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A novel neurotropic expression vector based on the avirulent A7(74) strain of Semliki Forest virus

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Semliki Forest virus (SFV), an enveloped alphavirus of the family Togaviridae, infects a wide range of mammalian host cells. Most strains are neurotropic but differ in virulence. The authors took advantage of the nonpathogenic properties of SFV strain A7(74), cloned recently in their laboratory, and constructed a replication-proficient expression vector to target the central nervous system (CNS) for heterologous gene expression. The vector, termed VA7, was engineered to drive expression of foreign inserts through a second subgenomic promoter inserted in the viral 3′ nontranslated region (NTR). Infectious virus was obtained by *in vitro* transcription and transfection into BHK cells, and was shown to direct synthesis of heterologous proteins in several mammalian cell lines. Although novel expression vehicle is not applicable for targeting specific cell populations within the CNS in its present form, in cultured rat hippocampal slices, VA7 encoding enhanced green fluorescent protein (EGFP) efficiently transduced pyramidal cells, interneurons, and glial cells. With prolonged time post infection, the number of EGFP-expressing neurons in hippocampal slices increased. Mice infected intraperitoneally with the recombinant virus remained completely asymptomatic but showed CNS expression of EGFP as evidenced by immunohistochemistry. SFV A7(74) is a nonintegrating virus, which gives rise to a randomly distributed, patchy infection of the adult CNS that is cleared within 10 days. With the advantage of noninvasive administration, the expression vector described in this work is thus applicable for short-term gene expression in the CNS. *Journal of NeuroVirology* (2003) **9,** 1–15.

Keywords: central nervous system (CNS); double subgenomic; enhanced green fluorescent protein (EGFP); gene transfer; hippocampus; replication-competent

Introduction

Viruses of the *Alphavirus* genus (family Togaviridae) are positive-strand RNA viruses consisting of an 11.5-kb genome packed in an icosahedral capsid, which in turn is surrounded by a lipid bilayer containing the envelope proteins. When exposed to the cytoplasm, the capped RNA genome of the alphavirus serves as mRNA and initiates synthesis of the viral proteins. First, the replicase is generated, which during the first hours of infection produces minus-strand RNA complementary to the viral genome. Next, a template switching takes place, converting minus-strand synthesis to the production of genomic RNA and 26S subgenomic RNA directed by an internal promoter located on the minus strand. The 26S RNA corresponds to the 3′ one third of the alphaviral genome and encodes the structural proteins. Once these have been synthesized, new virions assemble and exit by budding at the plasma membrane. For an

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in-depth review on alphavirus structure and replication, see Strauss and Strauss (1994).

In nature, alphaviruses are generally transmitted by mosquitos but are capable of infecting a wide variety of cell types *in vitro*. In particular, the neurovirulence of alphaviruses has been extensively studied, as many of the species are neurotropic. In this regard, the two prototype members of this family, the Sindbis virus (SIN) and the closely related Semliki Forest virus (SFV) have proven useful (reviewed by Atkins *et al*, 1994). Although neither virus is considered a serious human pathogen, SIN and SFV readily infect rodents and cause encephalomyelitis, sometimes with lethal outcome. However, strains of both species have been isolated that differ significantly in virulence. SIN AR339 and SFV A7(74) are both avirulent for adult mice, causing a limited infection of the CNS, which is cleared within 10 days post infection (p.i.) (Griffin and Johnson, 1977; Fazakerley *et al*, 1993).

Full-genome-length cDNA clones are available for a number of alphaviruses, including SIN (Rice *et al*, 1987), SFV prototype strain (Liljeström *et al*, 1991), Venezuelan equine encephalitis virus (VEE) (Davis *et al*, 1989), and Ross River virus (Kuhn *et al*, 1991), and the construction of these clones, together with knowledge of alphavirus replication and pathogenicity, has led to the development of novel viral expression vectors. Principally two different vector types are available: replication-deficient vectors, called replicons, and replication-competent vectors. The first replication-deficient system was based on a SIN cDNA in which the structural genes had been replaced by the insert of choice (Xiong *et al*, 1989). For SFV, a similar system was developed soon after (Liljeström and Garoff, 1991), and today a replicon system is also available for VEE (Pushko *et al*, 1997). Replicon particles capable of one round of infection can be produced by providing the structural genes from one or more cotransfected helper plasmids (Liljeström and Garoff, 1991; Smerdou and Liljeström, 1999), or in stably transfected cell lines (Polo *et al*, 1999). On the other hand, replication-competent vectors multiply autonomously and carry foreign genetic material in addition to the complete viral genome. Compared to replication-deficient constructs, they offer a significant advantage for *in vivo* applications as virus spread is not restricted to the site of injection.

Originally Raju and coworkers created a series of double subgenomic constructs to test relative promoter efficiencies (Raju and Huang, 1991). Next, an actual expression vector, called TE/3′2J, was developed based on a chimeric cDNA of several SIN strains, and has since then been applied in many experiments (Hahn *et al*, 1992). Over the years, a few novel cDNAs different from TE/3′2J have been developed and vectors based on these have also found use (Piper *et al*, 1992; Davis *et al*, 1996; Levine *et al*, 1996; Olson *et al*, 2000). The additional 26S promoter and the accompanying insert have been cloned either before or after the structural genes, but other strategies for virion production, gene expression, and vector modification have been devised, both for the replicon systems and the replication-proficient vectors (reviewed by Schlesinger, 2001).

SFV A7(74) was originally isolated based on its avirulence in mice (Bradish *et al*, 1971). We recently constructed a full-genome-length cDNA of A7(74) in order to investigate the mechanisms of neurovirulence and found that the attenuating determinants reside entirely in the replicase genes (Tuittila *et al*, 2000). The neuroinvasiveness and avirulence of the virus have made it an attractive candidate for vector development, and we report here the construction of a replication-proficient vector based on SFV A7(74). We inserted an additional 26S promoter into the 3′ nontranslated region (NTR) of the genome, immediately downstream of the E1 gene, and included a multiple cloning site (MCS) to facilitate insertion of heterologous genes. Based on experiments *in vitro* and on comparison of infected mouse brain tissue sections, we show that our expression vector, named VA7, has similar tropism and transduction capacity compared to the parental A7(74) strain. We show, using immunochemistry, that the vector drives synthesis of reporter protein in several different mammalian cell lines. Moreover, we show that VA7 efficiently transduces and drives expression of reporter protein in central nervous system (CNS) cells of rat hippocampal slices. Finally, we demonstrate immunohistochemically that VA7 expresses heterologous genes in the CNS of adult mice infected intraperitoneally (i.p.). Because avirulent SFV is cytotoxic *in vitro* but not in adult animal, and because the neuroinvasive infection is cleared within 10 days from the CNS in immunocompetent mice, the VA7 vector is applicable for transient gene expression in the adult CNS. To our knowledge, this is the first replication-competent expression vector based on SFV.

Results

Construction of expression vectors

Construction of the expression vectors FA7 and VA7 is depicted in Figure 1**A**. The triple subgenomic vector FA7, originally constructed to bear enhanced green fluorescent protein (EGFP) as a marker, in addition to a heterologous gene of interest, in order to more easily identify infected cells, is described here mainly as an intermediate step towards the actual expression vector VA7. The promoter sequences were chosen to include the fully active alphavirus promoter plus additional sequences thought to enhance translation (Raju and Huang, 1991; Hahn *et al*, 1992). The 3′ NTR of alphaviruses seems tolerant to extensive deletions and modifications (Raju *et al*, 1999), and the 3′ NTR of SFV A7(74) can be replaced by wild-type 3′ NTR without affecting

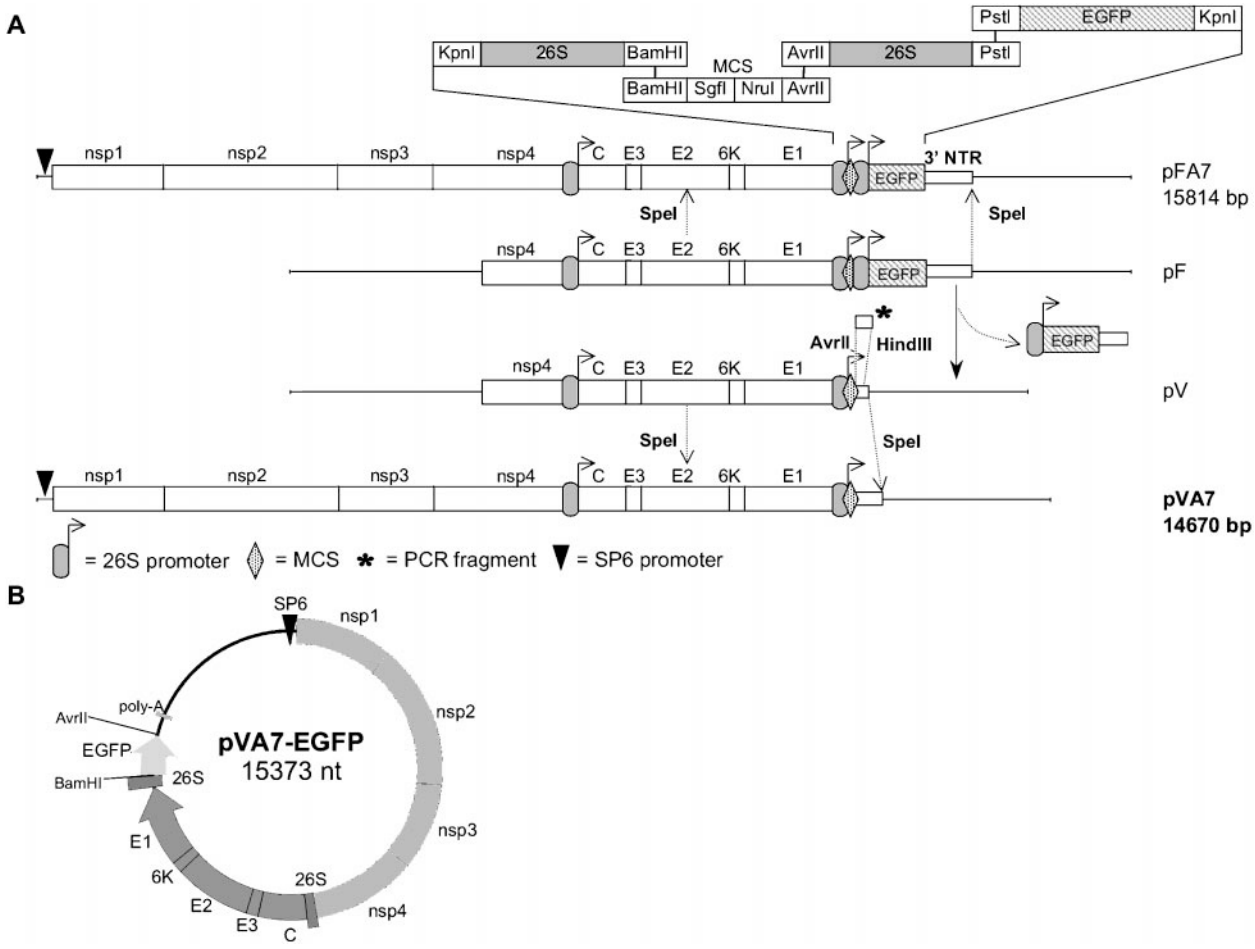

Figure 1 (**A**) Construction of the expression vectors FA7 and VA7. A cassette containing the viral 26S promoter, MCS, another 26S promoter, and the EGFP gene was inserted into a KpnI site in the 3′ NTR of rA774 (nucleotide 11193) in an intermediate plasmid pF. A SpeI fragment from pF was used to replace the corresponding region in prA774 (Tuittila *et al*, 2000). This gave a triple subgenomic construct pFA7. To create the double subgenomic vector VA7 the latter 26S promoter, EGFP and part of the 3′ NTR were excised from pF with AvrII and HindIII. This sequence was then replaced by a PCR fragment from prA774 (nucleotides 11440–11688), shortening the 3′ NTR by 247 nucleotides and forming intermediate plasmid pV. A region of pV was subsequently cut out with SpeI and used to replace the corresponding fragment in prA774 (nucleotides 9032–14800), completing pVA7. (**B**) A schematic drawing of plasmid pVA7-EGFP. Nsp1–4 depict the replicase genes of the virus, C, E3, E2, 6K, and E1 depict the structural genes.

virulence (Santagati *et al*, 1998). We have found that FA7 can maintain expression of inserts longer than 2 kb in many settings (not shown). However, removal of the EGFP gene, the latter 26S promoter, and part of the 3′ NTR of A7(74) provided an expression vehicle, VA7, with a larger insert capacity (2 to 3 kb). To characterize our expression vector, we inserted the reporter EGFP into the MCS of VA7, creating VA7-EGFP (Figure 1**B**).

VA7 efficiently infects several mammalian cell lines

To analyze the transduction efficacy of the double-subgenomic expression vector as a function of infected cells over time, and the stability of the construct as a function of infected cells versus cells positive for EGFP, we infected rat gliosarcoma (9L), rat glioma (BT4C), human malignant melanoma (A2058), mouse neuroblastoma (C-1300), mouse glial (MBA-13), baby hamster kidney (BHK-21), chinese hamster ovary (CHO), and mink lung epithelial (MLEC) cells with VA7-EGFP at a multiplicity of infection (MOI) of 0.1, 1, and 10 and fixed and stained the cells against SFV antigen at 5, 10, and 24 h p.i. We performed a double-immunofluorescence assay using a red fluorescent probe to distinguish between infected cells and cells infected and coexpressing EGFP. The results given in Table 1 show that under the current conditions, for all tested virus concentrations, BHK-21 cells seem the most permissive to infection, displaying the highest number of infected (red) cells 5 and 10 h p.i., and provide the most efficient replication environment, as assessed by the level of mean EGFP fluorescence at 24 h p.i. Nearly 100% of the infected cells were also EGFP-positive, as shown in Figure 2**A**, which is representative of the infection experiments described below.

Table 1 Transduction capacity of VA7-EGFP in mammalian cell lines

	% SFV-positive cells									
	MOI 0.1			MOI 1			MOI 10			
Cell line	*5 h*	*10 h*	*24 h*	*5 h*	*10 h*	*24 h*	*5 h*	*10 h*	*24 h*	*EGFP intensity*
9L Rat	0	1	60–70	<1	1–5	80–90	1	20–30	100	++
A2058	1–5	25–35	100	5–10	45–55	100	10–20	65–75	100	++++
BHK-21	5–10	50–60	100	15–25	70–80	100	30–40	90–100	100	+++++
BT4C	0	15–25	80–90	<1	25–35	90–100	1–5	35–45	100	+++
C-1300	0	0	1–5	0	<1	5–10	0	1	10–20	+/≈
CHO	1–5	30–40	100	5–10	50–60	100	10–20	70–80	100	++++
MBA-13	1–5	40–50	100	5–10	60–70	100	20–30	80–90	100	++++
MLEC	1–5	30–40	100	5–10	50–60	100	10–20	70–80	100	++++

Note. Infected monolayers of cells were fixed and stained for SFV antigens at 5, 10, and 24 h p.i. and observed under fluorescence microscope. Percentages are estimations of the number of SFV-positive cells versus total cells. Cells positive for SFV were also positive for EGFP. Mean EGFP fluorescence intensity at 24 h p.i. is given to indicate relative replication efficiency in each cell line.

In A2058, BHK-21, CHO, MBA-13, and MLEC cell lines, the virus spread rapidly, infecting the entire cell population irrespective of initial MOI. EGFP fluorescence could be detected as early as 4 h p.i., but generally manifested at 5 h p.i., correlating well with alphaviral subgenomic RNA synthesis (Strauss and Strauss, 1994). 9L and BT4C cells showed only weak EGFP fluorescence at this time, whereas the C-1300 neuroblastoma cells seemed unaffected. Indeed, in the few C-1300 cells that were infected, EGFP fluorescence was barely detectable at 24 h, owing probably to both low transduction capacity and reduced gene expression. This is in agreement with studies demonstrating that the C-1300 cell line is extremely refractory to infection by SFV A7 (Atkins, 1983). As shown by our results, the transduction capacity of VA7 varies in several mammalian cell lines, a feature common also for wild-type SFV and the closely related SIN (Wahlfors *et al*, 2000). The number of infectious particles did not alter the replication efficiency per se.

VA7 has similar tropism and replication rate compared to the parental strain in cultured cells

We wanted to investigate whether VA7 indeed behaves similarly to the parental strain A7(74). To further characterize the replication kinetics of VA7, and to compare with the parental strain rA774 (Tuittila *et al*, 2000), as well as wild-type SFV (Liljeström *et al*, 1991b), we infected with each virus at MOI 0.1, 1, and 10 in parallel three cell lines differing significantly in permissiveness, BHK-21, 9L, and C-1300, and fixed and stained the cells at 5, 10, 24, 32, and 48 h p.i. The results given in Table 2 clearly show that the infectivity and replication rate of VA7-EGFP are indistinguishable from those of rA774. Both viruses infected cells with the same preference and replicated equally fast in each separate cell line. No cell line–specific difference in the mean fluorescence intensity of the SFV-positive cells was found between VA7 and rA774, at any given sampling point. Interestingly, differences in replication rate could be detected by fluorescence microscopy between both VA7-EGFP and rA774 compared to SFV4 (Table 2). The differences were more apparent with low MOI and varied between different cell lines, an observation consistent with comparisons between SFV A7 and wild-type virus in cultured cells (Atkins, 1983; Atkins *et al*, 1990; Glasgow *et al*, 1997). Although all three viruses spread equally fast in BHK-21 cells, SFV4 replicated somewhat faster in 9L and C-1300 cells. Virus replication correlated with the appearance of cytopathic effects as shown in Figure 2**B** for 9L cells infected at MOI 1. No difference in cell type tropism could be detected between VA7-EGFP and rA774 compared to SFV4, which is in agreement with previous comparative studies of avirulent and virulent SFV (Atkins, 1983; Gates *et al*, 1985; Atkins *et al*, 1990; Glasgow *et al*, 1997, but see Ehrengruber *et al*, 2003).

VA7 is cytotoxic to cultured cells

Unlike in infected adult rodent CNS, in the CNS of neonatal mice and in cell culture, avirulent SFV strains give rise to apoptotic cell death (Glasgow *et al*, 1997; Allsopp *et al*, 1998). To analyze the cytotoxicity of VA7-EGFP and to compare with the parental A7(74) strain and wild-type SFV, we observed the infected cells (above) using phase-contrast optics. The results are summarized in Table 3 for infections with MOI 1, showing that the outcome varies from complete cell loss and dying cells to normal morphology (in uninfected control). Phase-contrast images of 9L cells infected with VA7-EGFP at MOI 1 are shown in Figure 2**B**, revealing severe cytotoxicity at later time points with all viruses. Taken together, the results in Table 2, Table 3, and Figure 2 show that the cytopathic effects result from virus replication and that cells permissive to infection are also susceptible to cell death. Using MOI 0.1, cytotoxicity was initially delayed, corresponding to the smaller number of infected cells, but later, by 48 h, resulted in a similar degree of morphological changes and cell loss as shown in Table 3. The C-1300 neuroblastoma cells

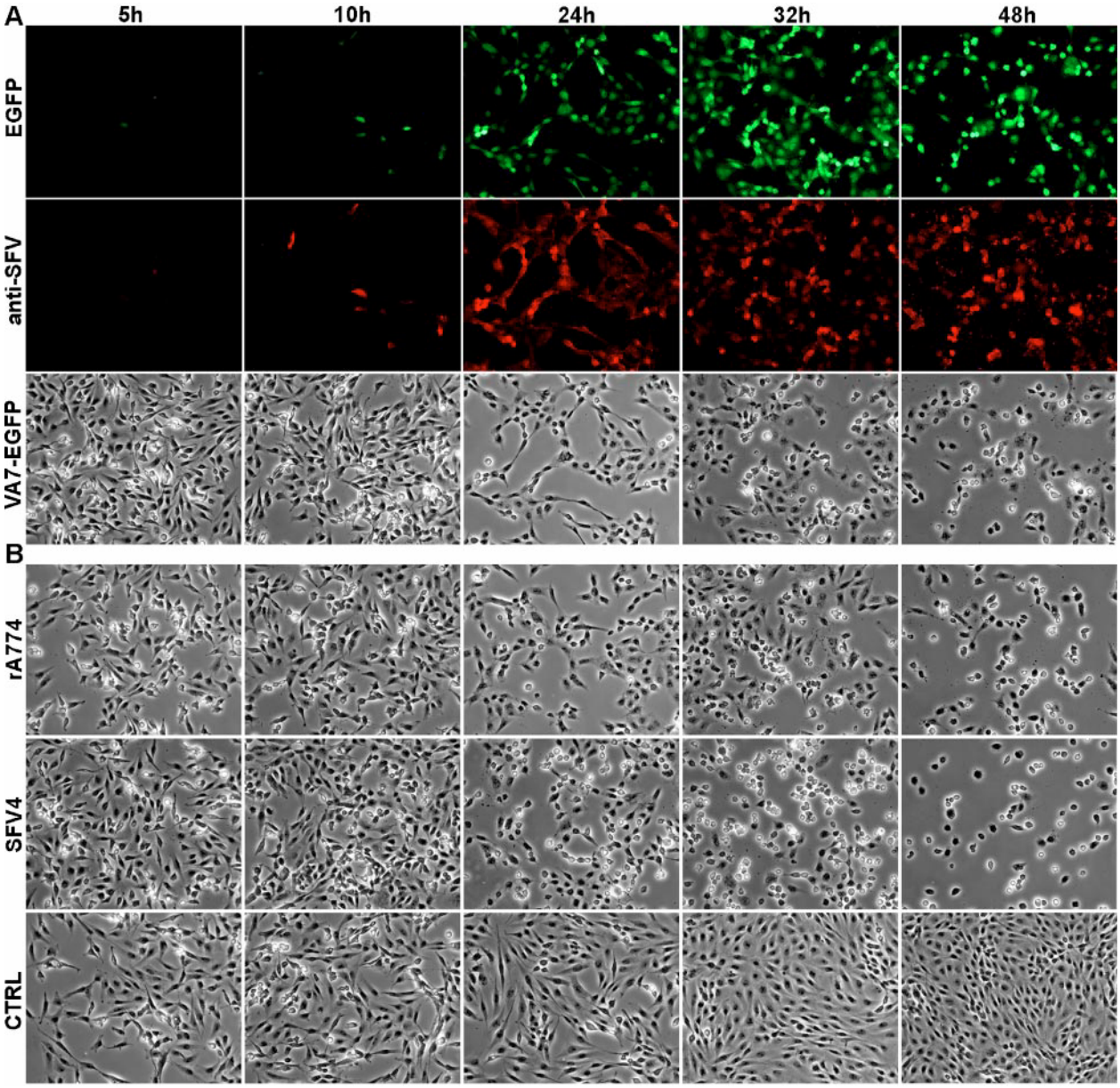

Figure 2 (**A**) Replication of VA7-EGFP in 9L cells infected at MOI 1. The cells were fixed and stained with SFV-specific antibody at indicated time points p.i. The number of cells expressing EGFP (*green*) equals the number of cells positive for SFV antigen (*red*). Phase-contrast images are given to show the total number of cells. With prolonged infection virus spreads and infects the entire cell culture. (**B**) Comparison of the cytotoxic effects caused by VA7-EGFP, rA774, and SFV4. The phase-contrast images demonstrate VA7 has similar cytotoxicity compared to parental rA774. As shown, SFV4 gives rise to cytopathic effects faster than the two avirulent strains, correlating with its higher replication rate in 9L cells (Table 2). Uninfected control is included.

were more resistant to infection and cell death by both wild-type SFV and the avirulent strains than the other cell lines tested. However, given that the viruses described here were replication competent and that an increasing number of SFV-positive cells was detected over time, we believe most cell types in culture, regardless of species or origin, eventually succumb to SFV infection. This claim is supported by experimental data from studies on SFV cytopathogenicity in cell culture (Atkins, 1983; Gates *et al*, 1985; Glasgow *et al*, 1997). We conclude that VA7-EGFP shares not only the tropism and replication kinetics of the parental strain, but also its cytotoxicity. Although it has been reported that EGFP is cytotoxic *in vitro* (Detrait *et al*, 2002), we did not observe any increased cytopathogenicity conferred by the heterologous gene product based on morphological changes and cell loss in culture.

Table 2 Comparison between VA7, rA774, and SFV4

	% SFV-positive cells								
	VA7-EGFP			rA774			SFV4		
	MOI			MOI			MOI		
Cell line/time point	*0.1*	*1*	*10*	*0.1*	*1*	*10*	*0.1*	*1*	*10*
9L Rat									
5 h	0	<1	1	0	<1	1	1–5	5–10	10–20
10 h	1	1–5	20–30	1	1–5	20–30	10–20	20–30	40–50
24 h	60–70	80–90	100	60–70	80–90	100	90–100	100	100
32 h	100	100	100	100	100	100	100	100	100
48 h	100	100	100	100	100	100	100	100	100
BHK-21									
5 h	5–10	15–25	30–40	5–10	15–25	30–40	5–10	15–25	30–40
10 h	50–60	70–80	90–100	50–60	70–80	90–100	50–60	70–80	90–100
24 h	100	100	100	100	100	100	100	100	100
32 h	100	100	100	100	100	100	100	100	100
48 h	100	100	100	100	100	100	100	100	100
C-1300									
5 h	0	0	0	0	0	0	0	0	<1
10 h	0	<1	1	0	<1	1	1–5	5–10	10–20
24 h	1–5	5–10	10–20	1–5	5–10	10–20	10–20	20–30	30–40
32 h	5–10	10–20	20–30	5–10	10–20	20–30	30–40	40–50	50–60
48 h	10–20	20–30	30–40	10–20	20–30	30–40	50–60	60–70	70–80

Note. Infected monolayers of cells were fixed and stained for SFV antigens at indicated time points and observed under fluorescence microscope. Percentages are based on the number of SFV-positive cells versus total cells. Note that no relative difference between VA7-EGFP and rA774 could be detected in any of the tested cell lines.

VA7 infects and replicates in CNS cells in vitro

Extensive studies employing electron microscopy and immunohistochemistry or *in situ* hybridization coupled with morphological analyses have shown that SFV A7(74) productively infects both neurons (Pathak *et al*, 1976, 1983; Fazakerley *et al*, 1993; Amor *et al*, 1996; Oliver *et al*, 1997; Scallan and Fazakerley, 1999) and glial cells (Pathak *et al*, 1976, 1983; Fazakerley *et al*, 1993) in the CNS of rodents. To test whether VA7-EGFP has a similar capacity, we used an *in vitro* preparation of brain tissue and injected 12 cultured rat hippocampal slices with different concentrations of virus. Figure 3**A** and **B** show a representative slice culture at 1 and 2 days, respectively, after injection of 100-fold diluted virus stock. Already at day 1 p.i., strongly EGFP-positive cells were found. Interestingly, as determined by the cellular morphology and location within the tissue slice, the majority of these cells were of non-neuronal origin, such as ependymal and glial cells that are normally not transduced in this culture system by wild-type SFV vectors (Ehrengruber *et al*, 1999, 2001). In agreement with these observations are reports showing that different SFV strains have preference for oligodendrocytes rather than neurons in infections of mouse and rat mixed primary brain cultures (Gates *et al*, 1985; Atkins *et al*, 1990). *In vivo* infection by SFV of ependymal cells has not been observed, although the underlying neuronal and glial cells have stained virus positive (Fazakerley *et al*, 1993). Besides the non-neuronal cells, apparent on the border of the slice in Figure 3**A** and in greater detail in Figure 3**F**, some EGFP-positive pyramidal cells could also be observed at day 1 p.i. (Figure 3**D**). With increasing time, VA7 infection spread to pyramidal cells rather than non-neuronal cells and led to a prominent EGFP staining of apical and basal dendrites in stratum radiatum and stratum oriens, respectively (Figure 3**B**). A cluster of EGFP-positive pyramidal cells at 2 days p.i. is shown in Figure 3**E**. Taken together, our results show that

Table 3 Cytotoxicity of VA7-EGFP, rA774, and SFV4

Cell line/ time point	% cell loss		
	VA7	*rA774*	*SFV4*
9L Rat			
5 h	0	0	0
10 h	1	1	10
24 h	25	25	50
32 h	50	50	70
48 h	80	80	95
BHK-21			
5 h	0	0	0
10 h	40	40	40
24 h	95	95	95
32 h	100	100	100
48 h	100	100	100
C-1300			
5 h	0	0	0
10 h	0	0	0
24 h	0	0	10
32 h	5	5	30
48 h	10	10	40

Note. Cell death was assessed by observing under microscope. The shown data are for infections with MOI 1. Cell death is directly relative to permissiveness (see Table 2).

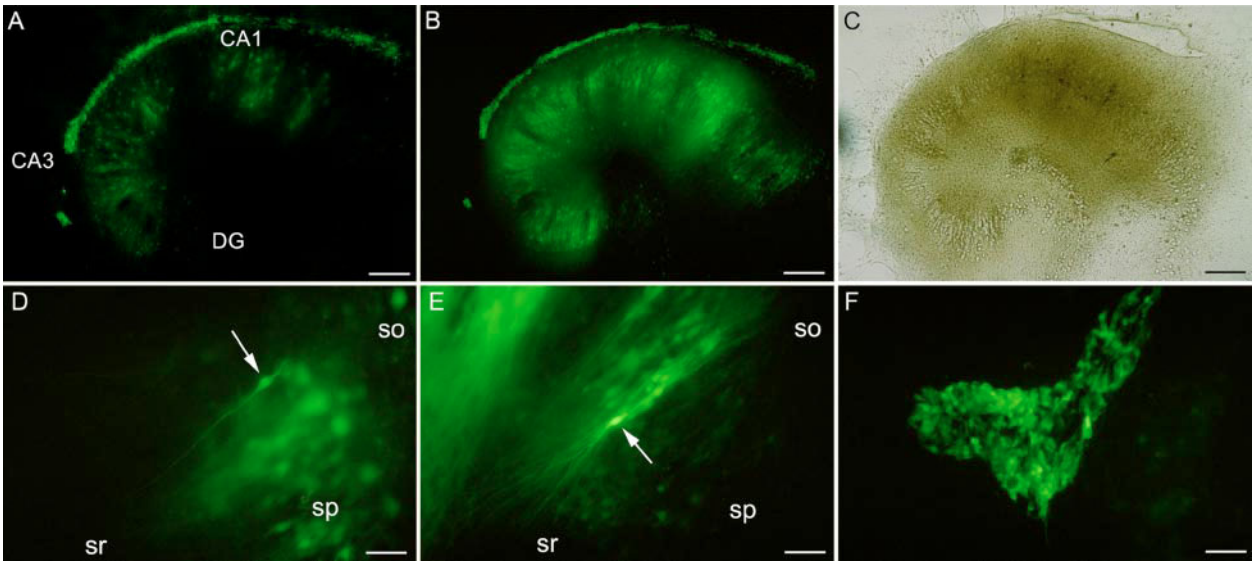

Figure 3 Infection of cultured rat hippocampal slices with VA7-EGFP. Fluorescence illuminations and bright field image of the same hippocampal slice at 1 (**A**) and 2 (**B**, **C**) days p.i. Note the EGFP-positive non-neuronal cells along the perimeter of the slice, which are not observed upon infection with wild-type SFV vectors expressing EGFP (Ehrengruber *et al*, 1999, 2001). (**D**, **E**) CA1 and CA3 pyramidal cells from different slices at 1 and 2 days p.i., respectively; selected EGFP-positive cells are indicated by the arrows. (**F**) EGFP-positive non-neuronal cells in the fimbria fornix from a slice at 1 day p.i. Abbreviations: DG, dentate gyrus; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Bars: 260 μm (**A**, **B**, **C**), 60 μm (**D**, **E**, **F**).

VA7 transduces both neurons and non-neuronal CNS cells *in vitro*.

Infectious virus is released from hippocampal slices

To investigate whether the spread of EGFP fluorescence in CNS tissue culture is accompanied by release of infectious particles, we collected culture medium from infected hippocampal slices at regular intervals and assayed fractions on monolayers of BHK-21 cells by counting the number of EGFP-positive cells after a 24-h incubation. Representative results from five separate slice cultures are shown in Figure 4, demonstrating that infectious virus is indeed released into the extracellular space. The rate and extent of virus release varied greatly between the cultures, a phenomenon correlating with the highly variable virus titers obtained from the CNS of infected adult mice (see below). Although the reason for this variability remains unclear, it can be speculated that in addition to nonspecific variation, caused by, e.g., differences in culture medium and slice preparation, the maturity and health of the CNS cells affects the outcome. The maturation state of CNS cells has been shown to greatly influence SFV A7(74) virus release *in vivo* and no free virus particles have been observed in the CNS of adult mice using electron microscopy (Pathak *et al*, 1976; Fazakerley *et al*, 1993). Interestingly, as far as could be detected, no virus was released at day 1 p.i., despite otherwise prominent infection (Figure 3**A**). It can be speculated that the cells initially restrict virus release but that eventually this restriction is overcome, possibly due to altered cellular homeostasis or the lack of more stringent environment present *in vivo*.

VA7 expresses heterologous genes in the CNS of mice

To determine if VA7 is able to mediate expression of heterologous protein in the CNS of mice, we infected i.p. 10 5-week-old female BALB/c mice with

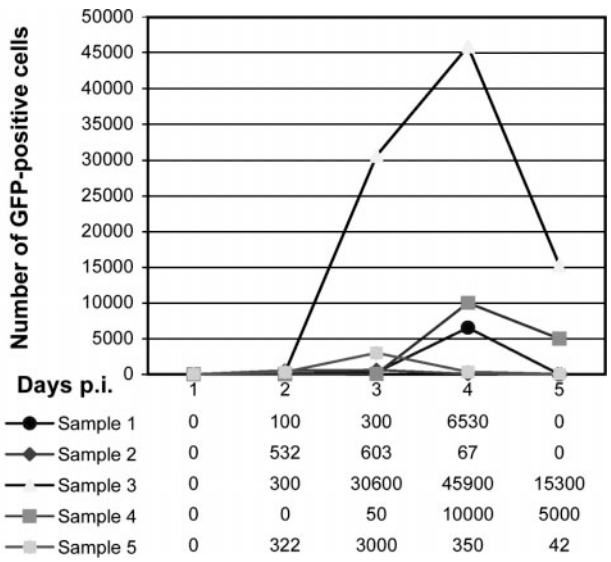

Figure 4 Time course of infectivity release from organotypic rat hippocampal slices infected with VA7-EGFP. Slice cultures at 17 days *in vitro* were infected with VA7-EGFP and cultured for an additional 5 days. At increasing time points p.i., indicated in the graph, 100 μl of slice culture medium was removed and assayed on BHK-21 cells for the release of infectious particles. Data points represent means ($n = 3$) of the number of EGFP-positive BHK-21 cells per 35-mm plastic Petri dish at 1 day after addition of 10 μl slice culture medium.

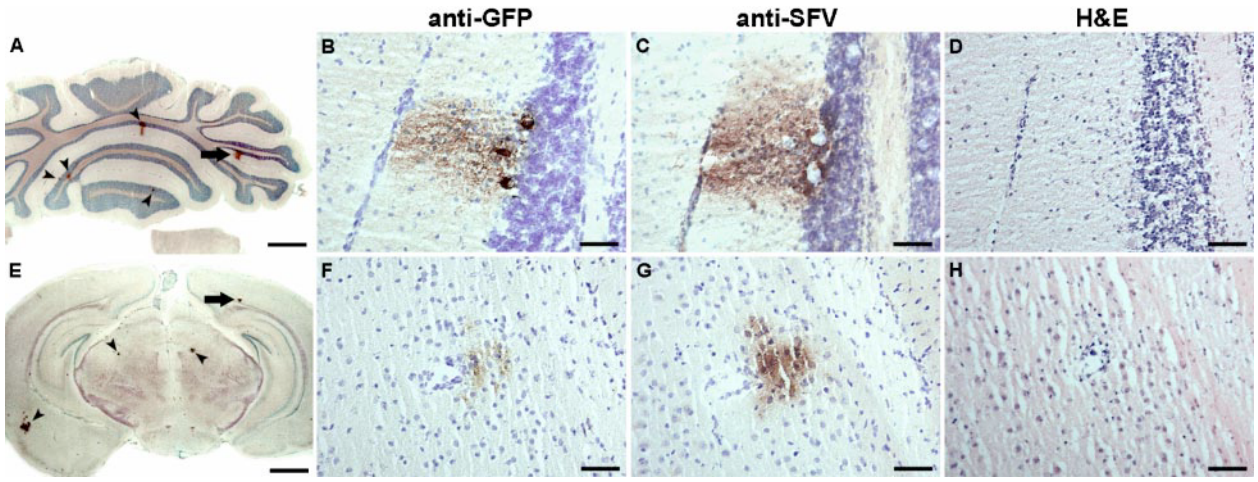

Figure 5 Immunohistochemical detection of SFV antigens (**C**, **G**) and EGFP (**B**, **F**) in CNS cryosections from VA7-EGFP–infected mice as well as hematoxylin/eosin (H&E) staining for detection of immune infiltrates (**D**, **H**). The pictures are representative of all mice analyzed from days 5 and 6 p.i. EGFP is detected both in cerebellum (**B**) and cerebrum (**F**), colocalizing with the SFV-positive foci (**C**, **G**). H&E shows no increased cellular staining, indicating a lack of immune infiltrate at the site of infection. To estimate nonspecific staining, controls without primary antibody were included (data not shown). Scale bar in (**A**) and (**E**) corresponds to 1 mm, in other parts to 100 μm. *Arrows* indicate the virus plaques magnified in (**B–D**) and (**F–H**). *Arrowheads* indicate other SFV-positive foci in the same section.

10^6 plague-forming units (PFU) of VA7-EGFP virus and sampled five of the infected mice at days 5 and 6 after challenge when CNS viral titers are peaking (Tuittila *et al*, 2000). Brains were removed and cryosectioned and the tissue was immunostained against SFV antigens as well as EGFP, and viewed under microscope. The results in Figure 5 clearly show the presence of EGFP colocalizing with characteristic SFV-positive foci formed in adult CNS by A7(74) infection (Fazakerley *et al*, 1993; Subak-Sharpe *et al*, 1993; Oliver *et al*, 1997; Scallan and Fazakerley, 1999; Tuittila *et al*, 2000). Viral antigen was detected throughout the brain in all sampled mice in apparently randomly distributed patches (Figure 5**A**, **E**). In addition, although not analyzed in the present study, we have detected colocalization of virus and heterologous gene product in the spinal cord using VA7 derivatives carrying inserts other than EGFP (not shown). A closer analysis of the foci of infection revealed their mainly perivascular location (Figure 5**B–D**, **F–H**). Approximately 50% of the SFV-positive foci stained positive also for EGFP, indicating that either the level of heterologous gene expression was significantly reduced in nonresponsive regions or that the virus no longer expressed functional EGFP. However, it must be pointed out that in all likelihood the sensitivity of the immunoassay is significantly lower for EGFP compared to SFV, as the latter antibody is polyclonal and reacts with multiple viral nonstructural and structural proteins, quantitatively exceeding the heterologous gene product. Supporting this claim, we also have observed variation in staining using inserts other than EGFP, with more than 80% of the SFV-positive foci staining positive for heterologous gene product (not shown).

Although the identity of the infected cells in the CNS of the mice remains unclear, based on the morphology of EGFP-positive cells in infected hippocampal slices (Figure 3) and on published data from infections with the parental A7(74) strain (Pathak *et al*, 1976, 1983; Fazakerley *et al*, 1993; Amor *et al*, 1996; Oliver *et al*, 1997; Scallan and Fazakerley, 1999), it is most likely that the infected cells were both neurons and oligodendrocytes. Because the expression vector is based on the genome of SFV A7(74), VA7 has the same envelope glycoproteins and recognizes the same cellular receptors as parental A7(74), lending further support to this assumption.

Replication of VA7 is restricted in adult mouse brain

To analyze the infection of VA7 in the adult mouse CNS, and to compare with the parental strain rA774, we plaque assayed on BHK-21 cells the brain homogenate from each of the 10 infected mice above. Titers ranged from $1 \approx 10^2$ to $4.6 \approx 10^4$ (average of $5.6 \approx 10^4$) PFU per gram tissue in mice sampled 5 days p.i. and $1.84 \approx 10^2$ to $6.9 \approx 10^5$ (average of $1.7 \approx 10^5$) PFU per gram in mice taken 6 days p.i. The numbers are similar to CNS titers obtained in several studies with SFV A7(74) (Fazakerley *et al*, 1993; Subak-Sharpe *et al*, 1993; Scallan and Fazakerley, 1999; Tuittila *et al*, 2000). As expected from these studies, the virus had already cleared from the blood by days 5 and 6 p.i. On closer inspection, approximately 40% of the plaques were nonfluorescing, indicating that during infection a portion of the virus particles had undergone deleterious changes in the EGFP gene or the 26S promoter. It is unclear whether the EGFP insert was lost or had accumulated mutations to render the protein nonfunctional.

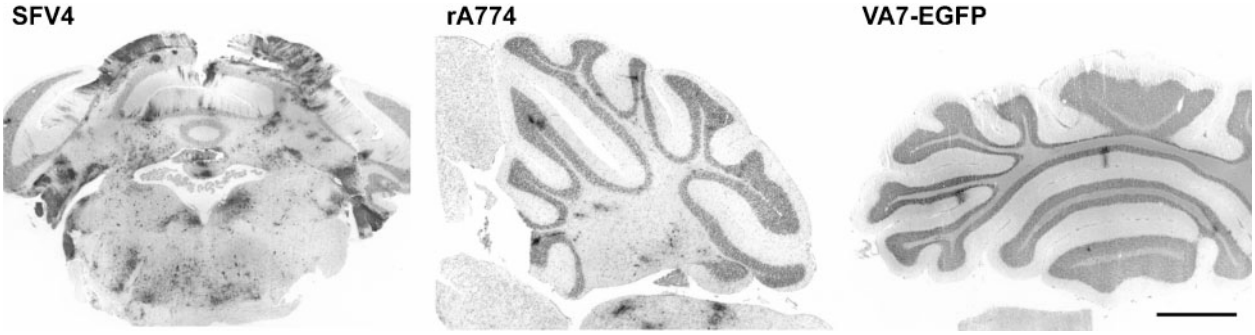

Figure 6 Comparison of viral spread in SFV4-, rA774-, or VA7-EGFP–infected mouse brain by immunohistochemistry using SFV-specific antibody. The infection patterns of VA7-EGFP and rA774 are highly similar, with randomly scattered SFV-positive foci throughout the brain, whereas extensive virus spread can be seen in SFV4-infected brain. Scale bar = 1 mm.

Given the mutation rate of the viral RNA polymerase (Strauss and Strauss, 1994), or the long-term instability of other reported alphaviral double-subgenomic expression vectors (Hahn *et al*, 1992; Chen *et al*, 1995; Pugachev *et al*, 1995), these results were not unexpected.

Importantly, none of the 10 VA7-infected mice (above) displayed clinical symptoms during the infection, which is in accordance with the avirulent nature of the parental virus established in previous analyzes (Fazakerley *et al*, 1993; Amor *et al*, 1996; Oliver *et al*, 1997; Tuittila *et al*, 2000). To further elucidate the pathogenicity of VA7-EGFP in the CNS, we stained the brain tissue section with hematoxylin/eosin to detect inflammatory reaction. The results are shown in Figure 5**D** and **H**, indicating that at 5 and 6 days p.i., no significant immune infiltrate is present, which agrees with studies showing SFV A7(74)-induced CNS demyelination, accompanied by infiltration of mainly macrophages, begins at day 11, peaks between days 14 and 21, and is usually cleared by day 35 p.i. (Pathak *et al*, 1983; Fazakerley *et al*, 1993; Subak-Sharpe *et al*, 1993). The cryosectioned tissue appeared structurally normal and we could not detect any marked alterations in cellular morphology due to presence of virus, as compared to SFV-negative cells in the VA7-EGFP–infected brain tissue and mock-infected control. Indeed, it has been shown by electron microscopy, morphological analysis, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay that SFV A7(74) causes only minimal or no cell death in the CNS of adult mice (Pathak *et al*, 1976, 1983; Fazakerley *et al*, 1993; Subak-Sharpe *et al*, 1993; Amor *et al*, 1996; Scallan and Fazakerley, 1999). These studies show mature neurons are consistently resistant to cell death, even during the demyelination following virus clearance, and that oligodendrocytes also survive, despite extensive myelin loss. The limited neuronal degeneration observed during infection with avirulent SFV seems to be restricted to the poorly differentiated neurons of the olfactory bulb and rostral migratory stream (Sammin *et al*, 1999). In addition, although EGFP is detrimental to cells *in vitro*, it has not been shown to be harmful *in vivo* (Detrait *et al*, 2002), giving us reason to believe our expression vector in itself is nonpathogenic in the adult CNS.

To confirm that VA7-EGFP infection is restricted in adult mouse brain, similar to infection with the parental virus, we compared brain tissue slices from mice infected with VA7-EGFP (Figure 5) with rA774-infected sections, as well as slices infected with wild-type SFV. Figure 6 shows representative pictures of the sections and gives an overview of the infection pattern of VA7-EGFP versus rA774 and SFV4. It is clear from these results that VA7-EGFP has a similar infection profile compared to the parental strain rA774 and that both avirulent viruses are restricted in CNS spread. The same restricted infection pattern has been observed for SFV A7(74) in numerous previous studies and can be compared with the present results (Fazakerley *et al*, 1993; Amor *et al*, 1996; Oliver *et al*, 1997; Scallan and Fazakerley, 1999; Tuittila *et al*, 2000). Taken together, the obtained CNS virus titers and the results in Figures 5 and 6 show that VA7-EGFP infection in adult mouse brain is restricted, yet productive, and similar to infection with parental A7(74).

Discussion

There were two possible locations in rA774 for insertion of the second subgenomic promoter and the multiple cloning site: one in the region between the nonstructural and structural genes and the other in the 3′ NTR. As the 3′ NTR of SFV A7(74) has a unique 101-nucleotide sequence immediately downstream of the E1-coding region, which does not affect viral pathogenic properties (Santagati *et al*, 1998), this site was chosen for insertion of the expression cassette (Figure 1). The properties of any replication-competent alphaviral expression vector, however, are determined not only by the properties of the viral backbone on which they are constructed, but also by the properties of the inserted sequence elements.

Potentially limiting the use of these vectors are both the size and stability of the insert. In this regard, FA7, carrying a third subgenomic promoter directing the reporter EGFP gene, would inevitably face restrictions. Small inserts in other alphaviral expression vectors have been reported to be more stable than large ones in cultured cells, with a maximum limit of 2 to 3 kb (Hahn *et al*, 1992; Chen *et al*, 1995; Pugachev *et al*, 1995). We therefore chose to use VA7, as it would most probably accommodate larger fragments than FA7. To characterize VA7, we chose to use EGFP, as it is convenient and accurate to detect, innocuous to cellular or viral function, and of smaller size than the supposed limit mentioned above. Although Detrait *et al* (2002) recently reported that EGFP causes apoptotic cell death when expressed in cultured mouse primary cortical neurons, the cytopathic effects of the protein manifested fairly slowly (over a period of 5 days) and have not been detected *in vivo*.

VA7 is built on the genome of rA774, a cDNA clone of SFV A7(74) (Tuittila *et al*, 2000), and the viruses have the same nonstructural and structural proteins. The only difference between the viruses resides in the 3′ NTR of the genome, in a nonconserved segment shown not to affect virulence (Santagati *et al*, 1998). Thus we expected VA7 to share the cell type tropism, neuroinvasiveness, and apathogenicity of the parental strain. Indeed, our results show, on one hand, that VA7-EGFP spreads in the same manner in cell culture as does rA774 (Figure 2, Tables 2, 3). The expression vector infected cells in culture with the same preference and speed as rA774, which also indicates that the replication efficacy of VA7 is not diminished due to the presence of the additional 26S promoter, the EGFP open reading frame (ORF) or the 247-nucleotide deletion in the 3′ NTR. Although measuring virus titer in culture supernatants and determining cellular RNA and protein levels would have offered a more accurate analysis, we feel tropism and replication kinetics can be measured accurately by comparing the number of infected cells, which reflects virus spread and thus the production of new virions, and fluorescence intensity of the infected cells, which is a marker of viral protein production.

On the other hand, neither the additional 26S promoter nor EGFP ORF increased virus cytotoxicity in cell culture (Figure 2, Table 3), which is important when the vector is considered for CNS gene targeting. We could not detect increased cellular infiltrate or morphological differences between SFV-positive and SFV-negative CNS cells in the brain tissue slices from mock-infected or VA7-EGFP–infected mice (Figure 5**D**, **H**), which indicates the expression vector per se is not more pathogenic than the parental strain. The pathogenesis of SFV A7(74) in animals has been studied extensively and in adult immunocompetent mice the infection remains subclinical (Fazakerley *et al*, 1993; Oliver *et al*, 1997). The virus initially replicates in the periphery, giving rise to a plasma viremia that is cleared by day 2 p.i. (Pusztai *et al*, 1971). It then crosses the blood-brain barrier and establishes small, perivascular foci of infection throughout the CNS (Fazakerley *et al*, 1993; Oliver *et al*, 1997; Tuittila *et al*, 2000). Consistent with these observations, we show that VA7-EGFP infects adult mouse CNS in a random, patchy fashion (Figure 5**A**, **E**), much like parental rA774 but quite unlike wild-type SFV (Figure 6). The SFV-positive foci seem to be located near blood vessels (Figure 5**G**). More detailed analyses have shown that the CNS resident neurons and oligodendrocytes are resistant to A7(74)-induced cell death, contrary to cultured cells, despite productive infection (Pathak *et al*, 1976, 1983; Fazakerley *et al*, 1993; Subak-Sharpe *et al*, 1993; Amor *et al*, 1996; Scallan and Fazakerley, 1999). Moreover, in these studies, the mortality rate of SFV A7(74) has been found to be practically zero, attesting to the extreme avirulence used as a criterium when this particular strain was originally isolated (Bradish *et al*, 1971). In agreement, none of the 10 infected mice in the present study displayed any clinical signs and all mice remained healthy throughout.

We have thus established that the modifications introduced to the genome of rA774, in creating the expression vector VA7, have not reduced the ability of the virus to replicate and spread, nor conferred an increase in cytotoxicity or virulence. Although the expression of the heterologous gene in the periphery or the longer time course of expression have not been analyzed, we are confident the short-term infection of VA7 warrants its applicability in CNS gene transfer. We base this assumption on our results shown in Figure 5, demonstrating that VA7 indeed delivers and drives expression of heterologous genes in the CNS of adult mice. The amount of expressed gene product remains to be investigated, but for applications such as treatment of degenerative neurological disorders, it has been shown that many protein modulators of CNS homeostasis, which are likely candidates for future therapy applications, are effective even in the pico- and femtomolar ranges (Steiner *et al*, 1997; reviewed by Thorne and Frey, 2001). The authors of these reports discuss the limitations of gene therapy of CNS disorders using potent immune or trophic regulators, pointing out that delivering these effectors in a widespread, non-invasive manner remains one of the largest obstacles of successful treatment. In this regard, the innate tropism of our expression vector may serve as an asset, as intraperitoneal infection with VA7 is CNS-wide. Naturally, certain heterologous gene products are expected to be toxic outside the CNS or have a negative impact on the peripheral immune system, in which case the expression vector would have to be administered via alternative routes. SFV can be administered both intranasally and intracranially, making little difference for the infection pattern in the CNS (Pathak *et al*, 1976; Sammin *et al*, 1999). In

any case, because the properties of the heterologous insert are likely to determine the altered phenotype of each new construct, compared to the backbone vector VA7, the infection characteristics and effects of each novel virus must be investigated individually and are as such not the focus of this paper.

Figuratively, we envision our expression vector to function as a shotgun-therapy tool for rapid, transient modulation of immune and trophic responses in the CNS. The CNS is considered a difficult site for gene targeting mainly because of the impermeability of the blood-brain barrier. Viral vectors have therefore received much attention as means for delivering effectors into neural cells. Work has been conducted with vectors based on adenovirus, herpes simplex virus type 1, adeno-associated virus, and human immunodeficiency virus (reviewed in Hermens and Verhaagen, 1998), but also alphaviral replicons have been used to target the CNS (Altman-Hamamdzic *et al*, 1997). VA7 differs from these vectors in that it is replication competent and neurotropic by nature. Because of this, it does not have to be delivered intracranially by stereotactic infusion as do most of the above-mentioned vectors, unless the properties of the heterologous gene product specifically require it. Nevertheless, attempts to target the CNS via alternative routes have been made with both adenoviral vectors (Boulis *et al*, 1999; Ghadge *et al*, 1995) and vectors based on herpes simplex type 1 (Dobson *et al*, 1990; Keir *et al*, 1995), and lentiviral vectors were recently shown to transduce CNS neurons following intramuscular injection (Mazarakis *et al*, 2001), but despite that these vectors are capable of partially spreading into the CNS, the infection patterns there remain very limited and restricted to specific areas. The overall efficiency of peripheral administration of these vectors compared to intracranial delivery is also very poor. In contrast, we demonstrate here that a single intraperitoneal injection of 10^6 PFU VA7-EGFP is adequate to cause a randomly scattered infection of the entire CNS of adult mice. Although some of the SFV-positive foci did not stain positive for EGFP, and a fraction of the viruses recovered from the brains were EGFP-silent, the total distribution and amount of heterologous gene expression is clearly detectable and should be sufficient for significant biological effect *in vivo*. Indeed, preliminary therapy of animal models of CNS disease, using VA7 derivatives carrying potent immune modulators, show significant amelioration of the clinical symptoms, coupled with positive transgene expression in the CNS (results to be published elsewhere).

A major hurdle for viral gene therapy in general is the immunogenicity and pathogenicity of the vectors used. Vectors based on adenovirus and herpes simplex type 1 elicit strong immune responses and may cause cytopathic damage to neurons, which restricts their applicability (McMenamin *et al*, 1998; Benihoud *et al*, 1999). In adult immunocompetent animals, parental A7(74) initially gives rise to a brief blood viremia (Pusztai *et al*, 1971) and later CD8+ T cell–mediated demyelination in the CNS of infected mice (Subak-Sharpe *et al*, 1993; Fazakerley *et al*, 1993; Amor *et al*, 1996). SFV A7(74) also elicits a protective immune response involving at least all subclasses of virus-specific immunoglobulin G (Fazakerley *et al*, 1993). Although not analyzed in the present study, the VA7 backbone virus is expected to induce a similar immune response in normal animal. However, neither the inflammatory reaction in the CNS nor the antibody response, which does not cause apparent illness, is likely to restrict the usefulness this vector system, as it is transient and aimed at short-term gene expression. The immune response eventually eliminates all traces of virus, guaranteeing the transitory nature of the VA7 vector system.

A7(74)-induced, immune-mediated demyelination is cleared within 35 days, leaving the CNS largely unscathed (Pathak *et al*, 1983; Subak-Sharpe *et al*, 1993). Also, because the virulence determinants of SFV A7(74) are polygenic, it is not likely that the vector converts to a virulent form (Tuittila *et al*, 2000). In this respect, mutant SFV replicons have recently been developed with reduced cytotoxicity, and such mutations may readily be introduced to the VA7 backbone to further attenuate the expression vehicle (Lundstrom *et al*, 2003). However, our novel vector is applicable also for other purposes, such as CNS cancer therapy. We have shown VA7-EGFP efficiently infects and kills several tumor cell lines (Figure 2, Tables 1–3). In addition, it is well known that SFV A7(74) infects and destroys immature CNS cells, such as neurons in neonatal mouse (Oliver *et al*, 1997; Allsopp *et al*, 1998). Given that SFV A7(74) is cytotoxic to immature cells in the CNS, but not to the fully differentiated mature cells found in adult CNS, a potential for preferential spread and destruction of proliferating cell populations, such as CNS tumors, exists. In these settings, the insert in VA7 would in all likelihood be chosen to enhance cancer cell killing or invoke anti-tumorific immune responses. Experiments of these types are ongoing in our laboratory.

Ultimately, the wide host cell range of VA7 may facilitate its utilization in settings other than CNS expression. For instance, attenuated VEE vectors have been successfully used to express antigens of human immunodeficiency virus in vaccination experiments (Davis *et al*, 1996; Caley *et al*, 1999). Also, because VA7 is in essence a replicative form of SFV A7(74), with the added ability to express heterologous genes of interest, it should prove useful in the study of different aspects of SFV virology. Finally, other replication competent alphaviral vectors have been used for successful *in vivo* gene transfer into the CNS, albeit through invasive intracranial administration (Levine *et al*, 1996; Liang *et al*, 1998), supporting the credibility of our novel system. Although VA7 in its present form is not applicable for long-term gene expression, or in settings where it is critical to avoid immune responses to the vector, it constitutes a valuable

addition to the growing panel of viral expression vehicles and expands the choices of tools available for specific gene delivery purposes. In conclusion, we would like to point out that despite its limitations, this system offers some benefits compared to existing viral vectors and it can be conveniently applied for rapid, noninvasive CNS gene transfer, as well as other purposes. It will be interesting to investigate in which settings this system proves to be most productive.

Materials and methods

Construction of expression vectors

For the construction of the replication competent vectors, the 3′ NTR of plasmid prA774, containing the full-length cDNA of SFV A7(74) (Tuittila *et al*, 2000) was used. Figure 1**A** shows the cloning steps and the general layout of the constructs. An MCS, containing recognition sites for enzymes BamHI, SgfI, NruI, and AvrII, was generated by annealing two complementary oligonucleotides. The 26S promoter used throughout this project comprises nucleotides ≈115 to +51 relative to transcription start and was obtained by polymerase chain reaction (PCR) from pSP6-SFV4 (Liljeström *et al*, 1991b). The EGFP gene was obtained by PCR from pIRES-EGFP (Clontech, USA). pVA7-EGFP, shown in Figure 1**B**, was created by inserting a PCR fragment of the EGFP gene from pIRES-EGFP into the MCS of pVA7, using BamHI and AvrII. All relevant constructs were verified by DNA sequencing.

Recombinant viruses, cells, and tissue culture

Viruses were generated by SP6 RNA polymerase transcription of plasmid templates followed by electroporation of infectious RNA into baby hamster kidney cells (BHK-21), as described previously (Santagati *et al*, 1995). The plasmids could not be linearized before transcription due to the presence of a second SpeI recognition site within the genome but this was of little significance as the vectors are self-replicating and capable of propagating in cell culture. The obtained virus stocks were plaque titered as described (Santagati *et al*, 1995). MBA-13 cells were sustained in Eagle's minimum essential medium (MEM) (GIBCO-BRL) supplemented with 5% inactivated fetal calf serum (FCS) (Autogen Bioclear, UK) and streptomycin/penicillin (10 μg/ml). BHK-21 cells were grown in Glasgow MEM (GIBCO-BRL) with 5% FCS, 5% tryptose phosphate broth (GIBCO-BRL), and streptomycin/penicillin (10 μg/ml). Rat gliosarcoma (9L Rat), rat glioma (BT4C), human malignant melanoma (A2058), and mouse neuroblastoma (C-1300) cells were cultured in Dulbecco's modified Eagle medium (DMEM) (GIBCO-BRL) supplemented with 10% FCS and streptomycin/penicillin (10 μg/ml). MLECs were grown in DMEM in the presence of gentamycin (20 μg/ml) and 10% FCS. CHO cells were maintained in RPMI 1640 (Sigma, USA) in the presence of 10% FCS, 1% (*w*/*v*) L-glutamine, and streptomycin/penicillin (10 μg/ml). All cultures were maintained at 37℃ in a humidified atmosphere containing 5% CO_2. A2058 cells were kindly provided by Dr. Veli-Matti Kähäri, University of Turku, and 9L and BT4C cells by Dr. Jarmo Wahlfors, University of Kuopio. MLECs were a gift of Prof. Daniel Rifkin, New York University. Hippocampal slices were prepared from 6-day postnatal rats and cultured in roller-tubes as described (Gähwiler, 1981).

Infection and analysis of cell lines and hippocampal slices

To characterize vector transduction capacity and replication kinetics *in vitro*, as well as to test stock viability, several cell lines were infected with VA7-EGFP at varying concentration and fixed and stained against SFV antigen at 5, 10, and 24 h p.i., as indicated in Table 1. The cells were grown as monolayers on coverslips in 24-well culture dishes. At the indicated time points, cells were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 min at 4℃. Infected cells were detected by immunofluorescence staining with polyclonal rabbit anti-SFV antibody (from immunized animal) at a dilution of 1:2500 and trimethylrhodamine (TRITC)-conjugated goat anti-rabbit antibody (Zymed Laboratories, Inc., USA) at a 1:50 dilution. The coverslips were mounted on microscope slides and observed under an epifluorescence microscope (Leica DM IRB).

To further analyze the transduction efficacy and growth kinetics of the double-subgenomic expression vector and to compare it with the parental strain as well as wild-type SFV, we infected 9L Rat, BHK-21, and C-1300 cells with VA7-EGFP, rA774 (Tuittila *et al*, 2000) and SFV4 (Liljeström *et al*, 1991b) at MOI 0.1, 1, and 10 and fixed and stained the cells at 5, 10, 24, 32, and 48 h p.i., as described. Cytopathic effects of the virus were followed by observing cell morphology using phase-contrast optics. Dying cells were contracted, had pycnotic nuclei, and had lost most cellular processes.

Hippocampal slices at 21 days *in vitro* were infected and analyzed as described (Ehrengruber *et al*, 1999). Upon infection, slices were incubated at 37℃. To measure virus release from infected tissue slices, 100 μl culture medium was harvested 1, 2, 3, 4, and 5 days p.i. and 10-μl aliquots were assayed on subconfluent monolayers of BHK-21 cells at each time point. The number of EGFP-positive cells was counted under fluorescence microscope as an average of 20 individual areas on a 35-mm culture dish 24 h p.i.

Infection of mice

Ten 5-week-old female BALB/c mice were infected i.p. with 10^6 PFU of VA7-EGFP in 100 μl PBS. Five mice were CO_2-anesthesized and PBS-perfused on days 5 and 6 p.i. and blood and brains were removed for virus titration and immunohistochemistry. The

brains were divided sagittally and one half was used for immunohistochemistry and the other for virus titration. Brain homogenate and blood serum was plaque titered as described (Santagati *et al*, 1995). For comparisons between VA7-EGFP, rA774, and SFV4, 4- to 6-week-old BALB/c mice were inoculated i.p. with 10^6 PFU rA774 or SFV4, in 100 μl PBS. Brains were processed as described.

Immunohistochemistry

CO_2-anesthesized mice were perfused with PBS, brains were removed, snap frozen in liquid nitrogen, embedded in Tissue Tek cooling compound (Miles, IN, USA), and frozen in $\approx$70°C isopenthane. Ten-micrometer coronal cryosections were attached onto Superfrost Plus microscope slides and fixed with acetone. After blocking of endogenous peroxidase activity, the sections were incubated with rabbit anti-SFV antibody, rabbit anti-GFP antibody (Santa Cruz Biotechnology, USA), or normal rabbit serum as control. The primary antibodies were detected with Vectastain ABC (Vector Laboratories, USA) according to the manufacturer's instructions. All tissue sections were counterstained with hematoxylin to increase contrast. A number of parallel sections were also stained with eosin in order to better distinguish cell morphology and cellular infiltrate.

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