected with SFV4 at low and high multiplicities of infection and incubated at a range of temperatures. Virus replication and antigen expression was determined on the basis of development of cytopathic effect (CPE) as characterized by swollen vacuolated cells and cell stranding.
†Cytopathic effect: +++, extensive; −, none.
‡Minus sign (−) indicates no virus titration.
§Immunocytochemical detection of SFV-specific antigens using a SFV polyclonal antiserum; ++++, > 70% positive; +++, 10%–20% positive; ++, 1%–5% positive; +, < 1% positive.

Expression of Luciferase in CHSE-214 Cells Infected with Replication-Defective SFV-luc Particles

A similar trend of temperature-restricted expression as that described for infectious SFV4 was displayed by SFV-luc particles following infection of CHSE-214 cells. Luciferase activity reached a maximum value of 1×10^5 RLU/s at an incubation temperature of 25°C but decreased significantly to values of 1×10^2 RLU/s or lower as the temperature was reduced to 20°C (Figure 1), providing further evidence to suggest that replication of SFV is blocked at some stage in the replication cycle at low temperatures in cultured fish cells.

Expression of LacZ and Luciferase Reporter Genes at Different Temperatures in Fish Cells Transfected with SFV-lacZ and SFV-luc RNA

The apparent temperature restriction of SFV replication and expression at low temperatures was investigated further by transfecting fish cells with SFV RNA encoding LacZ or luciferase reporter genes and examining their expression at selected temperatures (25°C, 20°C, and 15°C) up to 6 days posttransfection. Using optimized electroporation parameters CHSE-214 cells were successfully transfected with SFV RNA and expressed LacZ at 25°C, as determined by in situ

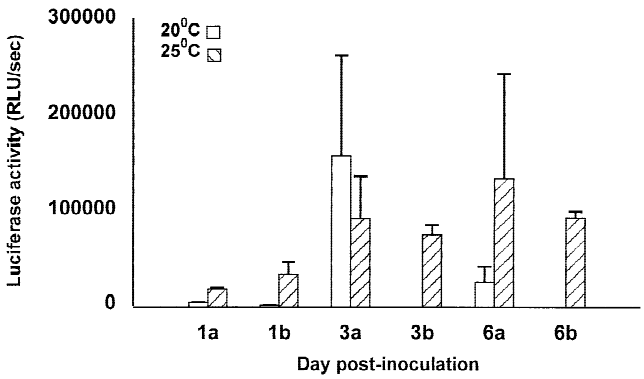

Figure 1. Temperature-dependent expression of luciferase in CHSE-214 cells transfected with SFV-luc RNA **(a)** or infected with replication-defective SFV-luc particles **(b).** CHSE-214 cells were transfected with SFV-luc RNA (approx. 25 μg) or infected with SFV-luc particles (1×10^6) and incubated at 25°C, 20°C, and 15°C. The luciferase activity in adhered cells and in cells in the culture supernatant was measured at various timepoints, and the sum was expressed as relative light units per second. SEM (+) values of triplicate samples are shown. Luciferase activities in cells infected with SFV-luc particles and incubated at 20°C and 15°C are not shown owing to the low levels of expression detected (below 1×10^3 RLU/s). Similarly, luciferase activities in cells transfected with SFV-luc RNA and incubated at 15°C are not shown.

staining with x-gal (Figure 2A). Transfection efficiencies of up to 55% were achieved, although levels of approximately 30% were more routine. As the posttransfection incubation temperature was reduced to 20°C or lower, however, there was a dramatic decline in the number of cells expressing β-gal in both CHSE-214 and F95/9 cell lines over a 72-hour period (Table 2). Levels of expression of luciferase were also found to decrease significantly in CHSE-214 cells transfected with SFV-luc RNA as the temperature was reduced from 20°C to 15°C (Figure 1), although high levels of luciferase activity were detected at 20°C. In fact, at 3 days postinoculation, luciferase activity was greater at 20°C than at 25°C, contrasting with results obtained for expression of β-gal. Even using the highly sensitive luciferase assay, luciferase activities in CHSE-214 cells maintained at 15°C reached a maximum of 7×10^2 RLU/s, compared with values ranging from 1–5 $\times 10^5$ RLU/s at 25°C and 20°C, respectively. Although luciferase activities were much lower at 15°C, levels detected were higher than background activities in mock-electroporated cells, calculated at approximately 0.05 RLU/s. These results indicate that expression from SFV RNA does occur, although to a much reduced level, in CHSE-214 cells at 15°C.

Expression of VP2 in BHK Cells Transfected with SFV-IPNV VP2 RNA

Expression of IPNV VP2 under the control of SFV expression elements (Figure 3) was confirmed by Western blot analysis and by metabolic labeling and immunoprecipitation of BHK cells transfected with SFV-VP2 RNA. Expression of a 58-kDa protein, the presumptive VP2 protein, was detected using IPNV polyclonal antiserum at 24 hours postelectroporation (Figure 4a). At 48 hours postelectroporation, a higher degree of cell death was observed, consistent with the lytic nature of SFV replication (Berglund et al., 1998). This resulted in a decrease in the amount of cellular material loaded onto the gel and may explain the absence of VP2 at this timepoint. Cross-reacting bands are also visible owing to anti-BHK antibodies present in the serum preparation. A 58-kDa protein, similar in size to the native VP2 antigen from partially purified IPNV particles, was also observed following metabolic labeling and immunoprecipitation with the IPNV polyclonal antiserum (Figure 4b).

Antigenic Characterization of SFV-IPNV VP2 and CMV-IPNV VP2 Constructs in BHK and Fish Cells

In BHK cells, the three different SFV-based constructs, SFV-VP2 RNA, replication-defective SFV-VP2 particles, and the layered pSFVL-VP2 construct encoding the 58-kDa gene product, were all found to express VP2 as determined by immunofluorescence (Figure 2). VP2 expressed from SFV-VP2 particles and pSFVL-VP2 DNA, but not SFV-VP2 RNA, were also found to react with the conformation-dependent neutralizing monoclonal antibodies H8G2 and B9 (Table 3). More intense staining patterns were obtained following infection of BHK cells with replication-defective particles compared with cells that had been transfected with SFV RNA by electroporation. The harsh electroporation conditions used to deliver the RNA into the cells may explain the inability of BHK cells transfected with SFV-VP2 RNA to react with the IPNV monoclonal antibodies.

The antigenicities of the proteins expressed by the SFV-IPNV constructs in CHSE-214 and F95/9 cells varied somewhat from those observed in BHK cells (Table 3). Only a weak response to fish cells transfected with SFV-VP2 RNA was displayed by the H8G2 and B9 monoclonal antibodies, and only a small percentage (approx. 1%) of fish cells transfected with the layered pSFVL-VP2 DNA plasmid reacted with the IPNV polyclonal, and negative results occurred with the H8G2 and B9 monoclonal antibodies. Further-

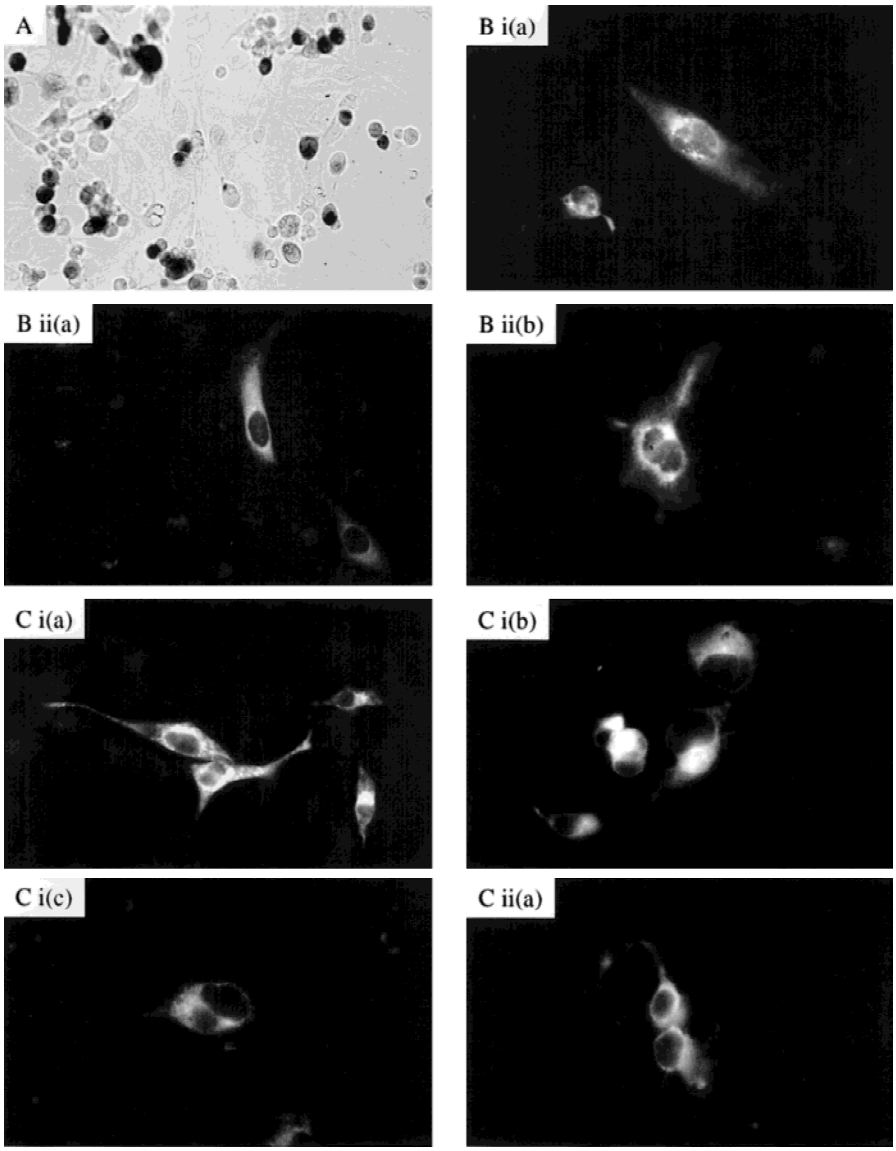

Figure 2. Expression of SFV-based constructs in animal and fish cells. **A:** Expression of lacZ in CHSE-214 cells transfected with SFV-lacZ RNA by electroporation. CHSE-214 cells were transfected by electroporation with SFV-RNA (25 μg) and harvested at 3 days posttransfection. Transfected cells expressing lacZ were identified as blue cells following in situ staining with x-gal. Magnification: ×40. **B:** Expression of IPNV-VP2 in CHSE-214 cells transfected with the layered pSFVL-VP2 DNA construct (*i*) or infected with replication-defective SFV-VP2 particles (*ii*). CHSE-214 cells were reacted with an IPNV polyclonal (PC) antiserum *(i-a)* and IPNV monoclonal antibodies F2 (*ii-a*) and B9 (*ii-b*). **C:** Expression of IPNV VP2 in BHK cells transfected with pSFVL-VP2 DNA (*i*) or infected with replication-defective SFV-VP2 particles (*ii*). Cells were reacted with the IPNV PC (*i-a* and *ii-a*) and the IPNV monoclonal antibodies H8G2 (*i-b*) and B9 (*i-c*). Cells expressing VP2 were identified by indirect immunofluorescent staining. Typical staining patterns are shown. Magnification: ×400.

more, a small number of F95/9 cells transfected with the pSFVL-VP2 DNA construct also appeared to react with the F2 monoclonal antibody. This variation in reactivity to the different IPNV monoclonal antibodies by VP2 expressed from the layered pSFVL-VP2 DNA construct may possibly be attributed to factors such as differences in the transfection efficiencies of BHK and fish cells and also the variable nature of the H8G2 and B9 epitopes (Melby and Christie, 1994; Frost et al., 1995). VP2 expression was also demonstrated from a DNA plasmid encoding the VP2 gene under the control of the CMV promoter. BHK and fish cells transfected with the CMV-VP2 DNA construct reacted with the IPNV polyclonal but failed to react with the IPNV monoclonal antibodies, with the exception of approximately 1% of CHSE-214 cells, which reacted only weakly with the H8G2 monoclonal antibody.

Discussion

Our study shows that SFV expression systems function in CHSE-214 and F95/9 fish cell lines at 25°C and 20°C as demonstrated by the ability of recombinant SFV RNA to express LacZ and luciferase reporter genes following transfection of CHSE-214 cells. The expression levels were low when compared with BHK cells, and could not be investigated by Western blot and pulse-chase analysis. This could be due to the lower efficiency of transfection achieved in CHSE-214 cells following electroporation with SFV RNA (approx. 30%) compared with values of up to 100% for BHK cells (data not shown). However, the levels of expression obtained would facilitate a study of antigenicity such as identification of a gene encoding a protective protein. Our finding that IPNV VP2 expressed from SFV-VP2 RNA in

Table 2. Temperature-Dependent Expression of LacZ in Fish Cells Transfected with SFV-lacZ RNA*

Cell type	Incubation temperature (%)	% cells expressing ß-gal†
F95/9	25	25
	20	3
	15	<0.5
	10	—
CHSE-214	25	30
	20	1.5
	15	—

*CHSE-214 and F95/9 cells were transfected with SFV-lacZ RNA (25 μg) and incubated at temperatures ranging between 10°C and 25°C.
†Cells were harvested at 72 hours posttransfection and tested for expression of lacZ by in situ staining with x-gal. The percentages of cells expressing lacZ at the different temperatures are given as the averages from 10 fields.

fish cells was more reactive with neutralizing monoclonal antibodies than that produced in BHK cells indicates that cell type and temperature may be important in protein processing. Moreover, recognition of the expression product by the different IPNV monoclonal antibodies, which are directed against conformation-dependent epitopes, suggests that the protein was correctly processed and its antigenicity conserved. This contrasts to the antigenicity of VP2 expressed by a CMV-based DNA plasmid in BHK and fish cells, which was found to react only weakly with one of the IPNV monoclonal antibodies. Interestingly, the differences in antigenicity were most apparent between VP2 expressed by the CMV-VP2 and the layered pSFVL-VP2 DNA constructs in BHK cells. Such variation in reactivity of VP2 expressed by the two systems may highlight differences between them and the potential advantage of using the SFV system for expression of certain fish proteins in fish.

SFV expression systems functioned inefficiently at temperatures of 15°C and below in fish cells. The low levels of LacZ and luciferase expression observed in CHSE-214 and F95/9 cells transfected with recombinant SFV RNA when the temperature was reduced to 15°C suggest that either translation of SFV RNA or its replication is inhibited. This may also be the case with infectious SFV, although we cannot rule out the possibility of blocks at penetration, assembly, or release steps also. Kielan and Helenius (1986) demonstrated that the uncoating step by which infectious alphavirus RNA is released into the cytoplasm was blocked at temperatures below 10°C. The use of SFV replication-defective particles as a tool for expression studies could be similarly restricted in fish cells.

SFV vectors may be an inappropriate delivery and expression system for development of a vaccine in fish. Farmed salmonid fish are maintained at temperatures below 15°C, and any SFV-based expression will be low. Levels of expression from eukaryotic expression vectors based on the CMV promoter are extremely low in cultured fish cells at temperatures below 20°C (unpublished data). However, following intramuscular inoculation of fish, antigen is expressed by these plasmids in sufficient amounts to be detectable by immunocytochemical procedures and to elicit protective immune responses (Anderson et al., 1996; Heppel et al., 1998; Lorenzen et al, 1998). It is therefore possible that adequate levels of SFV expression will take place in vivo to stimulate an immune response following inoculation of fish.

The layered SFV DNA construct displays more potential for vaccine development than use of replication-defective SFV particles owing to temperature restriction at an early stage in the replication cycle. In practical terms, this system offers a number of advantages over conventional CMV-based DNA vaccines. Immunization studies performed in mice have shown that inoculation with a DNA vector encoding a recombinant SFV replicon elicits stronger humoral and cell-mediated immune responses than that with a conventional plasmid vector vaccine (Berglund et al., 1998). In addition, higher proportions of mice responded to antigen expressed from the layered SFV DNA even when considerably lower doses of DNA were administered. These findings indicate that the amount of plasmid used for immunizations could be significantly reduced using alphavirus-based DNA vectors. Moreover, from a biosafety point of view, cells transfected with the layered SFV DNA plasmid will lyse owing to large-scale protein expression and be rapidly cleared by the immune system. This is in contrast to conventional DNA immunization, in which, depending on the antigen involved, expression can take place at much lower levels and cells harboring the plasmid can persist (Berglund et al., 1998). Thus, the transient expression and self-eliminating nature of the SFV-based DNA vector might represent a biosafety improvement in terms of reduced risks of DNA persistence, tolerance induction, chromosomal integration, and cell transformation.

As a eukaryotic expression system, the SFV system provides an alternative to existing viral vector and nucleic acid approaches for expression and analysis of fish proteins in

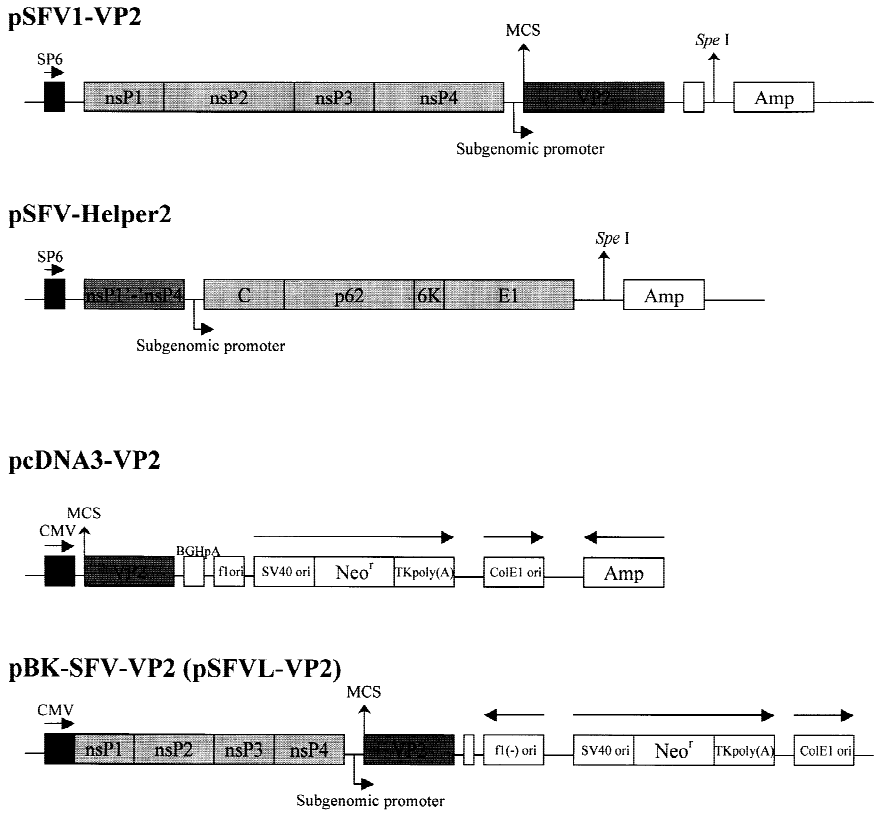

Figure 3. IPNV-VP2 expression constructs. pSFV1-VP2 and pSFV-Helper 2 DNA was transcribed in vitro using SP6 polymerase, and the RNA was cotransfected into BHK cells to produce replication-defective SFV-VP2 particles. DNA expression vectors were constructed in which VP2 was placed under the control of the eukaryotic CMV immediate early (IE) promoter (pcDNA3-VP2) or the CMV IE promoter and the SFV replicase (pSFVL-VP2).

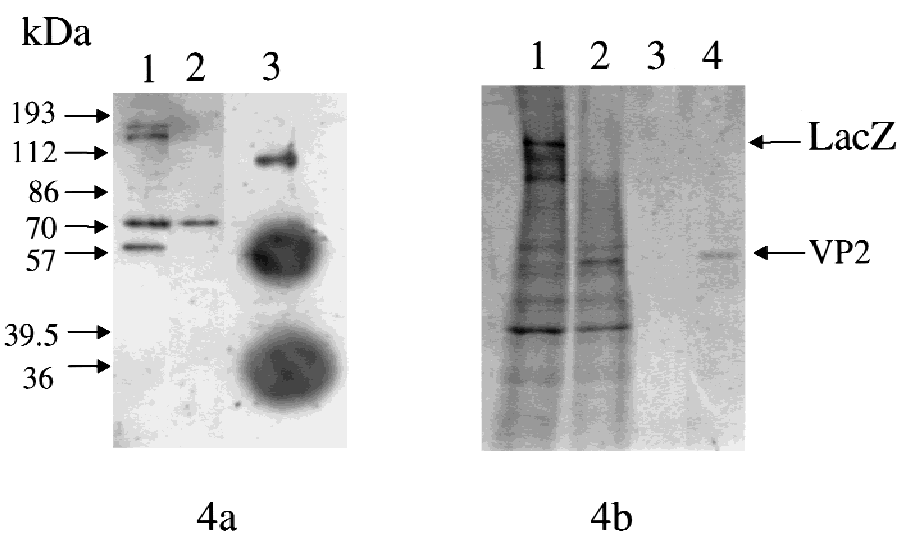

Figure 4. Expression of IPNV VP2 in BHK cells transfected with SFV-IPNV VP2 RNA by Western blotting and immunoprecipitation. **a:** Cell lysates prepared at 24 and 48 hours posttransfection were run on a 12% SDS-PAGE gel and probed with a rabbit polyclonal antiserum raised against IPNV (1:1000). Lane 1, pSFV-VP2 24 hours posttransfection; lane 2, pSFV-VP2 48 hours posttransfection; lane 3, purified IPNV (positive control). Positions of molecular weight standards are shown. **b:** Cell lysates prepared at 24 hours posttransfection were immunoprecipitated with an IPNV polyclonal antiserum and run on a 12% SDS-PAGE gel. Cell lysates from BHK-21 cells transfected with SFV-lacZ RNA (lane 1) and SFV-VP2 RNA (lane 2). Immunoprecipitates of cell lysates transfected with SFV-lacZ RNA (lane 3) and SFV-VP2 RNA (lane 4). The position of LacZ and IPNV VP2 are indicated.

Table 3. Antigenic Characterization of IPNV VP2 Expressed by CMV and SFV-Based Constructs in Animal and Fish Cells*

	BHK (37°C)				CHSE-214 (25°C)				F95/9 (25°C)			
	IPN PC	H8G2	B9	F2	IPN PC	H8G2	B9	F2	IPN PC	H8G2	B9	F2
CMV-VP2 DNA	+++	−	−	−	+++	+	−	−	+++	−	−	−
SFV-VP2 RNA	++	−	−	−	++	+[1]	+[1]	−	++	+[1]	+	−
SFV-VP2 particles	++	+[1]	+[1]	−	++	+	+[1]	−	++	+	+[1]	−
pSFVL-VP2 DNA	+++	++	+	−	+	−	−	−	+	−	−	+

*CHSE-214 and F95/9 cells were transfected or infected with the various CMV and SFV-based IPNV VP2 constructs, and the VP2 expression product was tested for reactivity against an IPNV polyclonal antiserum and the IPNV neutralizing monoclonal antibodies H8G2, B9, and F2: +++, 20% to 30% positive; ++, 5%–10% positive; +, 1% positive; +[1], cells faintly positive; −, negative.

cell cultures. In terms of detecting heterologous antigen expression in fish cells by immunocytochemical methods, replication-defective particles represent the delivery system of choice if processing of the protein concerned is not dependent on low temperatures. However, with regard to vaccine potential, it is possible that the SFV expression system is more suited for evaluation as a vaccine in warm-blooded fish (e.g., channel catfish). Further studies are necessary to establish the ability of the SFV-based constructs to function and deliver antigens to the immune system of fish under both cold and warm-water conditions.

Acknowledgments

The authors acknowledge the contribution made to this work by Mrs. K. Mawhinney and Mr. G. McKenna. The authors are grateful to Mr. Cliff Mason for production of the photographs. Financial support for this research was provided by EU FAIR grant CT.95.353.

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