Alphaviral Vectors for Gene Transfer into Neurons

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Abstract

Alphaviruses are small, enveloped positive-strand RNA viruses that have been successfully transformed into expression vectors in the case of Semliki Forest virus (SFV), Sindbis virus (SIN), and Venezuelan equine encephalitis virus. Compared to other viral vectors, their advantages are easy and fast generation of recombinant viral particles, rapid onset, and high-level transgene expression. When applied to neuronal tissue, SFV and SIN vectors possess the additional advantage of efficiently and preferentially transducing neurons rather than non-neuronal cells. This article gives an overview of the biology of SFV and SIN, their generation into expression vectors, and their application in neurobiology, with particular emphasis on the transduction of hippocampal neurons. In addition, it describes the more recent development of alphaviral vectors with decreased or absent cytotoxicity and lowered transgene expression, temperature-controllable gene expression, and altered host-cell specificity in the central nervous system (CNS). Finally, the review evaluates the use of SFV and SIN vectors in hippocampal tissue cultures vs recombinant lentivirus, adenovirus type 5, adeno-associated virus type 2, and measles virus.

Index Entries: Semliki Forest virus; Sindbis virus; RNA virus; expression vector; green fluorescent protein; hippocampus.

Introduction

Alphaviruses are enveloped (+)-strand RNA viruses of the togavirus family that are increasingly being applied as expression vectors. The most widely used alphaviral vectors are based on Semliki Forest virus (SFV), Sindbis virus (SIN), and Venezuelan equine encephalitis virus (VEE), which infect cells via the major histocompatibility class I molecule, laminin, and other receptors *(1)*. The original SFV isolate (strain L10) was obtained from mosquitoes from the Semliki Forest, Uganda, in 1944 *(2)*, while the original SIN isolate (strain AR339) stems from mosquitoes collected near Sindbis, Egypt, in 1952 *(3)*; VEE was originally isolated from the brain of horses in the Guajira region

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of Venezuela *(4)*. Since their initial identification, SFV and SIN have become the model alphaviruses, providing detailed knowledge of their molecular biology and replication in host cells. While initial viral vectors were based on DNA viruses (such as vaccinia virus, adenovirus, and adeno-associated virus), the molecular cloning of SFV and SIN as cDNAs has enabled the subsequent development of these RNA viruses into efficient, high-level expression systems.

In nature, alphaviruses cycle predominantly between mosquitoes and small mammals and birds. Wild-type SFV and SIN target neurons in the CNS, induce neuronal apoptosis, and cause encephalitis in rodents, sometimes with lethal outcome *(5)*. Data from studies in mice and rats with replication-competent SFV and SIN indicate that neurotropism and neuronal survival are determined by both the viral strain and the age of the injected animals. While all strains cause fatal disease in newborn and suckling rodents, several strains (including SFV A7, its derivative SFV A7 [74] and SIN [AR339]) are avirulent for adult animals and cause limited CNS infection, which is cleared within 7–10 ds post-infection *(6–9)*.

Alphaviral Replication

The first two thirds of the 11.8 kb long, capped RNA genome serve as mRNA for the polyprotein containing nonstructural proteins 1–4 (nsP1–4), which is post-translationally processed into the four proteins that are required for transcription and replication of the alphaviral RNA. NsP1 has methyl- and guanyltransferase activity; nsP2 is involved in the proteolytic processing of nsP1–4 and regulates viral RNA synthesis; and nsP4 is the viral RNA polymerase *(1)*. NsP3 is a phosphoprotein *(10)* involved in alphaviral RNA replication *(11)*, but its precise function remains unknown. Within the first hours of infection, the nsP123 polyprotein and nsP4 generate the negative-strand RNA that is complementary to the viral genome. Once the nsP123 polyprotein has been

cleaved (through nsP2), the negative-strand RNA serves as a template for efficient synthesis of both new positive-strand genomic RNA, and subgenomic 26S RNA that encodes the viral structural proteins. The subgenomic RNA is translated into the polyprotein that is then processed into the capsid and spike proteins, p62 and E1. Owing to an encapsulation signal in the *nsP1* gene for SIN and *nsP2* gene for SFV, capsid proteins assemble with genomic RNA molecules to form nucleocapsids, which then bud from the cell membrane where processed spike proteins have been inserted *(1)*. Figure 1 gives a schematic overview of the alphaviral replication and virus assembly.

Alphaviral Vectors: The Replicon System

Vectors for SFV and SIN as well as the closely related VEE have been developed to express high levels of foreign genes in vitro and in vivo *(12–15)*. Owing to their promiscuous usage of cell surface receptors for attachment, an extremely wide range of host cells, including most cell lines and many primary cultures from insect to mammalian cells, can be infected and successfully transduced *(16,17)*.

While fully replication-proficient alphaviral vectors have been used *(18)*, a more common approach is the replicon system (*see* Fig. 2) in which the transgene in the "vector RNA" replaces the viral structural protein genes *(13–15)*. Vector RNAs are self-replicating and are referred to as "replicons." They must be co-transfected with defective "helper RNA" to be packaged into infectious particles. Both vector and helper RNAs are obtained by in vitro transcription from plasmids containing their respective cDNAs. A vector RNA-encoding plasmid of the SFV system, pSFV2gen *(19)*, is shown in Figure 3. It is based on pSFV1 *(13)* but contains a multiple-cloning site with additional unique restriction sites.

Defective helper RNAs have a large deletion in *nsP1–4* and are not self-replicating, but can be replicated and transcribed by the nsPs pro-
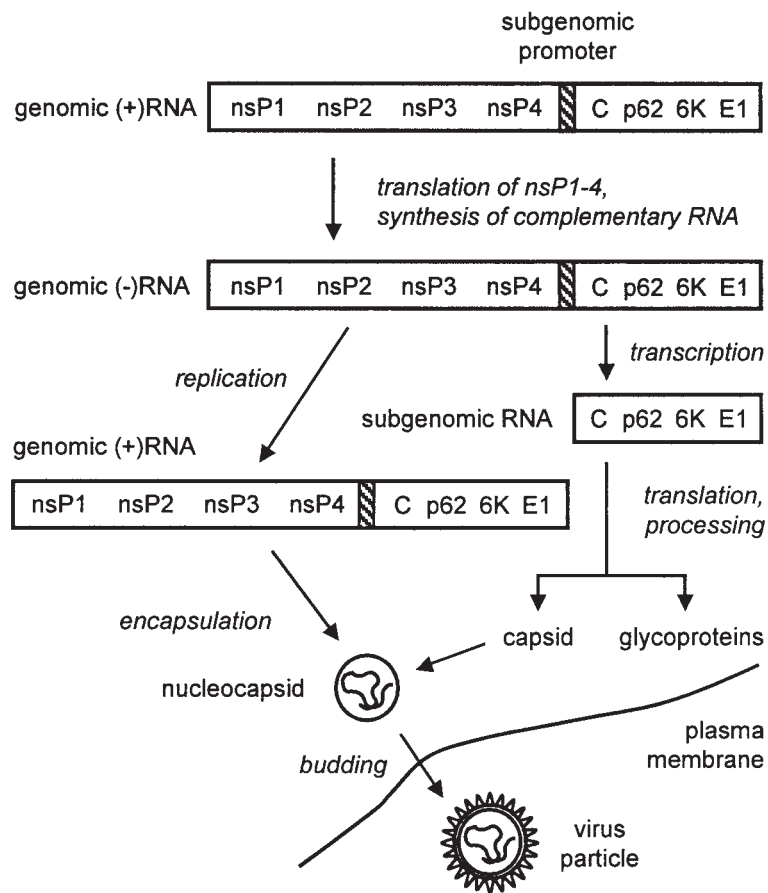
Fig. 1. Alphaviral replication and assembly. Upon attachment of the viral particle to the cell and entry via endocytosis, the viral nucleocapsid is released into the cytoplasm. Binding of the nucleocapsid to ribosomes then triggers the uncoating process that releases the genomic (+)-strand RNA into the cytoplasm. The genomic RNA is then translated into the nsP1–4 preprotein that synthesizes the complementary (–)-strand genome. When nsP1–4 is cleaved into the separate proteins, many copies of both (+)-strand genomic and subgenomic RNA are formed. The subgenomic RNA is translated into the capsid protein (C) and the envelope proteins (p62, the precursor of the E2 and E3 spike proteins; the 6K protein; and E1). Owing to the packaging signal in the nsP region of the genome, the genomic RNA is bound to capsid proteins, thereby forming the nucleocapsid. Interaction of the nucleocapsid with envelope glycoproteins (spikes), which have penetrated into the host cell membrane, liberates the virus particle from the host cell. (Modification of the figure kindly provided by Dr. Sondra Schlesinger, Washington University School of Medicine, St. Louis, USA.)

vided by the replicons. Helper subgenomic RNAs are translated to provide the capsid and spike proteins for replicon assembly under conditions in which helper RNAs are not packaged. Alternatively, the capsid and spike proteins can be supplied from stably transfected cells line *(20)*. Packaged replicons derived by

this approach have been termed "suicide vectors" *(21)* because they infect target cells without the capability of forming new infectious particles (as the introduced replicon genome is lacking the structural protein genes).

A fully DNA-based SFV expression system employs vector and helper plasmids with the
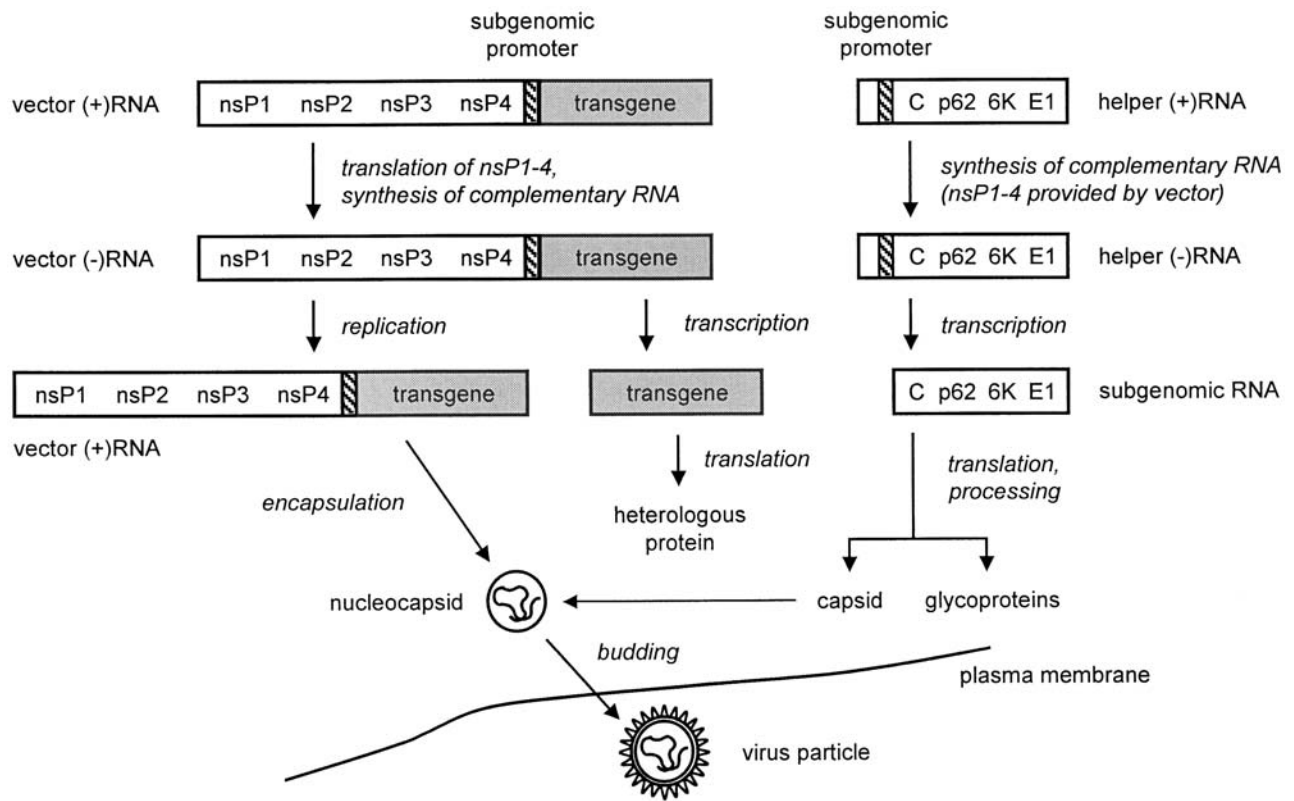
Fig. 2. Packaging of SFV and SIN replicons. Vector RNA encoding nonstructural proteins 1–4 *(nsP1–4)* and the transgene (shaded) under the control of the 26S subgenomic RNA promoter (hatched), and defective helper RNA (encoding the structural alphavirus proteins downstream of the subgenomic RNA promoter) are obtained by in vitro transcription from their respective cDNAs and co-transfected into BHK cells. Only the vector RNA contains the packaging signal required for encapsidation into the nucleocapsid. Within the cytoplasm, vector RNA replication occurs through the action of nsP1–4. In parallel, the defective helper RNA is replicated and transcribed by nsP1–4, and the capsid protein C, 6K protein, glycoprotein E1, and precursor protein p62 (which is later cleaved into the E2 and E3 spike proteins) are translated. Capsid proteins package only vector RNA, while spike proteins are incorporated into the cell membrane. Nucleocapsids dock to the cell membrane where spike proteins have been incorporated, thus allowing the budding of virus particles.

prokaryotic SP6 RNA polymerase promoter (for in vitro transcription) replaced by an RNA polymerase II-dependent promoter, permitting expression in eukaryotic cells *(22)*. The viral titers obtained by co-transfection of these plasmids into baby hamster kidney 21 (BHK) and COS-1 cells, however, are significantly lower than the titers obtained by co-electroporation of vector and helper RNAs.

In a different approach, the helper RNA, in addition to the vector RNA, similarly contains a packaging signal, resulting in the co-packaging of both vector and helper RNA into infectious particles. Virus particles derived by this method have been successfully used as an efficient expression cloning system for receptors and other membrane proteins, as well as for secreted and intracellular proteins *(23)*.
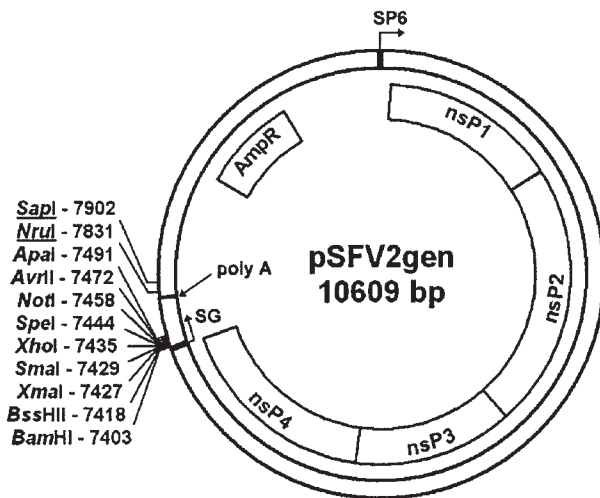
Fig. 3. Topological map of the pSFV2gen vector plasmid. Nucleotides are numbered with the first nucleotide of the cDNA derived from SFV4 genomic RNA defined as 1. The locations of the *nsP1–4* genes encoding the nonstructural proteins, the ampicillin resistance gene, the SP6 promoter for in vitro transcription, the alphaviral 26S subgenomic RNA promoter (SG), and the poly-A sequence are indicated. The graph contains the unique restriction sites of the multiple cloning site for inserting genes of interest, as well as the linearization sites (underlined) for in vitro transcription.

Biosafety

While other alphaviruses such as VEE and rubella virus are human pathogens, wild-type SFV and SIN normally cause less severe infections in humans, with SIN leading to mild fever, joint-pain, and rash *(5)*. For SFV, human infection is relatively common, but this has been linked to disease in only two occasions *(5)*. This explains why SFV is classified as biosafety level 3 in the USA, but with the condition that most activities (including the work with SFV vectors) can be carried out at level 2, which is also the biosafety level for SIN (US HHS Publication no. CDC 93-8395, 1993) *(24)*. In the European Union and in Switzerland, SFV and SIN are classified as biosafety level 2 (EC Council Directive 93/88/EEC, 1993; statement of the Swiss expert committee for biosafety on the classification of work with genetically modified viral vectors, August 2001).

SFV and SIN expression systems using replicons (cf, above) consist of two components: the expression vector and the helper plasmid. When the original SFV helper plasmid is used, fully replication-proficient viral particles may form by homologous recombination in BHK cells. In co-transfections of SFV expression vectors with the pSFV-Helper2 plasmid *(91)*, however, no infectious virus particles can arise due to point mutations in the structural genes, which prevent cleavage of the p62 precursor protein into functional E2 and E3 envelope glycoproteins. The resulting SFV particles are noninfectious until activated with α-chymotrypsin. Therefore, viral vectors packaged with pSFV-Helper2 have been grouped into biosafety level 1 classifications in several European countries, including Germany and Switzerland (http://www.rki.de/GENTEC/ZKBS/ZKBS.HTM; statement of the Swiss expert committee for biosafety on the classification of work with genetically modified viral vectors, August 2001).

Applications in Neurobiology

Transduction of Neurons

Both SFV and SIN vectors have been used successfully to transduce neurons in primary and tissue cultures (*see* Fig. 4) and in vivo (e.g., refs. *25–32*). Organotypic cultures of hippocampal slices *(33,34)* represent a highly applicable preparation that permits rapid tests for recombinant proteins in a system that is more similar to the in vivo situation than cultures of dissociated neurons. SFV and SIN vectors deliver genes efficiently to neurons in organotypic hippocampal slices prepared from neonatal and postnatal rats (*see* Fig. 4A,B; refs. *27,28,35*). In collaboration with my colleague Kenneth Lundstrom, I successfully established a standardized protocol for the application of alphaviral vectors in this test system *(36,37)*.
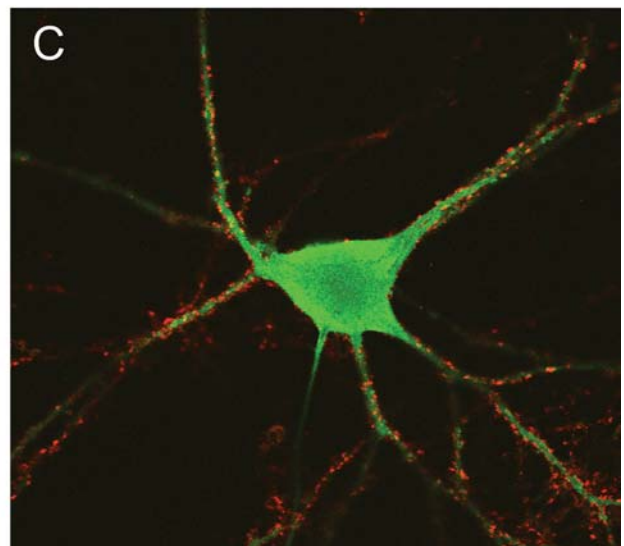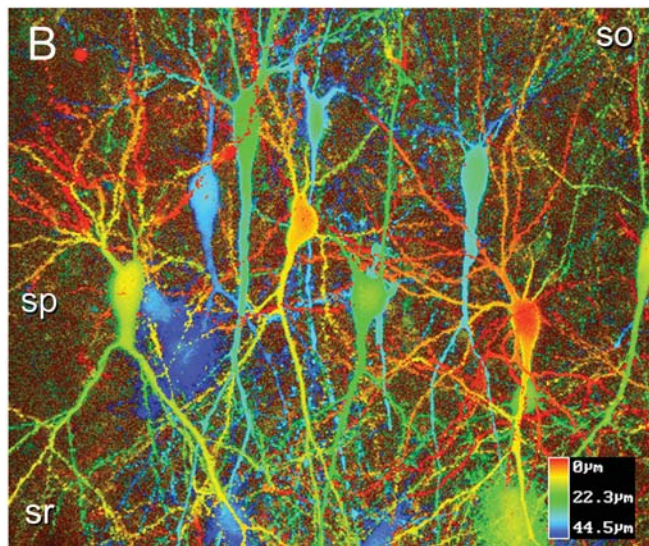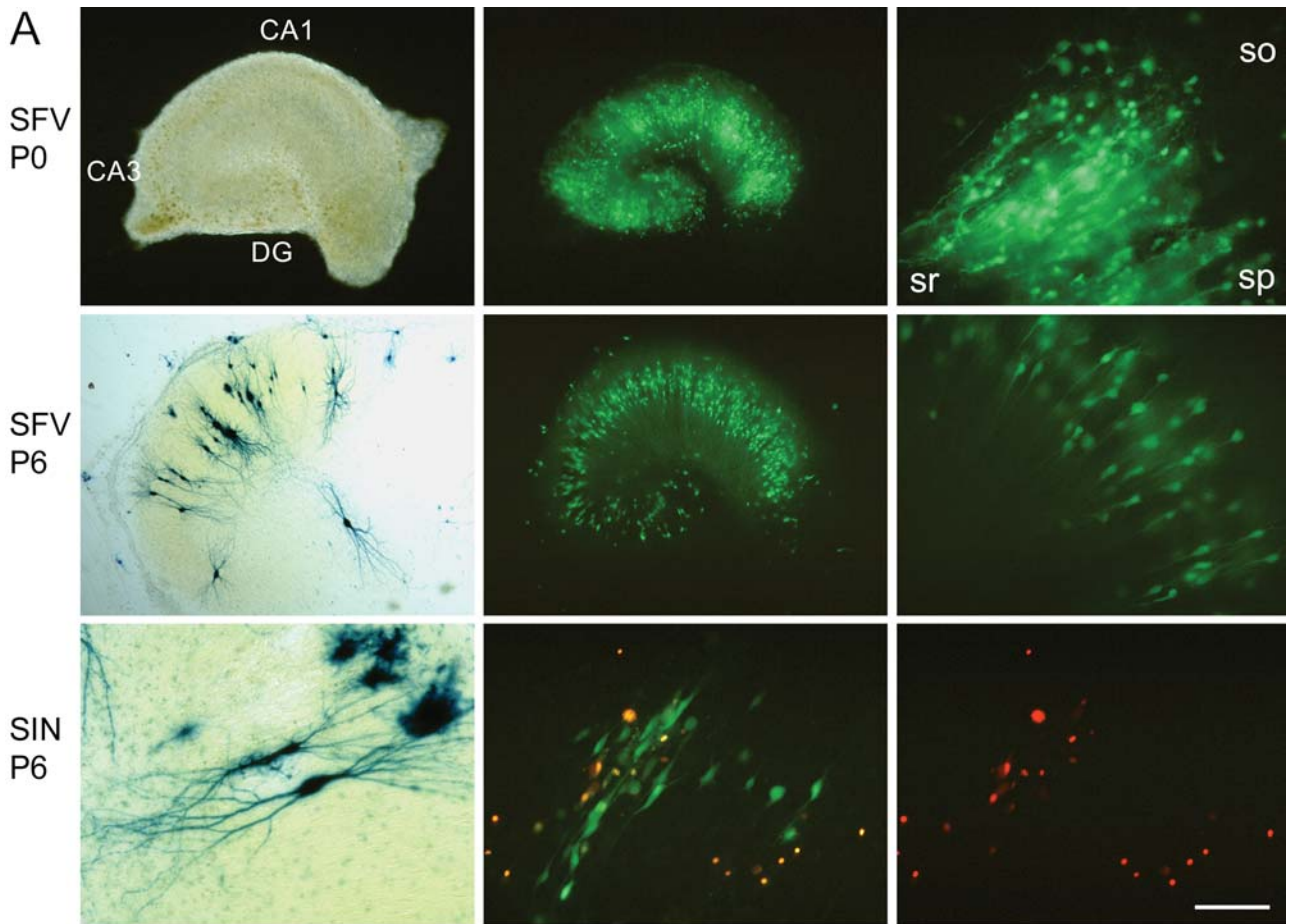
Table 1
Advantages and Disadvantages of Conventional SFV and SIN Vectors Compared to Other Viral
and Non-Viral Transduction Techniques

Advantages	Disadvantages
Rapid production	Inhibition of host cell protein synthesis and cytotoxicity
Extremely high viral titers obtained (stocks are therefore used at ≤10,000-fold dilution)	Not applicable for long-term gene transfer
Preferential and efficient infection of neurons	No choice of promoter
Fast gene expression (within hours)	
High transgene expression levels	

Note that more recently developed, mutant alphaviral vectors eliminate specific disadvantages (cf Table 3).

Fig. 4. Transduction of cultured rat hippocampal slices **(A,B)** and primary rat hippocampal neurons **(C)** by using SFV and SIN vectors. **(A)** Rat hippocampal slices from P0 and P6 rats were cultured for 1 and 45 d (P0 vs P6, respectively) and then infected with 100- to 10,000-diluted SFV and SIN encoding enhanced green fluorescent protein (GFP) or *E. coli* β-galactosidase (LacZ). Dark field (top left) and fluorescence micrographs of living slices were taken at 2 d post-infection, whereas bright field images were obtained from slices fixed and stained for LacZ expression at 1 d post-infection. Transduced cells appear in green and blue for GFP and LacZ, respectively. Propidium iodide staining was performed on living slices at 5 d post-infection; an FITC filter set with a long-pass emission filter revealed both infected cells and propidium iodide-positive (red), non-viable cells (bottom middle), whereas a rhodamine filter set revealed only propidium iodide fluorescence (bottom right). Note that most infected cells exclude propidium iodide, and therefore, appear to be intact. Abbreviations: DG, dentate gyrus; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Bar: 330 μm (left and middle panels of top and middle row) and 80 μm (remaining panels). Magnifications are from CA1 regions, except for the bottom left panel which shows infected CA3 pyramidal cells. **(B)** Confocal image of the CA1 region from a rat hippocampal slice culture at 2 d after infection with an SFV expressing GFP. The color code indicates the depth of the location of GFP-positive cells within the slice. Note that most GFP-positive cells are pyramidal cells (image kindly provided by Dr. R. Anne McKinney, University of Zurich, Switzerland). **(C)** Confocal image of a rat hippocampal neuron in culture, 1 d after infection with an SFV vector expressing GFP. GFP fluorescence is shown in green, while in vivo labeling of active boutons with an anti-synaptotagmin-I antibody is shown in red (image kindly provided by Dr. Antonio Malgaroli, Scientific Institute San Raffaele, Milano, Italy).

Table 2
Proteins that Have Been Functionally (Over)expressed with SFV and SIN Vectors in Neurons

Recombinant protein	Comments	Refs.
Intracellular proteins		
β-galactosidase	Reporter molecule	*(27,29,30,62)*
GFP	Reporter molecule	*(27,30,32,63)*
NEDD-2	Cysteine protease	*(64)*
Aspartylglucosaminidase	Enzyme	*(65)*
p16^{ink4},p21$^{waf/cip}$,p27^{kip1}	Cyclin-dependent kinase inhibitors	*(66)*
Cdk4, Cdk6 (dominant-negative)	G$_1$ cyclin-dependent kinases	*(66)*
CaMKII (truncated)	Kinase	*(67)*
Munc13-1, Munc 18-1	Synaptic vesicle priming proteins	*(68,69)*
Snapin	Binds synaptic protein SNAP-25	*(70)*
Syntaphilin	SNARE assembly regulator	*(71)*
Homer-1a and 1c	Postsynaptic scaffolding protein	*[a]*
Membrane proteins		
GABA$_A$ receptor α1 and β2 subunits	Ligand-gated ion channel	*(48,72)*
Serotonin 5-HT$_3$ receptor	Ligand-gated ion channel	*(73)*
P2X$_2$ receptor	Ligand-gated ion channel	*(74)*
Calcium channel β4 subunit	Calcium channel regulator	*(26)*
GluR1, GluR2, GluR4	AMPA receptor subunits	*(31,40,75–77)*
GluR2 C-term	Blocks Pick1-GluR2/3 interaction	*(78)*
VGLUT1/BNP1, VGLUT2/DNPI	Vesicular glutamate transporters	*(79,80)*
Connexin 36	Increases intercellular coupling	*[a]*
SNAP-25	Synaptic protein	*(81,82)*
SGT	Synaptic vesicle surface protein	*(83)*
GFP-tagged pleckstrin homology domain	Indicator of intracellular IP$_3$ dynamics	*(84)*
Amyloid precursor protein (APP)	Cleaved into β-amyloid peptide	*(85)*
Presenilin-1	Associated with familial AD	*(86)*
Catechol O-methyltransferase	Membrane-bound enzyme	*(87)*
Rab8	Membrane-associated GTPase	*(25)*
Neprilysin	Membrane-associated metalloendopeptidase	*(88)*
VIP21	Integral membrane protein	*(25)*
polymeric immunoglobulin receptor	Binds immunoglobulins A and M	*(89)*
F protein	Measles virus glycoprotein	*[a]*

[a] Own, unpublished data.

Compared to other viral vectors, biolistic and lipid-based transfection methods, SFV, and SIN vectors cause rapid, high-level, and efficient transduction (>95% in primary neurons, *see* Fig. 4C). It is also beneficial for many studies that conventional SFV and SIN vectors preferentially transduce neurons rather than non-neuronal cells, causing pyramidal cells and interneurons, in hippocampal slice cultures, to constitute approx 90% of all cells positive for green fluorescent protein (GFP; *see* Fig. 4B) *(27,28).*

Aside from their advantages (*see* Table 1), wild-type alphaviral vectors *(13,15)* retain the disadvantage of inhibiting host cell protein synthesis, which eventually causes cell death. While neurotoxic effects appear relatively fast (within 1–2 d) and are readily observed in cultures of dissociated hippocampal neurons *(25,38,39),* they are delayed by several days in

hippocampal slice cultures. Based on propidium iodide exclusion experiments (cf, Fig. 4A, bottom middle and right panels) and electrophysiological tests of passive and active membrane properties (i.e., resting membrane potential and conductance, action potential generation, firing accommodation, and hyperpolarization-induced currents), we found pyramidal cells in this preparation to survive for 2–5 d post-infection *(27,28)*. In any case, as alphaviral GFP expression in tissue cultures is fast (detectable within 4–6 h) and transient (peaking at 1 d post-infection) *(28)*, the time window to examine transgene effects suffices for many physiological studies, including the microscopical and electrophysiological analysis of synaptic function (e.g., Fig. 4C; ref. *40*), and the real-time investigation of activity-dependent morphological changes *(32)*.

Table 2 summarizes different transgenes that have been introduced into hippocampal and other neurons by using packaged SFV and SIN replicons. While it lists many intracellular and membrane proteins that could be expressed in a functional manner, our attempts to functionally overexpress the transcription factors EGR1 and EGR2 with the less cytopathic SFV(PD) mutant *(38,39)* were not successful. The analysis with an anti-EGR1 antibody revealed the presence of immunoreactive protein in the cytoplasm rather than the nucleus of infected cells; and functional tests for transactivation of EGR1-dependent luciferase reporter gene expression were not successful (M.J. Fend, K. Lundstrom, and M.U. Ehrengruber, unpublished data). By contrast, when inserted into adenoviral vectors, exactly the same EGR1 cDNA was able to induce EGR1-dependent gene expression *(41)*. These results indicate that the present alphaviral vectors may not be applicable for the functional study of specific transcription factors. Another report with a GFP-tagged transcription factor (ATF-4) overexpressed by SFV did not examine whether ATF-4 transactivating function was retained in infected cells *(42)*.

Heterologous Expression of Neuronal Proteins

Owing to the intracellular amplification processes inherent in alphaviral genome replication, SFV and SIN vectors generate extremely high transgene expression levels in infected cells. These levels can be problematic in cases where the transgene product has cytotoxic effects (as in the human neurokinin-1 receptor *[43]*) or because it does not match physiological levels. On the other hand, alphaviral vectors are particularly advantageous when applied in cell lines for generating high amounts of recombinant protein required (e.g., for the drug screening of receptors and the purification of proteins for structural studies and crystallization. Compared to the baculovirus system, which generates similar recombinant protein levels, alphaviral vectors possess the advantage of functioning well in mammalian cells.

Alphaviral vector systems have been particularly useful for the heterologous expression of receptors, including G protein-coupled receptors such as the metabotropic glutamate receptors mGluR2, 3, 4, and 8 *(19,44,45)*, the human neurokinin-1 receptor *(43,46)*, and the α_{1b}-adrenergic receptor *(47)*. Radioligand binding on intact cells and isolated cell membranes revealed extremely high expression levels, with 3–10 million receptors per cell and 50–200 pmol recombinant receptor per mg total protein. An intact functional coupling of the overexpressed receptors to G proteins in response to agonists has been tested via measurements of, e.g., GTPγS binding *(44)*, intracellular Ca^{2+} mobilization *(43)*, and inositol phosphate accumulation *(47)*.

In specific applications, the heterologous expression of distinct proteins, possibly at approximately equal ratios, within a given cell is required. For example, it has been shown for the $GABA_A$ receptor that $\alpha1$ or $\beta2$ subunits, when expressed alone, are retained in the endoplasmic reticulum, whereas co-expression of both subunits permits $GABA_A$ receptor access to the plasma membrane *(48)*. Separate

genes can be introduced into the same cell by co-infection with different viral vectors *(47,48)*, the use of internal ribosomal entry site (IRES) sequences *(67)*, and additional 26S subgenomic RNA promoters *(18)*.

Identification of Infected Neurons Using GFP

The function of transduced neurons is generally compared to one of uninfected control neurons (as well as to one of neurons infected with a control virus expressing, e.g., GFP) to determine the effect of the expressed transgene. This comparison is done ideally in the same population of cells, i.e., the same slice culture or the same Petri dish. Electrophysiological recordings, as well as other measurements from infected cells, thus require the identification of (living) cells that have been genetically modified. This is normally achieved by using GFP as a reporter molecule.

Three GFP-based approaches have been employed in combination with alphaviral vectors. The most common method is the generation of GFP fusion proteins where the gene of interest is fused to the GFP cDNA. This strategy, however, may be problematic as the attachment of the GFP domain can alter transport, localization, and function of the overexpressed protein. A second approach is the use of an internal ribosomal entry site (IRES) sequence between the gene of interest and the GFP cDNA, permitting separate expression of both genes. Our experiments using IRES-dependent GFP expression, however, revealed relatively weak GFP fluorescence and allowed identification of infected pyramidal cells in organotypic hippocampal slices at only 3 d post-infection. A third method is to drive GFP expression separately under the control of an additional subgenomic alphaviral 26S promoter downstream of the gene of interest. This procedure, in our hands, resulted in sufficiently high GFP fluorescence levels to allow for electrophysiological recordings from GFP-positive primary neurons at 1 d post-infection. The second and third approaches, of course, can generally be used to express two or more

transgenes as separate proteins, which may be required, e.g., for the expression of heteromeric proteins such as ligand-gated ion channels.

Alphaviral Vector Development

As summarized in Table 1, wild-type alphaviral vectors may be problematic because of 1) cytotoxicity; 2) short-term expression due to cytotoxicity and/or transient nature of the viral RNA; 3) no choice of promoter; and 4) in certain cases, extremely high expression levels. The development of alternative SFV and SIN vectors focuses on these negative properties by introducing specific mutations in the *nsP1–4* genes that control viral replication and expression. Tables 3 and 4 give an overview of the modified alphaviral vectors and indicate their transduction characteristics in cell lines and neurons.

Non-Cytopathic Vectors

Spontaneous mutations discovered in the nonstructural genes, particularly in nsP2 and nsP4, have dramatic effects on the viral pathogenicity. Mutations in *nsP2* of SIN have produced viruses *(49)* and replicons *(49–51)* with greatly reduced cytotoxicity in cell lines (*see* Table 3). The majority of the mutants had a single change at amino acid 726. For example, a change at this site from Pro to Ser produced replicons that had reduced levels of RNA replication, and therefore, also lower cytopathogenicity *(49)*. The change from Pro to Leu led to a further reduction in both RNA synthesis and cytotoxicity *(50)*. Replication-persistent replicons with ongoing viral multiplication were generated by random mutagenesis of *nsP* genes in SIN and SFV vectors containing the neomycin resistance gene; once again they had either deletions or point mutations in nsP2 *(51)*. All of these experiments were performed in cell lines (*see* Table 3), with mitosis of infected cells. In general, persistent infections appeared to be correlated with a lack of interferon production or the production of other potential cytokines.

Table 3
Mutant SFV and SIN Replicons and Their Transduction Characteristics in Cell Lines Compared
to the Conventional, Wild-Type Vectors SINrep5 (15) and SFV1 (13)

Vector	Characteristics	Refs.
SIN(nsP2$_{A1E}$)	Non-cytopathic in BHK cells, establishes persistent infection[a]	(51)
SIN(nsP2$_{P726S}$)	Less cytopathic in BHK cells, noncytopathic in mouse and human cell lines, higher expression in BHK cells	(49)
SIN(nsP2$_{P726L/T}$)	Noncytopathic in all cell lines tested, establish persistent infections in BHK, Vero and CHO cells[a]	(50,51,90)
SIN(nsP2$_{P726S}$nsP4$_{G153E}$), pCytTS	Temperature-inducible, DNA-based expression system in BHK, CHO-K1, C2C12, COS-7, and HEK 293 cells[a]	(57)
SFV(nsP2$_{L10T}$), SF2A; SFV(nsP2$_{\Delta D469}$), SF1B; SFV(nsP2$_{L713P}$), SF2C	Noncytopathic in BHK cells, establish persistent infection	(51)
SFV(nsP2$_{S259P,R649D}$), SFV(PD)	Less cytopathic in BHK, HEK 293, and CHO-K1 cells, higher expression, reduced viral titers	(38,39)[b]
SFV(nsP2$_{S259P,R650D,L713P}$), SFV(PD713P)	Noncytopathic in BHK cells, temperature-sensitive, expresses at 31°C but not at 37°, higher expression, low viral titers	[c]
SFV(nsP2$_{S259P,R649D}$nsP4$_{G153E}$), SFV(PDE$_{153}$)	Temperature-sensitive in BHK cells, expresses at 31°C but little at 37°	(39)
SFV(nsP2$_{S259P,R649D}$nsP4$_{G324E}$), SFV(PDE$_{324}$)	Temperature-sensitive in BHK cells, expresses at 31°C but little at 37°	(39)
SFV(nsP2$_{S259P,R649D,M780T}$nsP4$_{G153E}$), SFV(PDTE)	Temperature-sensitive in BHK cells, expresses at 31°C but not at 37°, achieving higher expression	(39)
SFV(A774nsP)	Temperature-sensitive in BHK cells, transduces ~100-fold more cells at 31 vs 37°C	[d]
SFV(26S-M5), SFV(26S-M1), SFV(26S-M3)	Decreased transgene expression levels (1, 3, and 30% of wild-type, respectively)	(58)

[a] This mutant has only been used as a nonpackaged replicon.

[b] Plus unpublished data by K. Lundstrom, A. Abenavoli, A. Malgaroli, and M.U. Ehrengruber.

[c] K. Lundstrom and M.U. Ehrengruber, unpublished data.

[d] M.U. Ehrengruber, M. Renggli, M.J.V. Vähä-Koskela, A.E. Hinkkanen, and K. Lundstrom, unpublished data.

With regard to neuronal infection (*see* Table 4), the introduction of known temperature-sensitive mutations into nsP2 and nsP4 reduced the cytotoxicity of SFV vectors in primary hippocampal neurons *(39)*. Furthermore, studies on replication-competent SFV revealed a point mutation (Arg649Asp) in the nuclear localization signal of nsP2 to confer lower neurovirulence in mice *(52,53)*. When we combined a similar mutation in the nsP2 nuclear localization signal (Arg650Asp) with an additional mutation in nsP2 (Ser259Pro), we obtained a vector, SFV(PD), that was less cytopathic and caused increased transgene expression in mammalian cell lines, primary hippocampal and cortical neurons, and organotypic hippocampal slices *(38,39)* (K. Lundstrom, A. Abenavoli, A. Malgaroli, and

Table 4
Transduction Characteristics of SFV Replicons in Hippocampal and Other Neurons

Vector	Characteristics	Refs.
Conventional SFV[a]	Infects primary rat hippocampal neurons, cytotoxic after >8–24 hs	(25,26)
	Targets pyramidal cells and interneurons in rat hippocampal slices, neurons tolerate infection for 2–5 d	(27,28)
	In vivo, infects striatal neurons in rat brain	(29)
SFV(nsP2$_{S259P,R650D}$), SFV(PD)	Less cytopathic in primary rat hippocampal and cortical neurons, higher expression	(39)[b]
	Targets pyramidal cells and interneurons in rat hippocampal slices	(28)
SFV(nsP2$_{S259P,R650D,L713P}$), SFV(PD713P)	Transduces pyramidal cells in rat hippocampal slices	[c]
SFV(nsP2$_{S259P,R649D}$nsP4$_{G153E}$), SFV(PDE$_{153}$)	Targets interneurons at 31°C, but both pyramidal cells and interneurons at 37°C in rat hippocampal slices	(39)
SFV(nsP2$_{S259P,R649D}$nsP4$_{G324E}$), SFV(PDE$_{324}$)	Temperature-sensitive in primary rat hippocampal neurons, expresses at 31°C but little at 37°C	(39)
SFV(nsP2$_{S259P,R649D,M780T}$nsP4$_{G153E}$), SFV(PDTE)	Transduces both pyramidal cells and interneurons of rat hippocampal slices at 31°C but not at 37°C	(39)
SFV(A774nsP)	Transduces selectively glial cells at 37°C, and only neurons at 31°C in primary rat hippocampal cells	[d]
	Transduces selectively glia and other non-neuronal cells at 37°C, but also neurons at 31°C in rat hippocampal slices	[d]

Note that SIN vectors also efficiently transduce hippocampal neurons in primary culture *(30,31),* in slices *(27,32),* and in vivo *(30).*

[a] The conventional vectors SFV1 *(13)* and SFV2gen *(19)* are based upon the cDNA for SFV4 that is derived from the wild-type, virulent L10 strain *(5).*

[b] Plus unpublished data by K. Lundstrom, A. Abenavoli, A. Malgaroli, and M.U. Ehrengruber.

[c] K. Lundstrom, and M.U. Ehrengruber, unpublished data.

[d] M.U. Ehrengruber, M. Renggli, M.J.V. Vähä-Koskela, A.E. Hinkkanen, and K. Lundstrom, unpublished data.

M.U. Ehrengruber, unpublished data). Furthermore, when a mutation in nsP2 known to confer replication-persistence (Leu713Pro; ref. *51*) was inserted into SFV(PD), the resulting vector SFV(PD713P) was noncytopathic in BHK cells and still infected neurons in hippocampal slice cultures (K. Lundstrom, and M.U. Ehrengruber, unpublished data).

Taken together, the less cytopathic SFV and SIN mutants will permit the application of alphaviral vectors under more physiological conditions. In addition to general overexpression of recombinant proteins, the vectors can also be used to study expression kinetics and signal transduction events *(54),* suppress genes by antisense and ribozyme approaches, and perform gene therapy trials with prolonged gene expression.

Vectors with Temperature-Dependent Regulation

In present SFV and SIN vectors, transgene expression relies on the endogenous 26S subgenomic RNA promoter. The minimal 26S subgenomic RNA promoter consists of 19 nucleotides upstream and 5 nucleotides down-

stream of the transcription start site for the subgenomic RNA, but its activity is enhanced when neighboring sequences are included *(1)*. The 26S subgenomic RNA promoter functions in the cytoplasm of infected cells, as it is the compartment where the transcription of alphaviruses occurs (cf Fig. 1). In contrast, the nucleus is the compartment where "non-alphaviral" promoters are functional that can be regulated and transcribe RNA from DNA. Thus, other means to control SFV and SIN-mediated transgene expression have to be found. Temperature-dependence is one of the few ways to regulate alphavirus vectors that replicate and are transcribed in the cytoplasm.

Temperature-sensitive mutations have been described for SIN in each of the *nsP* genes *(55,56)*, and those in nsP4 *(14)* have been introduced into SIN expression vectors *(57)*. Combination of the Pro726Ser change in nsP2 with a temperature-sensitive mutation in nsP4 led to the inducible expression of DNA-based vectors in a variety of cell lines *(57)*. Several of the known temperature-sensitive point mutations in *nsP2* and *nsP4* have also been introduced into the SFV expression vector, causing temperature-sensitive transgene expression in cell lines, primary hippocampal neurons, and organotypic hippocampal slices (*see* Tables 3 and 4) *(39)*. A quadruple-mutant vector, SFV(PDTE), with three point mutations in nsP2 and one in nsP4, permitted GFP expression at 31°C but not at 37°C. Similarly, SFV(PD713P) permitted transgene expression at 31°C rather than 37°C (K. Lundstrom and M.U. Ehrengruber, unpublished data). Interestingly, the triple-mutant vector SFV(PDE$_{153}$) when used at 37°C transduced interneurons, rather than pyramidal cells in rat hippocampal slices, characterized by a ratio of approx 0.6 between GFP-positive pyramidal cells and interneurons (the corresponding ratio is >10 for the wild-type SFV vector) *(28)*. When SFV(PDE$_{153}$) was used at 31°C instead, a wild-type phenotype was observed with a ratio of approx 10 *(39)*. A different study revealed another SFV mutant, SFV(A774nsP) encoding GFP downstream of the *nsP* genes from the

avirulent SFV strain A7(74), to transduce glial cells, and not neurons, in cultured hippocampal cells and slices at 37°C, whereas this was reversed at 31°C (M.U. Ehrengruber, M. Renggli, M.J.V. Vähä-Koskela, and K. Lundstrom, unpublished data). These results show that mutant alphaviral vectors are useful, at least in hippocampal tissue, to target transgene expression to a subset of neurons or even glial cells rather than principal neurons. At the non-permissive temperature, the host cellular environment presumably does not support alphaviral replication.

Vectors with Decreased Expression Levels

For many applications, the elevated expression levels obtained with alphaviral vectors are normally favorable or non-problematic. On the other hand, they may be detrimental under specific conditions—in particular when the overexpressed protein has cytotoxic effects or when more physiological levels of the expressed protein are essential (the functional expression of G protein-coupled receptors, e.g., may require a more physiological ratio of receptors vs G proteins). To achieve lower expression levels, several point mutations were introduced into the 26S subgenomic RNA promoter of SFV (*see* Table 3) *(58)*. The resulting levels of reporter protein (β-galactosidase and luciferase) expressed in BHK cells were only 1, 3, and 30%, as compared to the quantities expressed from wild-type SFV vectors. The down-regulated expression vectors will thus permit examination of recombinant protein function under conditions that are more similar to the *in situ* situation. The use of the GFP reporter in combination with the more down-regulated SFV vectors, however, will no longer be practical (transduced cells will not be recognized due to low GFP fluorescence).

Comparison with Other Viral Vectors

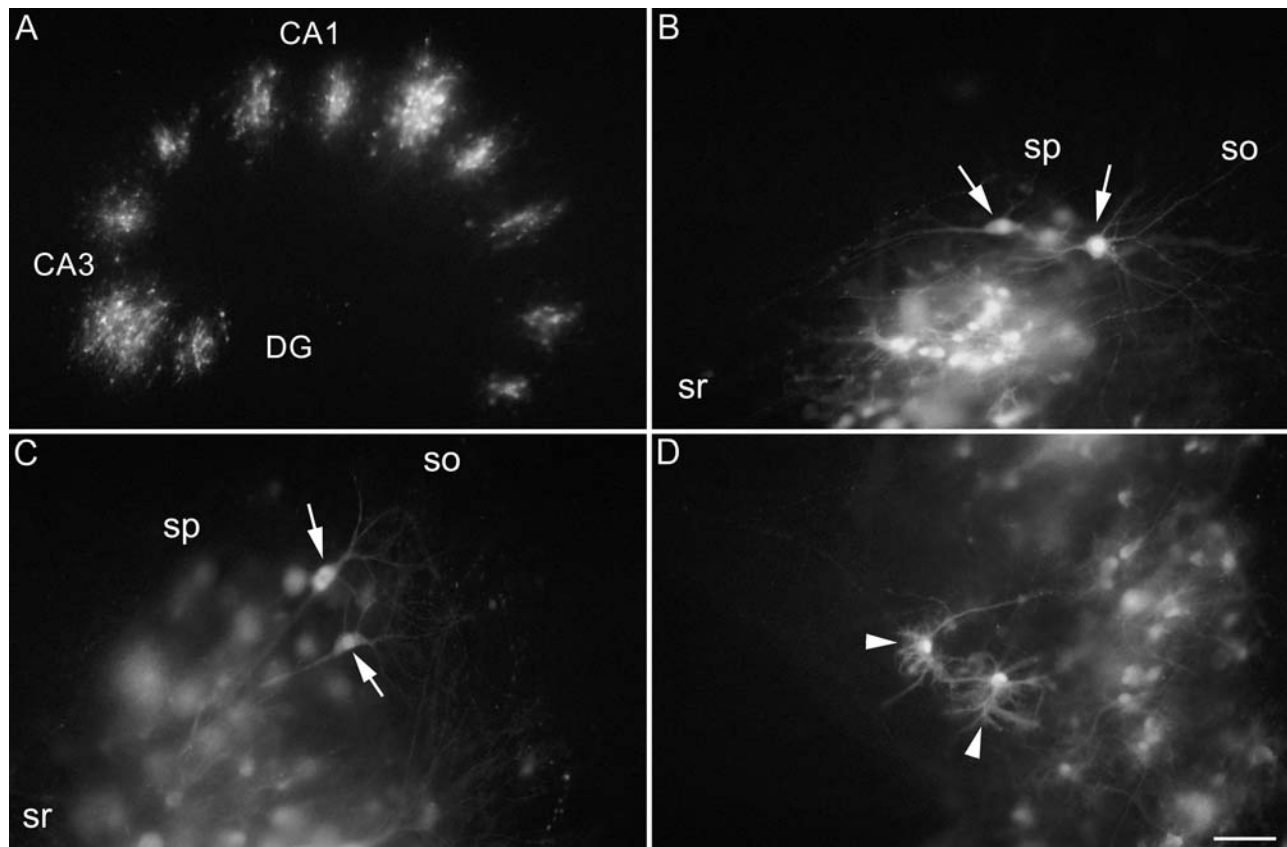Many viruses have been successfully employed to transfer genes into neurons, and

Fig. 5. Transduction of rat hippocampal slices with a lentiviral vector. P6 rat hippocampal slices at 28 d in culture were injected in the CA1 and CA3 pyramidal cell layer with two-fold diluted lentivirus expressing GFP under a CMV promoter. Slices were incubated at 37°C and fixed at 7 d post-infection. Fluorescence micrographs of a whole slice **(A),** CA1 neurons **(B),** arrows, CA3 pyramidal cells **(C),** arrows, and glial cells in the CA1 region **(D),** arrowheads. Abbreviations: DG, dentate gyrus; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Bar: 260 μm **(A),** 65 μm **(B–D).**

each viral vector has its advantages and disadvantages. As each vector system has normally been established in a different laboratory and tested under distinct conditions, it is sometimes difficult for a newcomer to choose a viral vector that is optimal for study. We have previously characterized wild-type SFV and SFV(PD) vectors, recombinant adenovirus type 5, adeno-associated virus type 2 (AAV), lentivirus, and measles virus by their expression of GFP in rat hippocampal slice cultures *(28).* Figure 5 illustrates the results obtained with a lentivirus expressing GFP under the

control of a cytomegalovirus (CMV) promoter. Both SFV vectors transduced more neurons (>90% of all GFP-positive cells) than AAV, lentivirus, and measles virus (71, 69, and 62%, respectively), while resting membrane potential and conductance, action potentials, firing accommodation, and H-current appeared normal in all infected CA1 pyramidal cells. No infected neurons were identified with adenovirus type 5 *(28).* This was an unexpected finding because this virus is useful to transduce primary rat hippocampal neurons *(59),* although relatively high titers (approx 10^8

plaque-forming units/mL) have to be applied to overcome the predominant glial cell infection. AAV-mediated GFP expression was restricted to neurons when the neuron-specific PDGF promoter (for platelet-derived growth factor β-chain) rather than the CMV promoter was used. Whereas transgene expression mediated by SFV was rapid but transient (as described above), it increased more slowly but remained stable with AAV and lentivirus, but was fast with measles virus. As no replication-impaired system is available for measles virus, this vector propagated in the hippocampal slices. It is noteworthy that spreading occurred to pyramidal cells rather than non-neuronal cells *(60),* which may facilitate the selective transduction of most pyramidal cells within a slice culture. In any case, SFV is useful for short-term and AAV and lentivirus for long-term transduction of hippocampal slices, while measles virus may permit both short-and long-term transduction.

A qualitative evaluation of the maximal GFP expression levels obtained in hippocampal slice cultures revealed the following order: measles virus ≥ SFV ≈ lentivirus > AAV. Compared to GFP-expressing lentivirus, the identification of neurons transduced with SFV and SIN vectors encoding GFP was easier (cf Fig. 4A vs Fig. 5). We also estimated the transduction efficiencies in hippocampal slices by comparing the number of GFP positive cells vs the number of applied virus particles. The following order was found: wild-type SFV ≈ SFV(PD) > measles virus > lentivirus ≈ AAV-PDGF-GFP > AAV-CMV-GFP *(28).* For SFV and SFV(PD), nearly every virus particle resulted in a GFP-positive cell. This high infection rate is due to the broad host range of the virus, and the fact that entry of one virus particle suffices to initiate viral replication in a host cell *(1,61).* For SIN vectors in organotypic hippocampal slices, similar results as with SFV vectors are obtained *(27).* In addition to the high infection rate, the easy generation of high-titer virus stocks *(36,37)* that contain a high ratio of infectious to physical particles *(61)* makes SFV and SIN vectors favorable for

gene transfer into neurons. In several countries, SFV vectors packaged with pSFV-Helper2 may be preferred as they are classified as biosafety level 1.

Acknowledgments

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