

The Semliki Forest virus vector induces p53-independent apoptosis

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Three deletion mutants of the structural protein region of the Semliki Forest virus (SFV) genome, including one which encompassed all the viral structural protein genes, induced apoptosis in BHK cells at 48 h after transfection, as shown by DNA laddering and TUNEL staining, as did the wild-type SFV4 RNA. A similar result was obtained for the SFV1 expression vector, which has a multicloning site inserted in place of the structural protein genes. However, in cells transfected with viral RNA containing a deletion of the nsP2 gene, neither viral RNA synthesis nor the induction of apoptosis occurred. Both SFV1 vector and wild-type SFV4 RNA induced apoptosis in human H358a lung carcinoma cells, which have a homozygous deletion of the p53 gene. It is concluded that the SFV vector encodes a function in the nonstructural coding region which induces p53-independent apoptosis and is dependent on viral RNA synthesis.

Programmed cell death or apoptosis is a process which is under the genetic and functional control of the cell, unlike necrosis which is death solely due to cellular injury or malfunction. It may be detected by the morphological appearance of cells undergoing apoptosis, or by the formation of the internucleosomal DNA fragments which are typical of the later stages of apoptosis (Hockenbery, 1995). It has recently been found that many viruses induce apoptosis in infected cells; the mechanism of induction can be either through the action of the cellular p53 gene or by a mechanism which is independent of p53 (Razvi & Welsh, 1995).

Semliki Forest virus (SFV) infection of mice has been used as a model to understand the molecular processes of virus pathogenesis (Atkins *et al.*, 1985, 1994). SFV is an enveloped, positive-stranded RNA virus of the genus *Alphavirus* of the family *Togaviridae*; it infects most types of animal cells and its

molecular biology has been intensively studied since it is a relatively simple virus, encoding only nine unique functional proteins (Strauss & Strauss, 1994). This virus and a related alphavirus, Sindbis virus, have previously been shown to kill cultured cells by induction of apoptosis (Levine *et al.*, 1993; Ubol *et al.*, 1994; Glasgow *et al.*, 1997; Scallan *et al.*, 1997). It has been suggested that expression of the anti-apoptotic gene *bcl-2* inhibits SFV-induced apoptosis (Scallan *et al.*, 1997), but recent work has shown that SFV encodes a function which leads to the inactivation of *bcl-2* by proteolytic cleavage, and is therefore able to multiply in *bcl-2*-expressing cells (Grandgirard *et al.*, 1998). Early gene functions concerned with viral RNA synthesis have been shown to be involved in inducing inhibition of cellular protein synthesis in cells infected with Sindbis virus, but synthesis of viral structural proteins is required for the full cytopathic effect (Atkins, 1976, 1977; Frolov *et al.*, 1994). A recent study of the effect of caspase inhibitors on Sindbis virus-induced apoptosis has indicated that apoptosis proceeds by a pathway similar to that induced by Fas or tumour necrosis factor- α and is unlikely to result from DNA damage (Nava *et al.*, 1998). Earlier work implicated double-stranded RNA synthesis in the cytopathogenicity of Sindbis virus, but this was not recognized at the time as apoptosis (Atkins, 1976, 1977; Atkins & Lancashire, 1976).

The SFV genome has been manipulated to construct a transient expression vector (Liljeström & Garoff, 1991; Berglund *et al.*, 1993). During the multiplication of SFV, a subgenomic 26S RNA species is formed which encodes the structural proteins of the virus only, and is a gene-amplification mechanism. In the vector, the structural protein-coding sequences have been deleted and a multicloning site inserted in their place; a foreign gene can therefore be inserted in this region. RNA transcribed *in vitro* from the plasmid encoding the vector is transfected into cultured cells, and viral RNA replication and expression occur as in a productive infection. However, unlike infection with virus particles, RNA transcribed from the SFV infectious clone or expression vectors and transfected into cells is not quickly expressed, but requires at least 16 h incubation before expression occurs (Suomalainen *et al.*, 1992). Here we show that this vector induces p53-independent apoptosis by a function which is encoded by the

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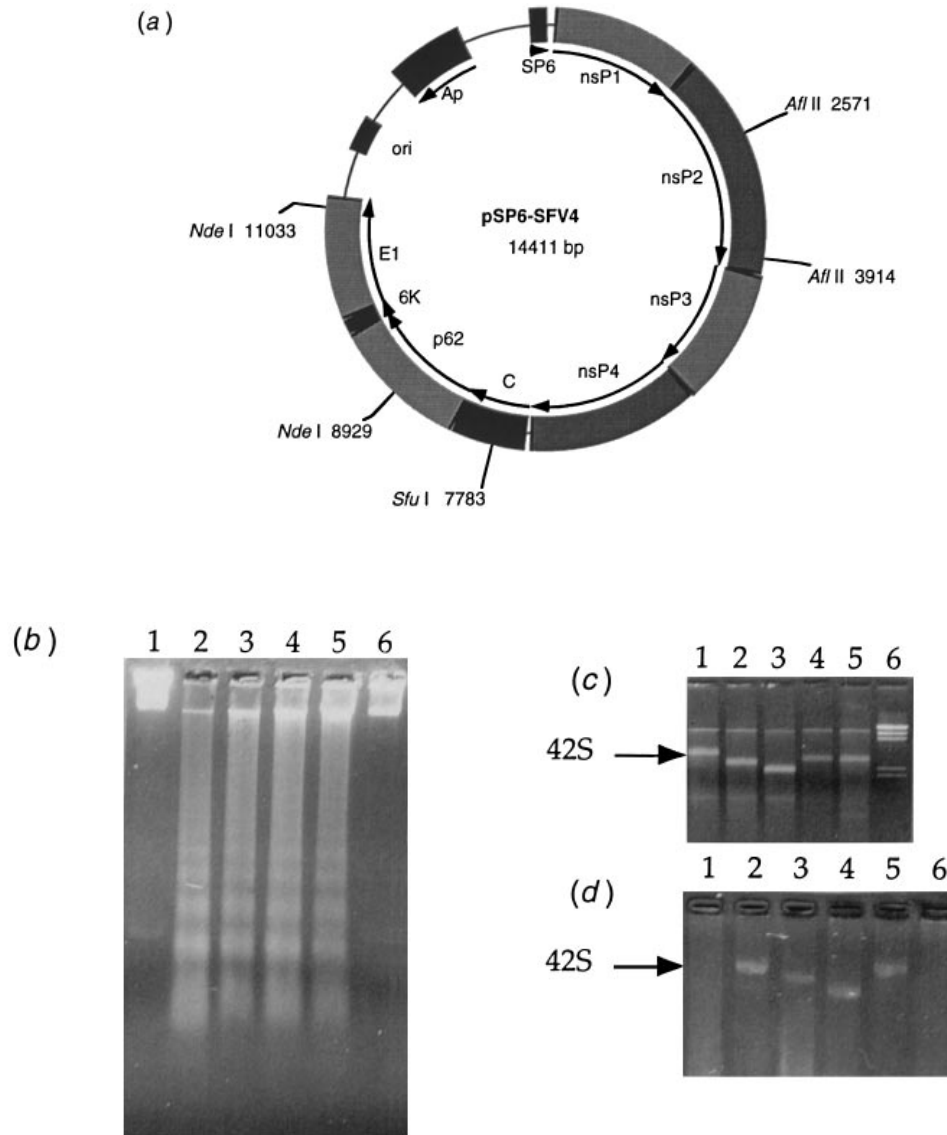


Fig. 1. (a) Diagram of the pSP6-SFV4 infectious clone of SFV showing the location of restriction sites used to make deletion mutants. (b) DNA ladders indicative of apoptosis formed by agarose gel electrophoresis of extracted DNA, 48 h after electroporation of BHK cells with viral RNA. Lanes: 1, untransfected control cells; 2, SFV4-transfected cells; lanes 3–6, cells transfected with deletion mutants: 3, $\Delta 8930/11033$; 4, $\Delta 7784/11033$; 5, $\Delta 7784/8929$; 6, $\Delta 2572/3914$. (c) Mini-gel of RNA transcribed from the SP6-SFV4 plasmid and deletion mutants. Lanes: 1, SFV4; 2, $\Delta 8930/11033$; 3, $\Delta 7784/11033$; 4, $\Delta 7784/8929$; 5, $\Delta 2572/3914$; 6, λ DNA markers. The position of the 42S viral genomic RNA is indicated. (d) Agarose gel electrophoresis of RNA isolated from transfected BHK cells at 48 h after transfection. Lanes: 1, untransfected control cells; 2, SFV4-transfected cells; lanes 3–6, cells transfected with deletion mutants: 3, $\Delta 8930/11033$; 4, $\Delta 7784/11033$; 5, $\Delta 7784/8929$; 6, $\Delta 2572/3914$. The position of the 42S viral genomic RNA is indicated.

nonstructural region of the SFV genome and is dependent on viral RNA synthesis. We first investigated the effect of deletion of the structural protein region of the SFV genome on apoptosis induction, and then showed that the vector construct, which has an inserted multicloning site, induces apoptosis in cells which do not express p53.

Transcription and electroporation of RNA from the pSP6-SFV4 infectious clone of SFV and its deletion mutants were carried out as previously described (Liljeström *et al.*, 1991). Transfection was carried out by electroporation of the

transcription mix, containing 100 μ g of RNA transcribed from 2 μ g of linearized plasmid DNA. Deletion mutants are labelled according to the extent of the deletion, e.g. $\Delta 2572/3914$ has a deletion from nucleotides 2572 to 3914 of the SFV4 genome. To construct plasmids expressing deletion mutants, the restriction sites shown in Fig. 1(a) were used. To construct $\Delta 2572/3914$, the pSP6-SFV4 plasmid was deleted between the two *Afl*III sites and ligated; a similar procedure was adopted for $\Delta 8930/11033$ for the two *Nde*I sites. To construct $\Delta 7784/11033$, the pSP6-SFV4 plasmid was deleted between the *Sfu*I

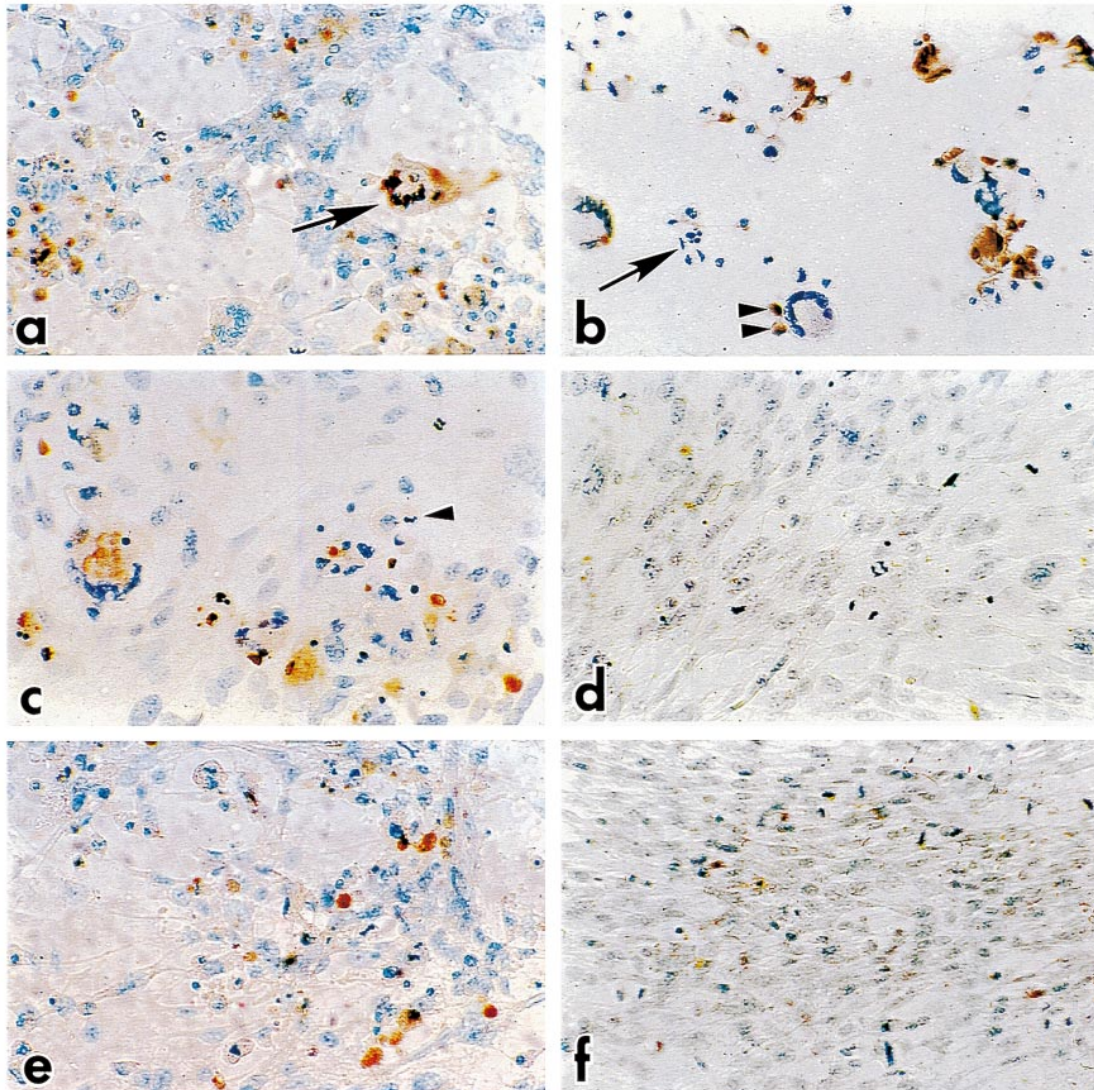


Fig. 2. Cytopathic effect and TUNEL staining of transfected cells, 48 h after electroporation. Positive TUNEL staining is indicated by the brown peroxidase stain and the cells are counterstained with methyl blue, $\times 400$. (a) BHK cells transfected with the SFV1 vector showing partial cytopathic effect, TUNEL staining of some cells, nuclear fragmentation and syncytial cells; the arrow indicates a TUNEL-positive giant syncytial cell. (b) BHK cells transfected with SFV4 RNA showing cytopathic effect and TUNEL staining of some cells; the arrow indicates fragmented nuclei which are negative for TUNEL staining; the arrowheads indicate TUNEL positive apoptotic bodies flanking a giant cell. (c) BHK cells transfected with $\Delta 7784/11033$ RNA showing partial cytopathic effect and TUNEL staining of some cells; the arrowhead indicates one of many fragmented nuclei which are negative for TUNEL staining. (d) BHK cells transfected with $\Delta 2572/3914$; note the absence of cytopathic effect and only occasional TUNEL staining. (e) H358a cells transfected with SFV1 vector RNA showing partial cytopathic effect and TUNEL staining of some cells. (f) untransfected control H358a cells subjected to the electroporation procedure.

and *NdeI* 11033 sites, the deleted plasmid treated with Klenow polymerase, and the blunt ends joined. To produce $\Delta 7784/8929$, the fragment between the two *NdeI* sites was inserted into $\Delta 7784/11033$ in the correct orientation. The presence of the required deletion in the plasmids was confirmed by automated sequencing.

For detection of apoptosis, cells were examined by agarose gel electrophoresis of extracted DNA for the DNA ladders formed by internucleosomal DNA cleavage (Fernandes &

Cotter, 1993). Where cell cultures were showing cytopathic effect after infection, the supernatant from the culture was centrifuged at 3000 *g* and the pellet pooled with the cells from the monolayer before extracting DNA. DNA was isolated by treating cells with lysis buffer (20 mM EDTA, 100 mM Tris pH 8, 0.8% w/v, sodium lauryl sarcosinate) followed by ribonuclease (10 mg/ml pancreatic ribonuclease; Sigma) at 37 °C for 4 h and proteinase K (20 mg/ml; Sigma) for 18 h. Samples were then heated to 50 °C for 2 h and the extract from

10^6 cells was loaded in each lane on 1.5% agarose gels, which were run for 5 h at 55 V in TBE (90 mM Tris pH 8.2, 90 mM boric acid, 2.5 mM EDTA). To extract RNA from transfected cells, an RNA isolation kit (Genosys) was used according to the manufacturer's instructions. Terminal deoxynucleotidyl transferase-mediated bio-dUTP nick end labelling (TUNEL; Gavrieli *et al.*, 1992) was carried out on transfected cells which had been seeded into glass chamber slides (Natale *et al.*, 1993). After fixing with 5% buffered formaldehyde, the ApopTag Plus kit (Oncor) was used to label apoptotic cells by TUNEL and peroxidase, according to the manufacturer's instructions. The results obtained for DNA laddering and TUNEL were checked by determination of cell viability by [3 H]uridine incorporation (Glasgow *et al.*, 1997).

Fig. 1(c, d) shows agarose gels of RNA produced by transcription of the pSP6-SFV4 plasmid and deletion mutants (Fig. 1c), and extracted from transfected BHK cells (Fig. 1d). The extent of the deletions is reflected in the size of the RNA detected in the transcription mix (Fig. 1c). RNA of the correct size could be detected from cells transfected with SFV4, Δ 8930/11033, Δ 7784/11033 and Δ 7784/8929, but no RNA could be detected for Δ 2572/3914 (Fig. 1d), although it was detected in the transcription mix for this mutant (Fig. 1c). This indicates that, whilst RNA synthesis occurred in transfected cells for SFV4, Δ 8930/11033, Δ 7784/11033 and Δ 7784/8929, it did not occur in cells transfected with Δ 2572/3914. This reflects the deletion in the nsP2 protein gene for Δ 2572/3914, since nsP2 is required for viral RNA synthesis (Strauss & Strauss, 1994). Fig. 1(b) shows DNA ladders, characteristic of internucleosomal DNA fragmentation, induced by transfection of BHK cells with the RNA from SFV4, Δ 8930/11033, Δ 7784/11033 and Δ 7784/8929 at 48 h after transfection. However, no DNA laddering could be detected for cells transfected with RNA from Δ 2572/3914 or for untransfected control cells which were subjected to the electroporation procedure only.

These results were confirmed by TUNEL. Representative examples of induced cytopathic effect and TUNEL, for cells fixed at 48 h after transfection, are shown in Fig. 2. For untransfected control BHK cell cultures, subjected to the electroporation procedure without added transcription mix, only occasional cells were positive for TUNEL at 48 h after electroporation and later. For BHK cell cultures transfected with SFV4 RNA, many of the cells had disintegrated before fixing. A proportion of remaining adherent cells showed positive TUNEL but many were negative (Fig. 2b). The latter may have included cells which did not receive RNA during the electroporation process. Control experiments involving electroporation of BHK cells with the SFV vector expressing LacZ have indicated that more than 90% of cells express RNA after electroporation. However, it is also clear that many cells show the characteristic nuclear fragmentation typical of apoptosis, but do not show TUNEL staining (Fig. 2b); this effect has been observed previously for SFV-infected cells (Scallan *et al.*, 1997).

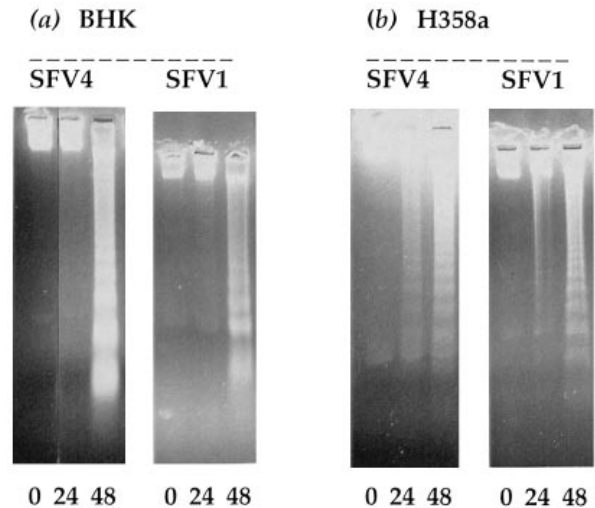


Fig. 3. DNA ladders indicative of apoptosis formed by agarose gel electrophoresis of extracted DNA at 24 and 48 h after transfection of (a) BHK cells and (b) H358a cells with SFV4 and SFV1 vector RNA. The 0 h sample consists of DNA from untransfected control cells subjected to the electroporation procedure.

Thus the TUNEL method fails to stain some cells which show morphological evidence of apoptosis. This could be due to specific substrate requirements for the TUNEL reaction which are not met in some cells undergoing the DNA fragmentation characteristic of apoptosis (Mundle & Raza, 1995). For the structural region deletion mutants, cytopathic effect in BHK cells at 48 h after transfection was less marked than for SFV4. More cells remained adherent, but many of these showed nuclear fragmentation. As with SFV4, many cells showed positive TUNEL staining (Fig. 2c), but some cells showed nuclear fragmentation without TUNEL staining. The formation of giant syncytial cells was a characteristic of all cultures undergoing cytopathic effect. At 72 h after transfection the structural protein region deletion mutants showed a cytopathic effect which was indistinguishable from SFV4. For Δ 2572/3914, which had a deletion in the nonstructural nsP2 gene and did not make RNA in transfected cells, no cytopathic effect and only occasional TUNEL staining was apparent at 48 h after transfection (Fig. 2d) or at later times; such cells were indistinguishable from untransfected cells. Both TUNEL and DNA laddering were detected at 48 h after transfection and later, when microscopically visible cytopathic effect was apparent, but were not detected at 24 h after transfection, when no cytopathic effect was apparent.

Similar experiments to detect apoptosis induction in BHK cells were carried out with the SFV1 vector (Liljeström & Garoff, 1991). Transfection of BHK cells with RNA from this vector construct resulted in the formation of DNA ladders similar to those produced by the wild-type virus (Fig. 3a) and deletion mutants (Fig. 1b). Induction of apoptosis in BHK cells by the SFV1 vector was confirmed by TUNEL staining (Fig. 2a).

In order to establish whether apoptosis induction by SFV requires p53, the H358a human lung carcinoma cell line was used. This tumour cell line carries a homozygous *p53* deletion (Takahashi *et al.*, 1989; Fujiwara *et al.*, 1994), and we confirmed in preliminary blotting experiments that no p53 mRNA can be detected in these cells. However, DNA laddering occurred following transfection both with the wild-type SFV4 RNA produced from the infectious clone and with SFV1 vector RNA (Fig. 3). Induction of apoptosis was confirmed by TUNEL staining (Fig. 2e), but untransfected control cells were negative (Fig. 2f). Similar results to those obtained for H358a cells were obtained for a number of other human lung carcinoma cells, obtained from the ATCC. These included H520 cells, which show reduced p53 expression, and H596 and H460 cells, which show normal p53 expression (Takahashi *et al.*, 1989). This is further evidence that apoptosis induction by SFV and the SFV vector occurs independently of p53 expression.

The results of this study indicate that p53-independent apoptosis can be induced by the nonstructural region of the SFV genome, and that apoptosis induction is dependent on viral RNA synthesis. This does not preclude the possibility that apoptosis could also be induced by one or more of the viral structural proteins, or that p53-dependent apoptosis may also occur. Since apoptosis is induced by transfected RNA, virus particles are not required. It is known that activation of the double-stranded RNA-dependent protein kinase PKR induces apoptosis (Lee & Estaban, 1994; Kibler *et al.*, 1997). Since alphavirus multiplication involves the synthesis of double-stranded RNA, it seems likely that this induces apoptosis. Double-stranded RNA synthesis has long been recognized as a factor in alphavirus cytopathogenicity which is linked to interferon induction, but the cell-death mechanism involved was not recognized at the time as apoptosis (Atkins & Lancashire, 1976; Atkins, 1977). It is also clear that double-stranded RNA synthesis is not the only contributory factor in the induction of alphavirus cytopathogenicity, and that structural protein synthesis is also involved (Atkins, 1977; Frolov *et al.*, 1994). Inhibition of cytopathogenicity, either by the action of the *bcl-2* gene, or by restriction of viral RNA synthesis by mutation, leads to persistent infection (Atkins, 1979; Barrett & Atkins, 1981; Ubol *et al.*, 1994; Scallan *et al.*, 1997).

The SFV vector has a deletion of the structural protein region of the viral genome and a multicloning site inserted in its place; the nonstructural region remains intact and is indeed essential for high-level expression of cloned genes (Liljeström & Garoff, 1991). The results obtained in this study for structural region deletion mutants indicate that expression of the nonstructural region of the SFV genome by the vector induces apoptosis, and that this is not the result of further manipulation to create the vector. Induction of apoptosis by the vector may be advantageous for purposes such as vaccine construction, where persistence of the viral genome following administration of the vaccine is not desirable; however, apoptosis induction

may also be a disadvantage since it may result in tissue damage. The presence of an apoptosis inducing function in the nonstructural region of the SFV genome may make SFV vectors useful vehicles for the destruction of cells with pathogenic potential, such as tumour cells.

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