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Recovery of a Recombinant Salmonid Alphavirus Fully Attenuated and Protective for Rainbow Trout

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Sleeping disease virus **(SDV) is a member of the new *Salmonid alphavirus* genus within the *Togaviridae* family. The single-stranded RNA genome of SDV is 11,894 nucleotides long, excluding the 3′ poly(A) tail. A full-length cDNA has been generated; the cDNA was fused to a hammerhead ribozyme sequence at the 5′ end and inserted into a transcription plasmid (pcDNA3) backbone, yielding pSDV. By transfection of pSDV into fish cells, recombinant SDV (rSDV) was successfully recovered. Demonstration of the recovery of rSDV was provided by immunofluorescence assay on rSDV-infected cells and by the presence of a genetic tag, a BlpI restriction enzyme site, introduced into the rSDV RNA genome. SDV infectious cDNA was used for two kinds of experiments (i) to evaluate the impact of various targeted mutations in nsP2 on viral replication and (ii) to study the virulence of rSDV in trout. For the latter aspect, when juvenile trout were infected by immersion in a water bath with the wild-type virus-like rSDV, no deaths or signs of disease appeared in fish, although they were readily infected. In contrast, cumulative mortality reached 80% in fish infected with the wild-type SDV (wtSDV). When rSDV-infected fish were challenged with wtSDV 3 and 5 months postinfection, a long-lasting protection was demonstrated. Interestingly, a variant rSDV ($rSDV_{14}$) adapted to grow at a higher temperature, 14°C instead of 10°C, was shown to become pathogenic for trout. Comparison of the nucleotide sequences of wtSDV, rSDV, and $rSDV_{14}$ genomes evidenced several amino acid changes, and some changes may be linked to the pathogenicity of SDV in trout.**

Sleeping disease in salmon was first observed in France in 1985 (1). In the rainbow trout (*Oncorhynchus mykiss*), the disease is characterized by an abnormal behavior of the fish, staying on their sides at the bottom of the tanks, reminiscent of a "sleeping state" that has provided the name of the disease (1). A related disease in farmed Atlantic salmon (*Salmo salar* L.) has also been reported (11). A viral etiology of these diseases was suspected (2) and confirmed a few years ago (4, 12, 14).

The viruses responsible for these diseases have been characterized and shown to be like alphaviruses (18, 19, 22), and the nucleotide sequences of the *Sleeping disease virus* (SDV) and *Salmon pancreas disease virus* (SPDV) genomes have been determined (21). Like all alphaviruses, the SDV and SPDV genomes consist of a positive-sense single-stranded RNA molecule of about 12 kb in length. The four nonstructural proteins (nsP1 to nsP4) are involved in virus replication and encoded by the 5′-terminal two-thirds of the genome, whereas the structural proteins (C-E3/E2-6K/E1) are encoded by the 3′-terminal one-third of the genome (for a review, see reference 17). SDV and SPDV have been classified as a new genus named *Salmonid alphavirus*. This classification is based on at least three main features. (i) The SDV and SPDV sequences are closely related to each other but differ substantially from those of other alphaviruses. (ii) Nonstructural and structural proteins are larger than the corresponding proteins of other alphaviruses. (iii) An arthropod-independent virus transmission to hosts has been demonstrated by cohabitation experiments (3).

The recovery of infectious virus from cDNA has been described previously for a number of mammalian alphaviruses, including Sindbis virus (13), Semliki Forest virus (9), Venezuelan equine encephalitis virus (5), and eastern equine encephalitis virus (16). The recovery of recombinant virus from cDNA is usually based on the transfection into cells of positive-stranded RNA generated by in vitro transcription from SP6- or T7-driven full-length viral cDNA constructs.

Similar approaches for SDV failed to produce infectious recombinant virus. Thus, we investigated the possibility of recovering infectious SDV by transfection of plasmid DNA into cells. As a first step, SDV-derived replicons in which the region coding for the structural proteins was replaced by the *Renilla* luciferase (LUC) or green fluorescent protein (GFP) gene were engineered as previously described for mammalian alphavirus (for a review, see reference 6). These replicons were validated through fish cell transfection and detection of expression of the reporter genes. Using these replicons, we show the effects of various targeted mutations in nsP2 on the level of synthesis of the subgenomic RNA. Finally, an SDV infectious cDNA clone was engineered and shown to be functional by the recovery of recombinant SDV (rSDV) following cell transfection. The growth kinetics of the rSDV in cell culture was comparable to that of the wild-type SDV (wtSDV). The use of rSDV to infect juvenile trout showed the following. (i) rSDV is infectious but nonpathogenic. (ii) rSDV is highly protective against a wild-type SDV challenge trial. (iii) The thermoresistant mutant of rSDV became pathogenic for trout.

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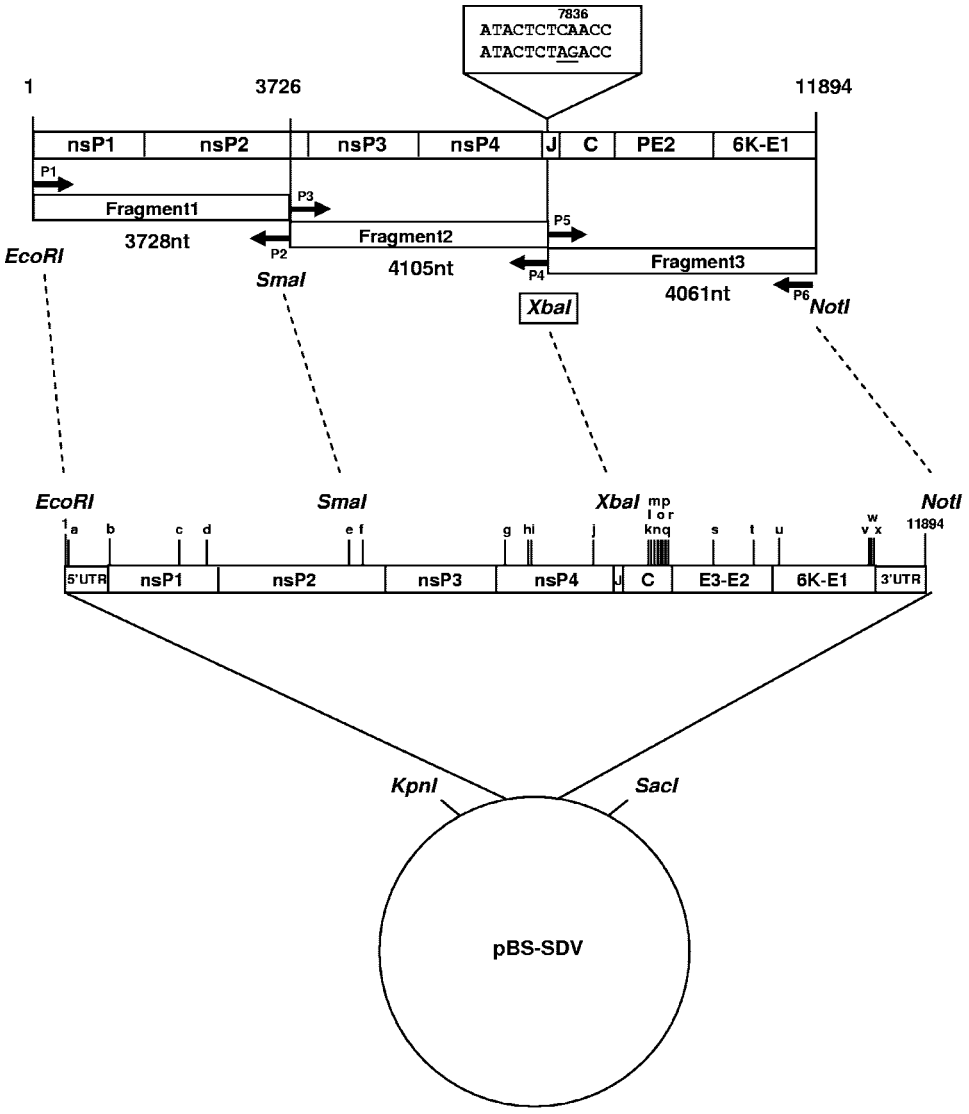

FIG. 1. Full-length SDV cDNA construct. Three cDNA fragments (fragments 1 to 3) covering the entire SDV genome were assembled by ligation into the multiple cloning site of the pBluescript plasmid using the EcoRI, SmaI, XbaI, and NotI restriction enzyme sites, yielding the pBS-SDV construct. An XbaI restriction enzyme site has been introduced into the junction region by changing 2 nucleotides (underlined) as indicated in the sequences in the box. The top and bottom sequences in the box are the wild-type and modified SDV sequences, respectively. Differences observed in comparison to the previously published sequences (18, 21) are indicated by letters (a to x) on the pBS-SDV construct. Nucleotide positions and amino acid changes are indicated in Table 2.

MATERIALS AND METHODS

Viruses and cell cultures. The SDV strain S49P used in this study has been described previously (4) and will be termed S49P-B (B for Brest, France). S49P-B (12 passages in cell culture) was plaque purified and then amplified, yielding the S49P-J isolate (J for Jouy en Josas, France). Virus was propagated in monolayer culture of bluegill fry BF-2 cells maintained at 10°C in Eagle's minimum essential medium (Sigma France) buffered at pH 7.4 with Tris-HCl and supplemented with 10% fetal bovine serum. Recombinant vaccinia virus expressing the T7 RNA polymerase, vTF7-3 (8), was kindly provided by B. Moss (NIH, Bethesda, Md.).

Cloning of the complete SDV S49P-J genome. A full-length SDV cDNA was assembled from cDNA fragments (numbered 1 to 3) covering the complete SDV genome into a pBluescript plasmid (Stratagene), yielding the pBS-SDV construct (Fig. 1). Individual fragments were amplified by reverse transcription-PCR (RT-PCR) using SDV genomic RNA as the template. The RNA had been extracted from supernatants of SDV-infected cells, concentrated by high-speed centrifugation, using the QIAamp viral RNA purification kit (QIAGEN). Primers (P1 to P6) used for RT and PCR amplification are shown in Table 1. As depicted in Fig. 1, an XbaI restriction enzyme site was artificially introduced to facilitate further cloning steps. By nucleotide sequencing of the pBS-SDV construct, a large number of nucleotide differences, including frameshifts, were identified (Table 2), compared to the published sequence (21).

Fusion of a hammerhead ribozyme sequence to the 5′ end of the SDV cDNA. The full-length SDV cDNA genome insert was recovered from pBS-SDV by digestion with EcoRI and NotI restriction enzymes and inserted into a pcDNA3 plasmid (Invitrogen) digested with the respective restriction enzymes. A hammerhead ribozyme sequence was fused to the 5′ end of the SDV cDNA as follows: part of the SDV cDNA encoding the structural proteins was first removed by XbaI digestion, yielding an. intermediate construct, p-nsP. A HindIII fragment containing the first 2 kb of the SDV cDNA was removed from p-nsP and subcloned into a pUC19 plasmid (pUC-SDV HindIII). A BamHI/NaeI DNA fragment containing the 5′ end of the SDV genome was removed from pUC-SDV HindIII and exchanged with a synthetic DNA fragment generated by the annealing of 2-, 79-, and 80-nucleotide (nt) partially complementary oligonucle-

TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)[a]	Restriction enzyme site
P1	CCGAATTCGTTAAATCCAAAAGCATACATATATCAATGATGC	EcoRI
P2	CCCGGGGCGGCCCCAAGGTCGAGAACTGAGTTG	SmaI
P3	CCCGGGAGGAGTGACCGACTACTGCGTGAAGAAG	SmaI
P4	GGTCTAGAGTATGATGCAGAAAATATTAAGG	XbaI
P5	CCTCTAGACCAACCATGTTTCCCATGCAATTCACC	XbaI
P6	CCGCGGCCGCATTGAAAATTTTAAAAACCAATAGATGACTCA	NotI
5′RIBO	GGATCCTGGATTTATCCTGATGAGTCCGTGAGGACGAAACTATAGGAAAGGAATTCCTA TAGTCGATAAATCCAAAAGC	BamHI
3′RIBO	GCCGGCGGAAGGGTTAGCTGTGAGATTTTGCATCATTGATATATGTATGCTTTTGGATT TATCGACTATAGGAATTCCTT	NaeI
5′SanDI	CCTCGTCAGCGGGACCCATAATGCC	SanDI
3′ΔXbaIBlpI	CCGCTGAGCGGTTGGTTGAGAGTATGATGC	BlpI
5′GFP	CCAACCGCTGAGCATGGTGAGCAAGGGCGAGG	BlpI
3′GFP	*GTGGCTAACGGCAGGTGATTCACGC***TTAAGCTCGAGATCTGAGTCCG**	
5′nsp4	GCGTGAATCACCTGCCGTTAGCCACAATGGCGATGGCCACGCTCG	
3′Jun	CCATGCTGAGCGGTTGGTTGAGAGTATGATGC	BlpI
nsP4-F	GGCGGCTTCCTGTTACTCGACACGG	
GFP-R	TTAAGCTCGAGATCTGAGTCCGG	
5′ProGFP	ATCGATGAACGATATC*GGCCGCCGCTACACGCTATGGCG*	EcoV
3′ProGFP	CCGGAATGCTAGC**TTAAGCTCGAGATCTGAGTCCG**	NheI
3′UTR	**CGAGCTTAA**GCTAGCATTCCGGTATACAAATCGC	NheI
T7t	GGCTAGGTCGGCGGCCGCAAAAAACCCCTCAAGACCCG	NotI
GSP1	CCGCCGAGTCGCTCCAGTTGGCG	
GSP2	CGGGTTCTCCAGGACGTCCTTCAAG	
5′RACEseq	GGCGGCGGCATGGTCGTTGGACGACCGG	
Cap-R	CCTTCAGCATAGTCATGGCCTTCTTTGG	

[a] Restriction enzyme sites are underlined. The italic sequence is part of the nsP4 sequence, and the bold sequence is part of the GFP sequence.

otides containing the hammerhead ribozyme sequence fused to the 5′ end of the SDV genome (Fig. 2A). Oligonucleotide primers 5′RIBO and 3′RIBO (Table 1) were annealed, filled in using the Klenow fragment of the *Escherichia coli* DNA polymerase I (15), and digested with the respective restriction enzymes. Finally, this modified HindIII fragment was inserted back into the p-nsP construct, yielding the pHH-nsP construct.

Construction of an SDV replicon expressing reporter genes and a full-length SDV cDNA. The pHH-nsP plasmid was linearized by digestion with XbaI restriction enzyme. *Renilla* luciferase and green fluorescent protein genes were amplified by PCR from the phRL-CMV (Promega) and pEGFP-C1 (Clontech) vectors, respectively. Primers were designed so that the final PCR products would have the following characteristics: at the 5′ end, a XbaI restriction enzyme site followed by part of the SDV junction region and a BlpI restriction enzyme site upstream from the GFP and LUC gene initiation codons; and at the 3′ end, an EcoRV restriction enzyme site downstream of the GFP and LUC gene stop codons. The SDV 3′ untranslated region (3′UTR) sequence was amplified by PCR from the pBS-SDV plasmid so that an EcoRV restriction site was added at the 5′ end and a poly(A) tract was followed by NotI and XbaI restriction enzyme sites at the 3′ end. By a three-fragment ligation of XbaI-digested pHH-nsP with the GFP or LUC and 3′UTR-poly(A) PCR products digested by XbaI/EcoRV and EcoRV/XbaI restriction enzymes, respectively, the pHH-nsP-XbaI-GFP and pHH-nsP-XbaI-LUC constructs were generated. Finally, the XbaI restriction enzyme site located in the junction region was removed by replacing the SanDI/BlpI DNA fragment from the pHH-nsP-XbaI-GFP and pHH-nsP-XbaI-LUC constructs by the same DNA fragment, generated by PCR using primers 5′SanDI and 3′ΔXbaIBlpI (Table 1) but without the XbaI restriction enzyme site. The final constructs were termed pnsP-GFP and pnsP-LUC (Fig. 2B). Finally, the pnsP-LUC construct was further modified by replacing the LUC gene by the SDV structural coding region through BlpI and EcoRV restriction enzyme digestion and ligation. A T7 terminator sequence was then introduced at the 3′-end poly(A) tract, yielding pSDV (Fig. 2C).

Luciferase activity assay and GFP detection. BF-2 cells in 24-well plates (6×10^5 cells/well) were transfected with 0.8 μg of the pnsP-LUC construct using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Every day posttransfection, two wells were washed with phosphate-buffered saline (PBS), and the cells were lysed with 75 μl of 1× lysis buffer (25 mM Tris-phosphate [pH 7.8], 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100). Lysates were clarified by low-speed centrifugation. Samples were normalized by Bradford quantification. Aliquots of clarified lysates were supplemented with 50 μl of luciferase assay reagent (Promega). Luciferase activity was monitored using a luminometer (Berthold France SA). For cell transfected with the pnsP-GFP construct, GFP expression was monitored by direct observation using a UV light microscope (Carl Zeiss, Inc., Thornwood, N.Y.).

Recovery of recombinant SDV. Approximately 1.2×10^6 BF-2 cells per well were grown in 12-well plates and infected with the recombinant vaccinia virus vTF7-3 at a multiplicity of infection of 5 (8). After 1 h of adsorption at 37°C, cells were washed twice and transfected (as described above) with 1.6 μg of the pSDV construct. The cells were incubated for 6 h at 37°C, the medium was replaced with fresh medium, the temperature was shifted to 10°C, and the cells were incubated for 7 to 10 days. At each time point, supernatants were removed, clarified by centrifugation at 10,000 × *g* in a microcentrifuge, and used to inoculate fresh BF-2 cell monolayers in 24-well plates at 10°C. In some experiments, transfections were carried out following the same procedure except that the cells had not been infected with vTF7-3.

GFP-expressing SDV construct. The pSDV infectious cDNA was engineered further by inserting an additional transcription unit encoding the GFP. The pnsP-GFP construct (Fig. 2B) served as the DNA template to generate two separate PCR products, the GFP PCR product using primers 5′GFP and 3′GFP and the SDV subgenomic PCR product using primers 5′nsP4 and 3′Jun (Table 1). The SDV subgenomic promoter was then linked downstream of the GFP sequence in a fusion PCR by mixing both PCR products and 5′GFP and 3′Jun amplification primers. Following BlpI restriction enzyme digestion, the resulting PCR product was inserted into the BlpI-digested pSDV construct, yielding the pGFP-SDV construct (see Fig. 6A). An additional construct named pSDV-GFP (see Fig. 6A) in which the "GFP unit" was inserted downstream of the structural genes was also engineered. Two separate PCR products were generated: the subgenomic promoter fused to the GFP (PCR1) using primers 5′ProGFP and 3′ProGFP (Table 1) and the pnsP-GFP plasmid construct as the DNA template (Fig. 2B) and the SDV 3′UTR fused to a poly(A) tract and the T7 terminator sequence (PCR2) using primers 3′UTR and T7t (Table 1) and the pSDV plasmid construct as the DNA template (Fig. 2C). PCR1 and PCR2 were linked together by a fusion PCR using primers 5′ProGFP and T7t. The resulting PCR product was digested with EcoRV and NotI and inserted in the pSDV construct, digested with the respective restriction enzymes.

TABLE 2. Nucleotide and amino acid changes in SDV compared to the published sequence (20)

Region	Nucleotide position	Position[a]	Nucleotide change[b]	Amino acid change[b]
5′UTR	2	a	T→A	
nsP1	35	b	T→A	L→Q
	1123	c	G→A	D→N
	1519	d	G→A	G→R
	1531	d	C→A	L→I
	3728	e	G→C	R→P
	3934	f	CG→GT	R→V
	3938	f	G→T	R→L
	3941	f	C→T	S→F
nsP4	6107	g	T→C	L→P
	6471	h	A→C	E→D
	6505	i	A→G	K→E
	7467	j	A→C	E→D
Capsid	8337	k	T→A	F→I
	8383	l	T→A	V→D
	8415	m	T→A	C→S
	8469	n	G→T	G→W
	8482	o	A→C	N→T
	8486	o	T→	Frameshift
	8490	o	G→	Frameshift
	8504	p	T→G	L→L
	8506	p	T→C	Y→A
	8510	p	T→A	S→Y
	8539	q	G→	Frameshift
	8553–8555	r	CCA→GCC	P→A
	8556	r	T→A	F→I
E2	9310	s	C→T	T→M
	9937	t	T→G	L→W
6K	10422	u	GCG→AGC	A→S
E1	11709	v	A→G	R→G
	11722	w	A→	Frameshift
	11739	w	T→	Frameshift
	11751	x	G→	Frameshift

[a] Position on the pBS-SDV construct as shown by the letters (a to x) in Fig. 1.
[b] The nucleotide or amino acid before the arrow is the nucleotide or amino acid in the published sequence (21); the nucleotide or amino acid after the arrow is the nucleotide or amino acid in the cDNA sequence.

Immunofluorescence assays. Plasmid-transfected or rSDV-infected cells (in six-well plates, 2.5×10^6 cells/well) were fixed with a 1:1 (vol/vol) mixture of alcohol and acetone at −20°C for 15 min. SDV nonstructural and structural proteins were detected by incubating infected cells with different monoclonal antibodies (MAbs) directed against SDV (10) diluted to 1:1,000 in phosphate-buffered saline–0.05% Tween 20. After 45 min of incubation at room temperature, cells were washed and incubated with fluorescein-conjugated anti-mouse immunoglobulins (P.A.R.I.S., Compiègne, France). Finally, cells were washed and examined for staining with a UV light microscope (Axiovert 200 M; Carl Zeiss, Inc., Thornwood, N.Y.).

RT-PCR on rSDV. Aliquots of supernatants of BF-2 cells infected with rSDV were clarified by low-speed centrifugation, and viral RNA was directly extracted using the QIAamp viral RNA purification kit (QIAGEN) according to the manufacturer's recommendations. Part of the RNA genome was reverse transcribed and amplified by PCR (RT-PCR) with specific primers in the nsP4 gene (nsP4-F) and the capsid gene (Cap-R) (Table 1). RT-PCR products were analyzed in a 1% agarose gel with or without BlpI restriction enzyme digestion and were also subjected to nucleotide sequencing.

Nucleotide sequencing of rSDV, wtSDV-B, and wtSDV-J genomes. The RNA genomes of the three SDV isolates were amplified by RT-PCR as overlapping DNA fragments using pairs of primers along the genome. Each PCR product was gel purified and either directly sequenced using specific primers or subcloned into a pGEM-T vector (Promega). In the latter case, after *E. coli* transformation with the respective recombinant plasmids, 10 recombinant *E. coli* clones were selected for each plasmid construct, and plasmids were extracted and subjected to nucleotide sequencing using universal SP6 and T7 primers and specific primers. Nucleotide sequences were analyzed using Sequencher software (GeneCodes).

Experimental fish infection and virus recovery. One hundred virus-free juvenile rainbow trout (*Oncorhynchus mykiss*) (mean weight, 0.5 g) were infected by immersion in tanks filled with 3 liters of freshwater with either wtSDV (B and J) or rSDV (final titer, 5×10^4 PFU/ml) for 2 h at 10°C. Tanks were then filled up to 30 liters with freshwater. Controls were fish mock infected with cell culture medium under the same conditions. Infected fish started to die 3 weeks postinfection, and mortalities were recorded over a 2-month period of time. At 2 to 3 weeks postinfection, some fish were sacrificed and individual fish were homogenized in a mortar with a pestle and sea sand in 9 volumes of minimum essential medium containing penicillin (100 IU/ml) and streptomycin (0.1 mg/ml). After centrifugation at $2,000 \times g$ for 15 min at 4°C, the supernatant was used to infect BF-2 cells. Virus was detected by immunofluorescence assay (see above). The virus titers were determined by plaque assays.

RESULTS

Full-length SDV genome construct and replicon functionality. The complete SDV RNA genome was amplified by reverse transcription and PCR as three fragments using primers derived from the previously published sequence (18, 21). The three fragments were assembled together in a pBluescript plasmid, pBS-SDV, as depicted in Fig. 1. Comparison of the nucleotide sequence of the full-length cDNA clone with the published sequence evidenced 33 differences (Table 2). The full-length SDV cDNA was transferred from pBS-SDV into a pcDNA3 vector between the EcoRI and NotI restriction enzyme sites, downstream from the immediate-early cytomegalovirus (CMV) promoter and a T7 RNA polymerase promoter. The construct was further engineered to fuse to a hammerhead ribozyme sequence (Fig. 2A) at the first 5′-end nucleotide of the SDV cDNA. Introduction of a ribozyme sequence allowed precise cleavage at the extreme SDV 5′-end nucleotide by transcription. To evaluate the functionality of such a construct in fish cells, a replicon system was elaborated by replacing the cDNA part coding for the SDV structural proteins by LUC or GFP reporter genes. The final constructs, pnsP-GFP and pnsP-LUC (Fig. 2B), were separately transfected into BF-2 cells and incubated at 10°C. In these experiments, we expected that SDV RNA replicons would be expressed through transcription of the DNA templates by RNA polymerase II using the CMV promoter. Every day posttransfection, cells were either directly observed under a UV light microscope for pnsP-GFP-transfected cells or lysed for detection of LUC activity using a luminometer in the case of pnsP-LUC-transfected cells. Significant luciferase activity was detected as soon as 2 days posttransfection and increased until 8 days posttransfection (Fig. 3A). High LUC activity was still detectable 11 days posttransfection. GFP expression was visible at 5 days and optimal 10 or 11 days posttransfection (Fig. 3B). These data demonstrate that the SDV replicase complex nsP1, nsP2, nsP3, and nsP4 expressed from the pnsP-LUC or -GFP construct is biologically active and able to replicate and transcribe a subgenomic RNA containing the reporter genes.

Recovery of recombinant SDV from cDNA. To generate an infectious SDV cDNA, the complete region encoding SDV structural proteins was inserted back into the pnsP-LUC plasmid, and a T7 terminator sequence (T7t) was added after the

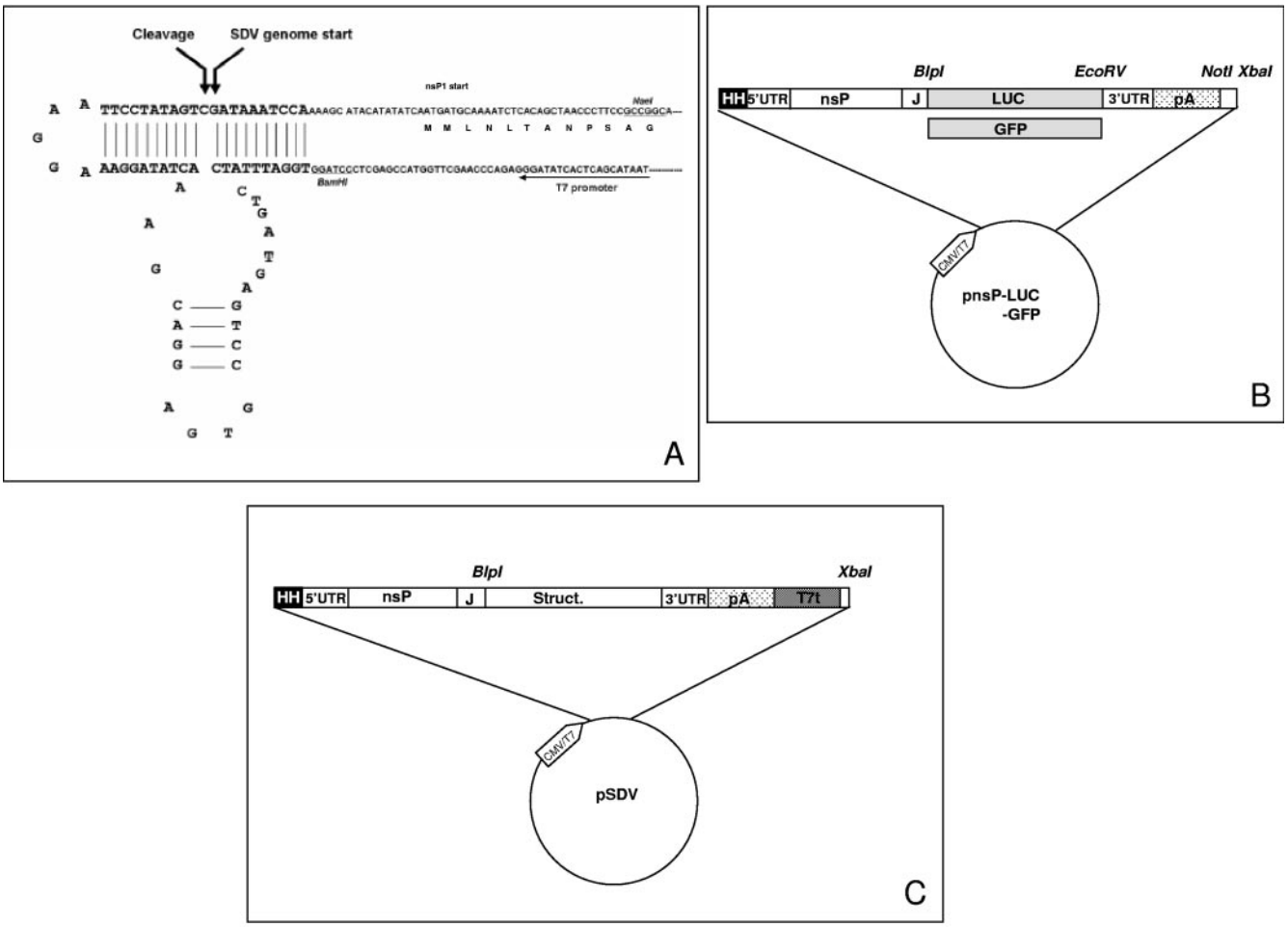

FIG. 2. SDV replicons and infectious cDNA constructs. (A) Hammerhead ribozyme nucleotide sequence. The BamHI and NaeI restriction enzyme sites and the T7 promoter sequence are underlined. The ribozyme cleavage site and the beginning of the SDV genome are indicated by arrows. (B) The entire SDV cDNA was transferred from the pBluescript backbone into a pcDNA3 plasmid and fused to the hammerhead ribozyme sequence (HH) (see Materials and Methods). The gene encoding the structural protein was removed by BlpI/EcoRV restriction enzyme digestion and replaced by the reporter gene LUC (pnsP-Luc) or GFP (pnsP-GFP). (C) The LUC gene was removed from the pnsP-LUC construct and exchanged with structural (Struct.) genes by BlpI and EcoRV restriction enzyme digestion and ligation. A T7 terminator sequence (T7t) was added downstream of the poly(A) tail (pA), yielding the final construct, pSDV.

poly(A) tail into the blunt-ended NotI restriction enzyme site, yielding the pSDV plasmid (Fig. 2C). Infectivity of the full-length cDNA clone pSDV was tested by transfection into vTF7-3-infected BF-2 cells. At 7 and 10 days posttransfection, fixed cells were subjected to indirect immunofluorescence assay using a panel of MAbs directed against the nonstructural and structural SDV proteins (10). At 7 days posttransfection, some small foci became apparent and increased in size at 10 days (not shown), probably reflecting the cell-to-cell spreading of the recombinant SDV. To confirm that rSDV was produced, supernatant from transfected cells 10 days posttransfection was used to infect fresh BF-2 cells, and positive detection of infected-cell foci by indirect immunofluorescence assay demonstrated the recovery of rSDV (Fig. 4A). In addition, we observed that live rSDV-infected cells were also stained by two anti-E2 neutralizing 17H23 and D20 MAbs (10; also data not shown), demonstrating the full maturation of the E2 glycoprotein. We then investigated whether the BlpI restriction enzyme site, which had been introduced in the SDV genome as a genetic tag, was present in the rSDV RNA genome. Genomic RNA was extracted from rSDV-infected cell supernatants after one passage and used as the template for RT-PCR using primers surrounding the BlpI site. BlpI restriction enzyme digestion of the PCR product generated two bands of the expected sizes, in contrast to a PCR product generated from the wild-type SDV RNA genome, which remained undigested (Fig. 4B). This demonstrated the successful recovery of rSDV from pSDV.

Identification of mutations in nsP2 involved in reduced growth of rSDV. During cloning of the full-length SDV genome, a number of mutations had been introduced and were corrected. Some of the mutations were located in the nsP2 coding region. The mutations located at nt 2345 (G210E), nt 2600 (M295T), and nt 3013 (I435V) were introduced separately into the pnsP-LUC construct, and the resulting mutated constructs were transfected into fish cells to measure luciferase activity. As shown in Fig. 5, these mutations had a decreasing effect on the synthesis of the subgenomic RNA encoding the LUC reporter gene. Compared to the wild-type SDV-LUC

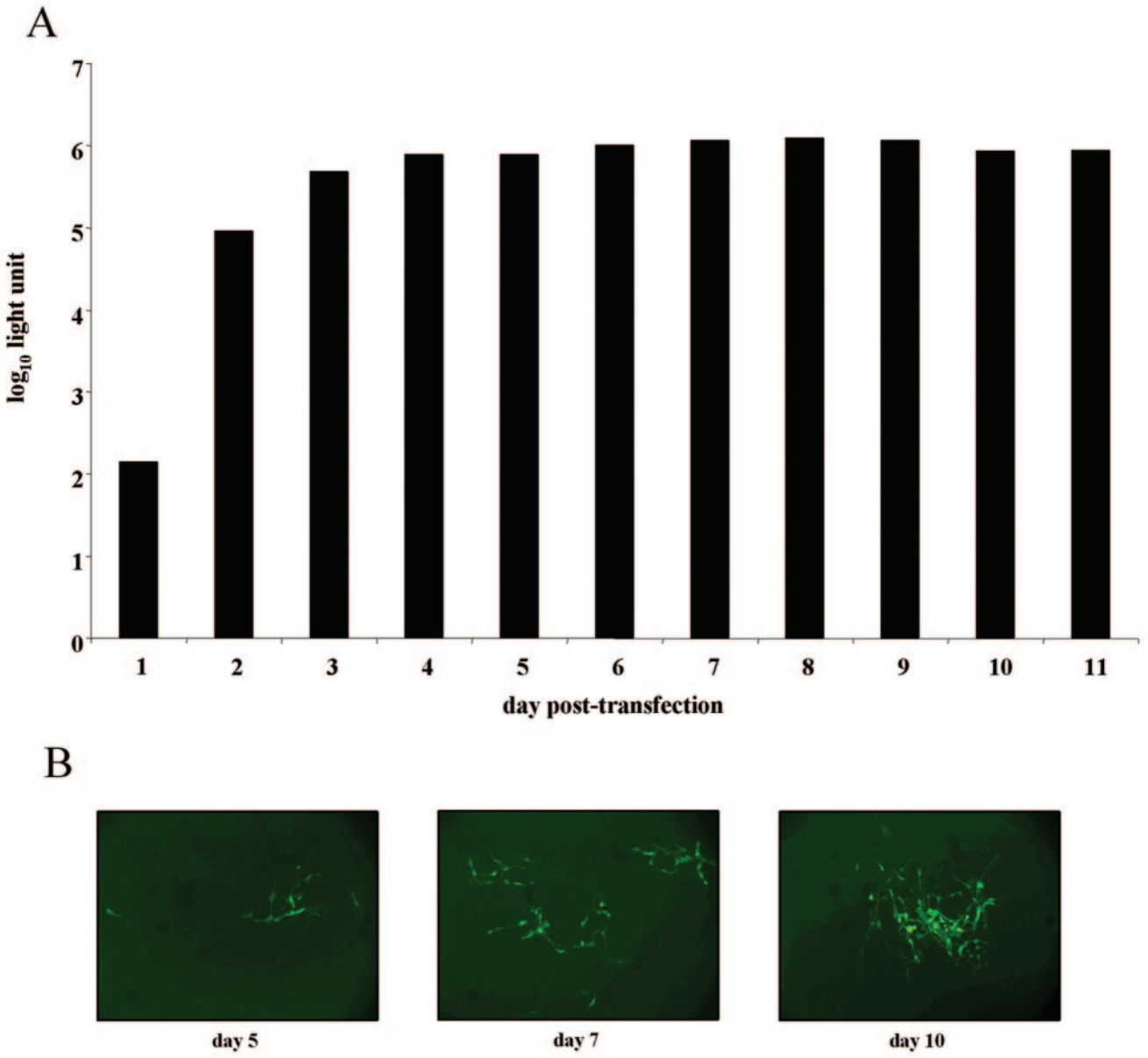

FIG. 3. Expression of the reporter genes in cells by the SDV-derived replicons. BF-2 cells were transfected with either pnsP-LUC (A) or pnsP-GFP (B) construct and incubated at 10°C. (A) At different days posttransfection, cell lysates were incubated with the luciferase substrate. Emitted light was measured using a luminometer (Berthold) and quantified as light units. (B) Live cells were directly observed with a UV light microscope.

replicon, expression of LUC activity increased from G210E to M295T to I435V to wild type (Fig. 5). When these mutations were introduced into pSDV, the growth of the recombinant viruses generated exhibited some discrepancies compared to that observed using LUC-expressing replicons, since the virus titers after one passage in cell culture were 2×10^2, 1.4×10^8, 2×10^5, and 3×10^8 PFU/ml for G210E, M295T, I435V, and rSDV, respectively (Fig. 5).

Recombinant SDV can be used as a gene vector and is able to accommodate at least 2.7 kilobases of additional sequence. To produce an infectious SDV expressing the GFP as reporter protein, the pSDV construct was modified in two ways. (i) An additional SDV RNA subgenomic promoter was fused to the 3′ end of the GFP gene and inserted upstream of the structural genes, yielding pGFP-SDV. (ii) The SDV subgenomic promoter was fused to the 5′ end of the GFP gene and inserted downstream from the stop codon of the structural genes, yielding pSDV-GFP. Both constructs are depicted in Fig. 6A. As the minimal size of the SDV subgenomic RNA promoter sequence has not yet been characterized, it has been assumed that a 100-nucleotide-long DNA fragment in the nsP4 sequence upstream of the junction region contains the promoter sequence. The pSDV-GFP and pGFP-SDV constructs were transfected into vTF7-infected BF-2 cells, and GFP expression was monitored every day with a UV light microscope. As shown in Fig. 6B, GFP expression was detectable 7 days post-

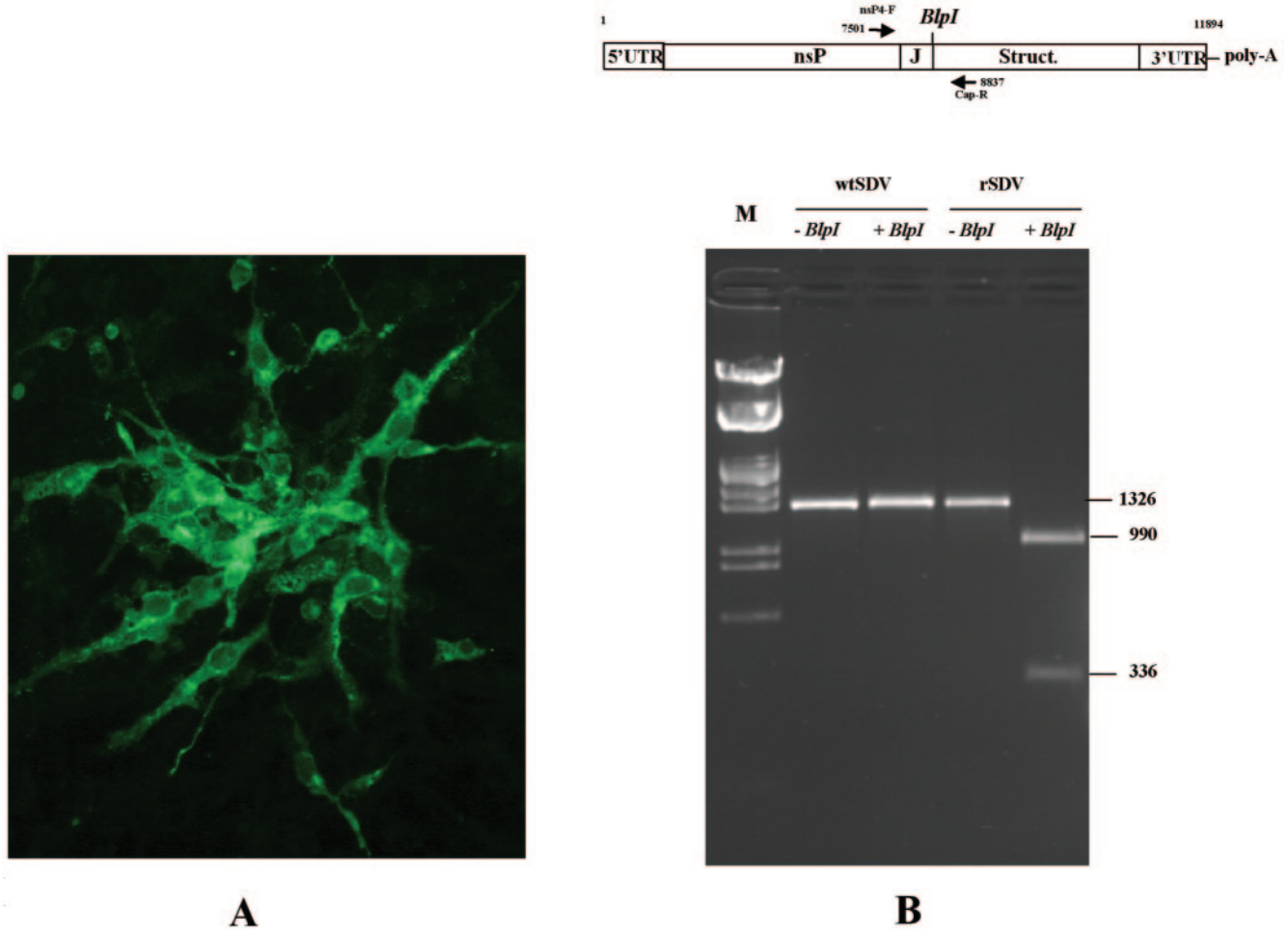

FIG. 4. Recovery of rSDV. Supernatant from cells transfected with the pSDV construct was harvested and used to infect fresh BF-2 cells. (A) At 4 days postinfection, the cells were fixed and subjected to an indirect immunofluorescence antibody technique using a mixture of SDV MAbs. (B) Viral RNA was extracted from either wtSDV- or rSDV-infected cell supernatants and used as the template in RT-PCR using a pair of primers that span the additional BlpI restriction enzyme site created in the junction region upstream from the start codon of the structural (Struct.) genes. Both 1,326-nucleotide-long PCR products were either mock digested (−) or digested (+) with restriction enzyme BlpI, yielding the expected 990- and 336-nt DNA fragments for the rSDV PCR product, but leaving the wtSDV cDNA fragment intact. Lane M contains molecular size markers.

transfection for both pSDV-GFP and pGFP-SDV constructs. However, we observed that GFP expression was more intense when the GFP gene was located downstream of the structural genes in the SDV genome. The same observations were made and quantified using similar constructs containing LUC instead of GFP (not shown). We also engineered a recombinant SDV cDNA genome in which three copies of the "GFP unit" were inserted upstream of the structural genes ($pGFP_3$-SDV [Fig. 6A]). Altogether, the three added "GFP units" represent a 2.7-kb DNA fragment. Recombinant $rGFP_3$-SDV was recovered and after one passage of the virus in cell culture, we confirmed by RT-PCR using a pair of primers (nsP4-F and GFP-R, Table 1) that three "GFP units" were indeed present in the viral genome (Fig. 6C) and that the GFP was expressed in the infected cells (not shown). These data demonstrated that SDV is able to package a genome that is more than 20% longer than the wild-type virus genome. However, expression of the GFP was no more detectable after one additional passage of the rSDV-GFP_3 in cell culture although the cells were shown to be readily infected by virus in an immunofluorescence assay (not shown). This indicates the instability of the chimeric virus.

In vivo infectivity of the recombinant SDV. Although replication of rSDV was demonstrated in vitro, the abilities of the recombinant virus to infect and to replicate in trout had to be confirmed. Juvenile rainbow trout were infected by immersion in a water bath with rSDV or wtSDV (B and J), and mortalities were recorded every day over a 2-month period of time (Fig. 7). Some fish ($n = 4$) were sacrificed 2 weeks postinfection, and organ homogenates from individual fish were used to infect BF-2 cells. Demonstration of virus-infected cells was assessed by immunofluorescence assays (data not shown). All sacrificed fish were positive for virus, and virus titer was approximately 10^7 PFU/ml for both rSDV and wtSDV (B and J). Mortalities started at day 21 in trout infected with wtSDV-B and -J, and cumulative mortality was 78 and 8%, respectively, at day 60. No mortality was registered in trout infected with rSDV during the same period of time,

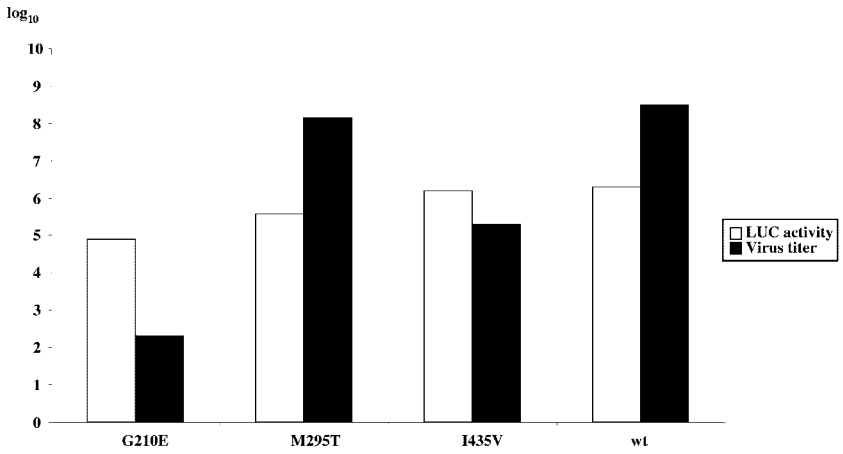

FIG. 5. Effects of mutations in nsP2. The effects of three mutations in nsP2 were evaluated using either a replicon expressing the luciferase gene (white bars) or mutated infectious cDNA clone (black bars). Data are presented as $\log_{10}$ of arbitrary emitted light units or PFU/ml for LUC activity and virus titer after one passage in cell culture. Amino acid mutations are indicated in the form of XnY, where X is the wild-type amino acid, n is the amino acid position in the nsP2 protein, and Y is the mutated amino acid. The wild-type nsP2 sequence (wt) is indicated. The results presented are the means of two individual experiments.

demonstrating the complete attenuation of rSDV (Fig. 7A). In addition, when rSDV-infected fish were challenged after 3 months with wtSDV-B by immersion in a water bath, again no mortalities appeared over a 2-month period of time (Fig. 7B, left). The latter data demonstrate that rSDV is totally attenuated and fully protective. Moreover, a long-lasting protection was established, because when rSDV-vaccinated fish were challenged 5 months later with wtSDV-B, the fish were totally protected (Fig. 7B, right).

Identification of amino acid residues involved in the pathogenicity of SDV. We previously showed that when SDV was propagated on CHSE-214 cells, a temperature of 10°C was required, while at 14°C no replication of the virus could be observed (18); however, when BF-2 cells are used, SDV can efficiently replicate at 14°C. To determine whether the temperature shift induced some changes in the SDV phenotype, rSDV and wtSDV-J were amplified at 14°C through five passages in BF-2 cells. Inocula were then used to infect trout by immersion in a water bath. In contrast to the rSDV grown at 10°C ($rSDV_{10}$), rSDV amplified at 14°C ($rSDV_{14}$) induced mortalities, starting day 21 postinfection, in the infected fish (Fig. 8). The cumulative mortality rate reached 24% 2 months postinfection. Similarly, $SDV\text{-}J_{10}$ induced only 7% of cumulative mortality, while $SDV\text{-}J_{14}$ induced 30%. To obtain insight into the differences observed between viruses grown at 10°C and 14°C, part of the rSDV14 genome encoding structural proteins was amplified by RT-PCR and subjected to nucleotide sequencing. Comparison of $rSDV_{10}$ and $rSDV_{14}$ nucleotide sequences emphasized six major changes in the structural polyprotein. Three mutations appeared in the E2 glycoprotein (C217Y, E294G, and F352L), two mutations were present in the 6K protein (F19L and R51G), and one mutation was present in the E1 glycoprotein (I193F).

DISCUSSION

A few years ago, the complete nucleotide sequence of the *Sleeping disease virus*, a new member of the alphaviruses, now grouped together with the *Salmon pancreas disease virus* in the genus *Salmonid alphavirus*, was published (18, 21, 22). Although the genome structure was similar to that of classical alphaviruses, such as Sindbis virus and Semliki Forest virus, the nucleotide and predicted amino acid sequences of SDV and SPDV are only distantly related to those of other alphaviruses, and the 5′UTR and 3′UTR are the shortest described so far. Indeed, the 5′UTR of SDV is only 27 nucleotides long, a half or a third of the 5′UTR size of all other alphavirus genomes sequenced to date. Thus, it should be considered that at least for the salmonid alphaviruses, there is no requirement for a stem-loop structure in that region to serve as a recognition signal by the replicase complex (17). SDV and SPDV replicate at a low temperature (10°C) in fish-derived cells, such as RTG-2, CHSE-214, and BF-2 cells. We observed that BF-2 cells are the most appropriate cell line to produce SDV at high titers ($>10^8$ PFU/ml [M. LeBerre, unpublished observation]). In addition, BF-2 cells could also be used to replicate SDV at a higher temperature (14°C) (see below). A cDNA clone for SDV has been engineered and was shown to be infectious but only when a hammerhead ribozyme sequence was fused at the 5′ end. Indeed, all the attempts to generate infectious RNA through in vitro transcription of SDV cDNA directly fused to a T7 or SP6 promoter, as routinely done for mammalian alphaviruses (17), failed to produce recombinant virus in the transfected cells. Similarly, when a plasmid construct, in which a T7 promoter was fused to the SDV cDNA, was transfected into vTF7-3-infected cells, no recombinant SDV was recovered.

SDV-derived replicons were shown to efficiently express the GFP or LUC protein in fish cells. Moreover, using the SDV-derived replicon expressing luciferase, we have shown that some mutations introduced into the nsP2 protein have a direct impact on the synthesis of subgenomic RNA, as measured by the luciferase activity. For example, changing a glycine to glutamic acid (G210E) reduces notably the expression of luciferase (2×10^6 versus 8×10^4 units) and the replication of the SDV-derived replicon, while changing isoleucine to valine (I435V) has only a limited impact (2×10^6 versus 1.6×10^4 units). However, interestingly, when recombinant SDV with

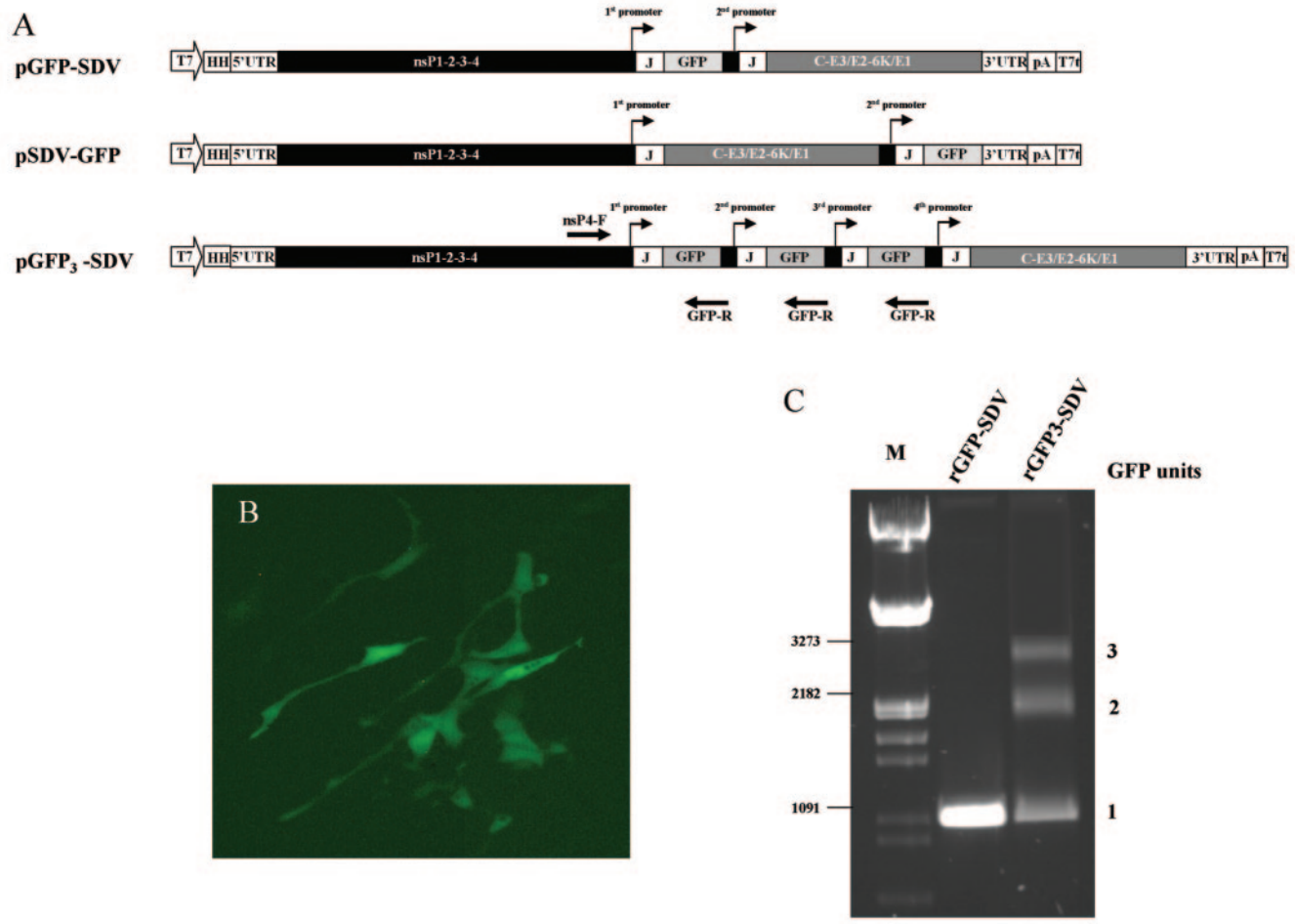

FIG. 6. rSDV with an additional subgenomic promoter. (A) An additional transcriptional unit expressing the GFP as a reporter gene was inserted into the SDV genome either upstream or downstream of the structural genes (pGFP-SDV and pSDV-GFP), or three additional transcriptional GFP units were inserted upstream of the structural genes (pGFP$_3$-SDV). HH, hammerhead ribozyme sequence; pA, poly(A) tail. (B) GFP expression in either rSDV-GFP- or rGFP-SDV-infected BF-2 cells was monitored by UV light microscopy, and typical results for both rSDVs 7 days postinfection are presented. (C) Confirmation by RT-PCR of the three additional transcription units on RNA extracted from infected cells after one passage of the cell supernatant. Lanes: M, DNA molecular weight markers; rGFP-SDV, recombinant SDV containing one additional transcription unit; rGFP$_3$-SDV, recombinant SDV containing three additional transcription units. The sizes of the RT-PCR products (in nucleotides) are indicated to the left of the gel. The primers used for the RT-PCR are depicted in panel A.

these changes in the genome are produced, we observed that the single change I435V had an attenuating effect on virus production (3×10^8 versus 2×10^5 PFU/ml). As this change has only a limited effect on genome replication and synthesis of a subgenomic RNA but a marked effect on the virus production, it can be hypothesized that this mutation is located in a region in nsP2 that is important as a packaging signal, as has been reported for Ross River virus and Semliki Forest virus (7) and more recently for Venezuelan equine encephalitis virus (20). All three mutations are located in the N-domain of nsP2 which contains the 5′-triphosphate and nucleoside triphosphatase regions, the G210E mutation being localized just upstream of the nucleoside triphosphatase motif; therefore, it may also be hypothesized that this mutation has a direct impact on the replication of the viral RNA genome.

The potential use of rSDV as a gene vector has been investigated by adding a "GFP unit" in the viral genome. Positive detection of the GFP in the rSDV-GFP-infected cells confirmed, as previously described for mammalian alphavirus (for a review, see reference 17), that SDV replication is not drastically impaired when an extra gene is present in the viral genome and that a second subgenomic promoter is being used for synthesis of the GFP RNA. However, the size and/or number of heterologous genes that can be added into the rSDV genome is limited, since it is shown here that although a rSDV expressing three additional "GFP units" was recovered, expression of the GFP was no longer visible after additional passage of that particular recombinant SDV in cell culture.

Recombinant SDV produced from the pSDV infectious cDNA clone was totally attenuated in rainbow trout when administered by either immersion in a water bath or injection (10^4 PFU/trout [data not shown]), although high titers (10^7 to 10^8 PFU/ml) of replicative virus could be recovered from sacrificed fish 2 to 3 weeks postinfection. It is of interest to emphasize that naïve trout infected with the wild-type SDV-B strain or -J strain exhibited different cumulative mortality rates

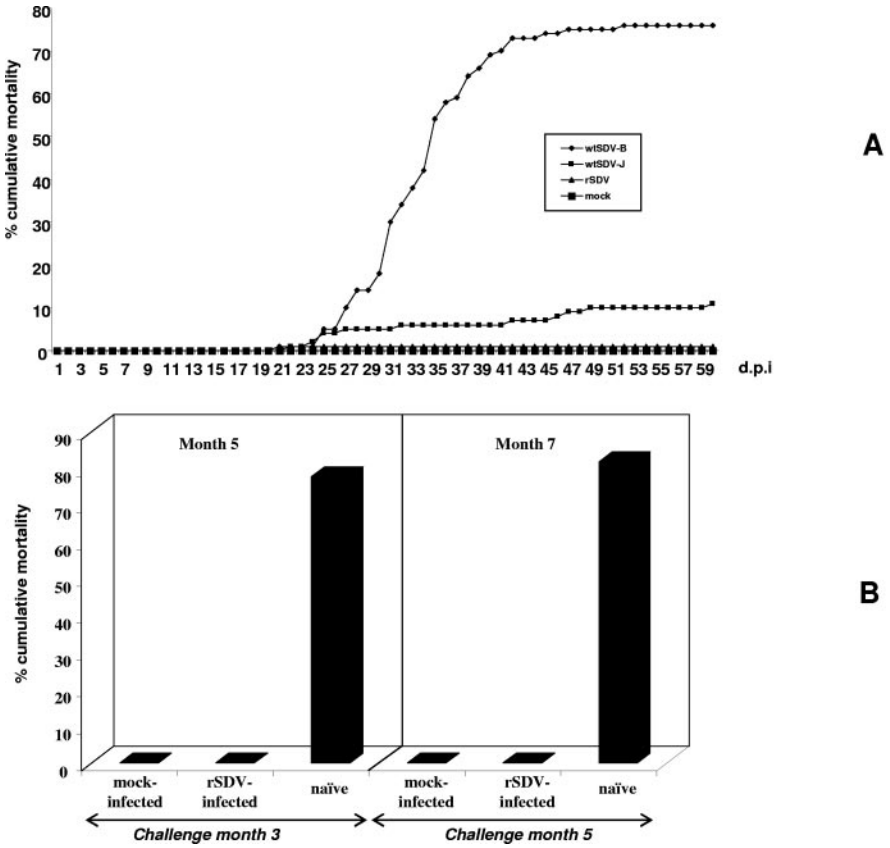

FIG. 7. Attenuation and protection of rSDV in trout. (A) Juvenile trout ($n = 100$; mean weight, 0.5 g) were infected by immersion in a water bath with 5×10^4 PFU/ml of the wild-type SDV strain B or J (wtSDV-B or wtSDV-J) or with the recombinant SDV (rSDV) or mock infected. Mortalities were recorded every day and are expressed as a percentage of cumulative mortality. d.p.i, days postinfection. (B) Trout infected with rSDV 3 months (left) or 5 months (right) earlier were challenged with the wtSDV-B, and cumulative mortality was recorded 2 months later (months 5 and 7, respectively). Naïve trout are trout infected with the wtSDV-B (positive infection control). Mock-infected trout are trout treated under the same conditions except that cell culture medium was used.

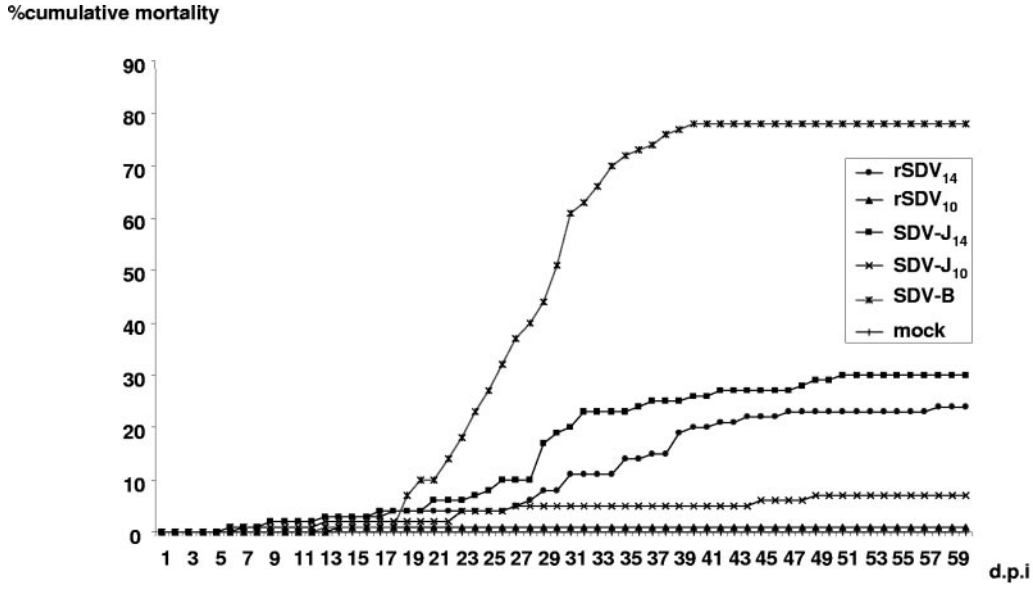

FIG. 8. Influence of temperature on the pathogenicity of SDV in trout. Juvenile trout ($n = 100$; mean weight, 0.5 g) were infected by immersion in a water bath with 5×10^4 PFU/ml of each virus grown at 10°C or 14°C or mock infected. Mortalities were recorded every day and are expressed as a percentage of cumulative mortality. d.p.i, days postinfection.

2 months postinfection, roughly 80 and 8%, respectively. As the SDV-J strain was derived from the SDV-B strain by plaque purification, it could be assumed that the SDV-B strain is a viral quasispecies population pathogenic for trout and that the viral heterogeneity was greatly reduced by plaque purification (SDV-J) and even more for rSDV derived from a single cDNA clone. Preliminary data on the nucleotide sequencing of the genomes of the three virus strains reinforce that idea, since a large number of amino acid changes, roughly 60 and 20, were observed in the nonstructural part (nsP1 to nsP4) of the SDV-B and SDV-J genomes, respectively, compared to the counterpart region in rSDV (data not shown). A long-lasting (at least 7 months) and complete protection was induced in the rSDV-vaccinated trout challenged with the wtSDV-B by immersion in a water bath and injection. In addition, rSDV-vaccinated fish were also protected from a challenge with the wild-type SPDV, a closely related but distinct salmonid alphavirus (not shown). The link between the temperature at which the viruses are produced and pathogenicity has been established, since rSDV and wtSDV-J became either pathogenic or more pathogenic for trout when the virus was produced at 14°C instead of 10°C. The shift in the temperature was shown to be associated with the appearance of amino acid changes in the SDV structural proteins and more specifically in the E2, 6K, and E1 proteins. Whether all these amino acid changes or only some of these changes are involved in pathogenicity should be studied in more detail. Nevertheless, the recombinant SDV already generated represents a very attractive live vaccine which can be produced at very high titer and can be used by immersion in a water bath.

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