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Sites of Simian Foamy Virus Persistence in Naturally Infected African Green Monkeys: Latent Provirus Is Ubiquitous, Whereas Viral Replication Is Restricted to the Oral Mucosa

Valeria Falcone,* Jörg Leupold,* Jörn Clotten,* Enikö Urbanyi,* Ottmar Herchenröder,* Wolfbernhardt Spatz,† Benedict Volk,‡ Norbert Böhm,§ Antonio Toniolo,¶ Dieter Neumann-Haefelin,* and Matthias Schweizer*,[1]

*Department of Virology, Institute for Medical Microbiology and Hygiene, University of Freiburg, Freiburg, Germany; †Department of Oto-Rhino-Laryngology, ‡Department of Neuropathology, and §Department of General Pathology, University of Freiburg, Freiburg, Germany; and ¶Department of Clinical and Biological Sciences, University of Pavia, Varese, Italy

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Foamy viruses (FV), retroviruses of the genus Spumavirus, are able to infect a wide variety of animal species and replicate in nearly all types of cultured cells. To identify the cells targeted by FV in the natural host and define the sites of viral replication, multiple organs of four African green monkeys naturally infected with simian FV type 3 were investigated for the presence of FV proviral DNA and viral transcripts. All organs contained significant amounts of FV proviral DNA. In addition to proviruses containing the complete transactivator gene *taf,* proviral genomes carrying a specific 295-bp deletion in the *taf* gene were detected in all monkeys. As in the case of human foamy virus the deletion leads to the formation of the *bet* gene that is regarded to be instrumental in the regulation of viral persistence. FV RNA was detected by RT-PCR and *in situ* hybridization only in the oral mucosa of one monkey. No other samples contained detectable levels of viral transcripts. Histopathological changes were not observed in any of the tissue samples analyzed. Our results show that the natural history of FV is characterized by latent infection in all organs of the host and by minimal levels of harmless viral replication in the oral mucosa. The broad host cell range *in vivo* further encourages the development of FV-derived vectors for therapeutic gene delivery.

INTRODUCTION

Simian foamy viruses (SFV) are complex retroviruses able to establish lifelong persisting infections in various mammalian species including nonhuman primates (Hooks and Gibbs, 1975). Despite their strong *in vitro* cytopathicity, no association of infection of foamy viruses (FV) with any clinical symptom has ever been demonstrated in naturally infected nonhuman primates or accidentally infected humans (Schweizer *et al.,* 1995; Heneine *et al.,* 1998). Even experimental cross-species infection of laboratory animals did not result in any disease (Brown *et al.,* 1982; Schmidt *et al.,* 1997; Saib *et al.,* 1997). In a transgenic mouse model the expression of a FV transgene, localized mainly in the central nervous system (CNS) and in the striated muscle, was followed by encephalopathy and myopathy (Bothe *et al.,* 1991; Aguzzi *et al.,* 1996). FV are characterized by a very broad host cell range *in vitro* but little is thus far known about target cells and viral gene expression *in vivo.* It has recently been shown that $CD8^+$ lymphocytes constitute a major target for foamy viruses in the peripheral blood; nevertheless, viral gene expression was not detected in this T-cell subpopulation (Von Laer *et al.,* 1996). Accordingly, infectious virus can be isolated from lymphocytes of infected individuals only after *in vitro* activation (Schweizer *et al.,* 1997); on the contrary, conventional virus isolation is continuously possible from the oropharynx of naturally infected monkeys (Neumann-Haefelin *et al.,* 1993; Schweizer *et al.,* 1997).

The mechanisms allowing this virus to establish apathogenic persistent infections *in vivo* are still an open question. The ability of the spumavirus prototype human foamy virus (HFV) to establish persistent infections in cell culture has been related to the presence of defective forms of the provirus (ΔHFV). These defective genomes carry a specific deletion in the transactivator gene *bel-1* generated by a single splicing event in the genomic RNA (Saib *et al.,* 1995), leading to the formation of the *bet* gene, whose functions are still unknown. However, the involvement of ΔHFV in the establishment of persistence has not been confirmed by others (Yu *et al.,* 1996) and the role of this defective form in the biology of FV remains unclear.

In the present study, tissues of four African green monkeys (AGMs) naturally infected with AGM FV (SFV-3) were molecularly analyzed to define the sites and mode of FV persistence *in vivo.* Perfusion of the animals was carried out in order to clear tissues from FV-infected

[1] To whom correspondence and reprint requests should be addressed at Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg, Hermann-Herder-Strasse 11, D-79104 Freiburg, Germany. Fax: +49-761-203-6634. E-mail: mschweiz@ukl.uni-freiburg.de.

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TABLE 1

Detection of SFV-3 *pol* and *taf* DNA in Various Organs of Naturally Infected AGMs

						taf PCR in AGM No.									
	pol PCR in AGM No.					A1		A4		A5		A7		A8	
Organ	A1	A4	A5	A7	A8	W[a]	D[a]	W	D	W	D	W	D	W	D
Bladder	+	n.d.	n.d.	+	−		n.d.		n.d.		n.d.		n.d.		n.d.
Bone marrow	+	+	n.d.	n.d.	−		n.d.		n.d.		n.d.		n.d.		n.d.
Bowel	+	+	+	+	−	+	+		n.d.	+	+		n.d.		−
Diaphragm	+	+	+	+	−	+	−	+	+	+	−	+	+		−
Heart	+	+	+	n.d.	−	+	+		n.d.	+	−		n.d.		n.d.
Kidney	+	+	+	+	−		n.d.		n.d.		n.d.	+	−		−
Liver	+	+	+	+	−	+	+	+	−	+	+	+	−		−
Lung	+	n.d.	+	+	−	−	+		n.d.	+	−	+	+		−
Lymph nodes	+	+	+	+	−	+	+	+	−	+	+	+	+		−
Oral mucosa	+[b]	+	+	+	−	+	+	+	+	+	+	+	−		−
Pancreas	+	+	n.d.	+	−		n.d.		n.d.		n.d.		+		n.d.
Parotid	+	n.d.	+	+	−		n.d.		n.d.		n.d.		+		−
PBL	+	+	+	+	−	+	+	+	−	+	+	+	−		−
Prostate	n.d.	+	n.d.	n.d.	−		n.d.	+	−		n.d.		n.d.		−
Skeletal muscle	+	+	+	+	−	+	+		n.d.	+	+	+	+		−
Spleen	+	+	+	+	−	+	+	+	−	+	+	+	+		−
Stomach	+	+	+	+	−		n.d.		n.d.		n.d.		n.d.		n.d.
Testis	+	+	n.d.	n.d.	−		n.d.	+	−		n.d.		n.d.		−
Tonsils	+	n.d.	+	n.d.	−		n.d.		n.d.		n.d.		n.d.		−
Uterus	n.d.	n.d.	+	+	−		n.d.		n.d.	+	−	+	−		−
CNS															
Cerebellum	+	+	+	+	−		n.d.		n.d.		n.d.		n.d.		n.d.
Frontal cortex	+	+	+	+	−	+	+		n.d.	+	+		n.d.		−
Occipital cortex	n.d.	n.d.	+	+	−	+	−		n.d.		n.d.	+	−		−
Temporal cortex	n.d.	+	n.d.	+	−		n.d.		n.d.	+	−		n.d.		−
Hippocampus	+	+	+	+	−	+	+		n.d.		n.d.	+	+		−
Spinal Cord	+	+	+	+	−	+	+		n.d.	+	−	+	+		−
Thalamus	+	+	n.d.	+	−		n.d.		n.d.	+	−		n.d.		n.d.

[a] *taf* PCR gives rise to a 486-bp fragment in the wild-type virus (W) and to a 191-bp in the defective form (D).

[b] All samples were also investigated for SFV-3 *pol* and *taf* transcripts by RT-nested PCR. Oral mucosa from AGM A1 was the only sample that repeatedly revealed detectable levels of *pol* and *taf* mRNA (Fig. 3a).

circulating blood cells. Using nested PCR, RT-nested PCR, and *in situ* hybridization (ISH), FV DNA was detected in all organs investigated, whereas FV RNA could exclusively be found in cells of the oral mucosa of one animal. In addition to complete genomes, defective proviruses carrying a deletion in the transactivator gene *taf* could be detected in several organs. This study provides data on the *in vivo* targets of SFV. Our results show that natural infection with SFV-3 is not restricted to particular body compartments and that proviral genomes persist in all organs. However, viral replication occurs at detectable levels only in a minimal number of cells.

RESULTS

Localization and quantification of FV DNA in different organs

When SFV-3 *pol*-specific nested PCR was performed on DNA extracted from perfused organs, all samples listed in Table 1 were found to be positive for FV proviral DNA. Negative controls (DNA from tissue samples of a seronegative monkey, AGM A8) were run in parallel to exclude false-positive results due to contamination with a laboratory source of FV nucleic acid, and the controls always stayed negative. To compare the amount of proviral DNA present in the CNS and in other compartments, a semiquantitative analysis was performed. Ten-fold serial dilutions of DNA extracted from cerebellum, cerebral frontal cortex, liver, skeletal muscle, oral mucosa, and lymph nodes of the four AGMs were amplified by *pol*-specific nested PCR and the minimal amounts of cellular DNA essential to give a positive result were compared. Considering that the sensitivity of the *pol* nested PCR used was about five copies of viral DNA (Schweizer *et al.,* 1995; Von Laer *et al.,* 1996) the viral copy number per nanogram of cellular DNA present in each sample was calculated. As shown in Fig. 1, most tissue samples harbored 1 viral copy per nanogram of cellular DNA. Only

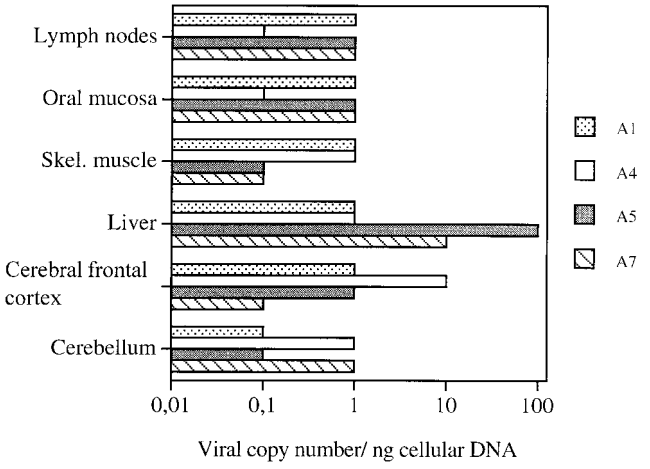

FIG. 1. Semiquantitative analysis of proviral load in some positive organs of infected AGMs. Bars represent the copy number of viral DNA per nanogram of cellular DNA.

in the liver of AGM A5 and AGM A7 and in the frontal cortex of AGM A4 were larger amounts of proviral DNA detected. However, the biological significance of this finding is unclear since not all monkeys showed the same pattern.

Detection of FV proviral genomes with *taf* deletions

Amplification of viral DNA using *taf*-specific primers was done on the organs listed in Table 1 to establish whether proviral genomes carrying deletions in the *taf* gene (Fig. 2A) occur after natural infection with SFV-3. Indeed, proviruses containing a complete and/or a deleted *taf* gene were detected. In all four monkeys investigated, amplification with *taf* primers gave rise in all AGMs to the same amplification products, namely to a 486- and a 191-bp fragment in the case of the complete and deleted *taf* genes, respectively. A distinct distribution of both forms was observed in each of the four animals. In some of the organs both forms were concurrently present, in others only the wild-type form could be detected, and in a single case (lung of AGM A1) the deleted form exclusively was found. Southern blot hybridization with an SFV-3-specific probe of some representative samples of AGM A5 showed that the ratio between the two forms is variable and that a predominance of the wild-type form is often observed (Fig. 2B). Amplification products from one sample (DNA from skeletal muscle of AGM A1) corresponding to the undeleted and to the deleted forms of the *taf* gene were cloned and sequenced. As shown in Fig. 2A, a 295-bp deletion was found. The deletion is localized between nucleotides 10,271 and 10,567; its margins correspond to the splice donor and acceptor sites of the intron of the *bet* gene; thus, as in the case of HFV, the deletion generates a new open reading frame encoding the Bet protein. The same deletion was also identified by sequencing the *taf* region of SFV-3 obtained from human fibroblasts infected *in vitro* (not shown). Alignment of the AGM A1 sequence with the proviral SFV-3 prototype sequence (Renne *et al.*, 1992) revealed three substitutions: an A instead of a G at positions 10,340 and 10,460 in the undeleted *taf* gene and a G instead of an A at position 10,267 in the deleted gene.

Detection of viral transcripts by RT-PCR

To identify the sites of active FV replication, RT-PCR was performed on the organs listed in Table 1. To reduce RNA degradation most of the samples for RNA extraction were collected before PFA perfusion (AGM A1 and A4) and snap-frozen immediately after excision in liquid nitrogen. All organs were analyzed with *pol*-, *taf* (deleted and undeleted)-, and c-*myc*-specific primers able to generate small amplification products (106, 391/96, and 99 bp, respectively). Thus, we also succeeded in the detection of viral sequences in samples showing partial degradation of RNA. Eighty-five percent of the samples were positive for the presence of c-*myc* transcripts. When these samples were analyzed with *pol*- and *taf*-specific primers, a positive signal was detected only in the oral mucosa of AGM A1. Both deleted and wild-type *taf* transcripts were found (Fig. 3a). However, a single round of

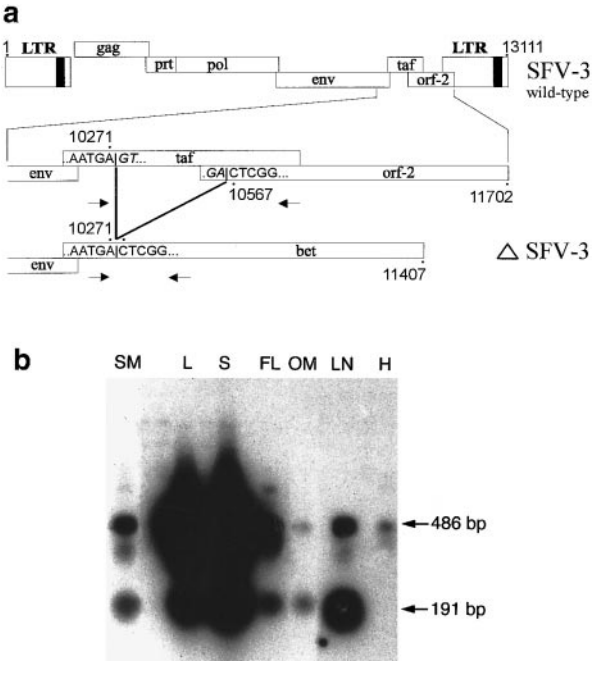

FIG. 2. Detection of complete and defective proviral DNA. (a) Schematic organization of SFV-3 genome. The *taf*/*orf*-2 region is enlarged to compare the complete (SFV-3 wild-type) and defective (ΔSFV-3) proviruses; nucleotide numbers refer to the sequence of SFV-3 strain LK-3, Accession No. M74895. Primers used for PCR analysis are represented by arrows. (b) Southern blot analysis of *taf*-PCR amplification products from different organs of AGM A5. SM, skeletal muscle; L, liver; S, spleen; FL, cortex of cerebral frontal lobe; OM, oral mucosa; LN, lymph nodes; H, heart.

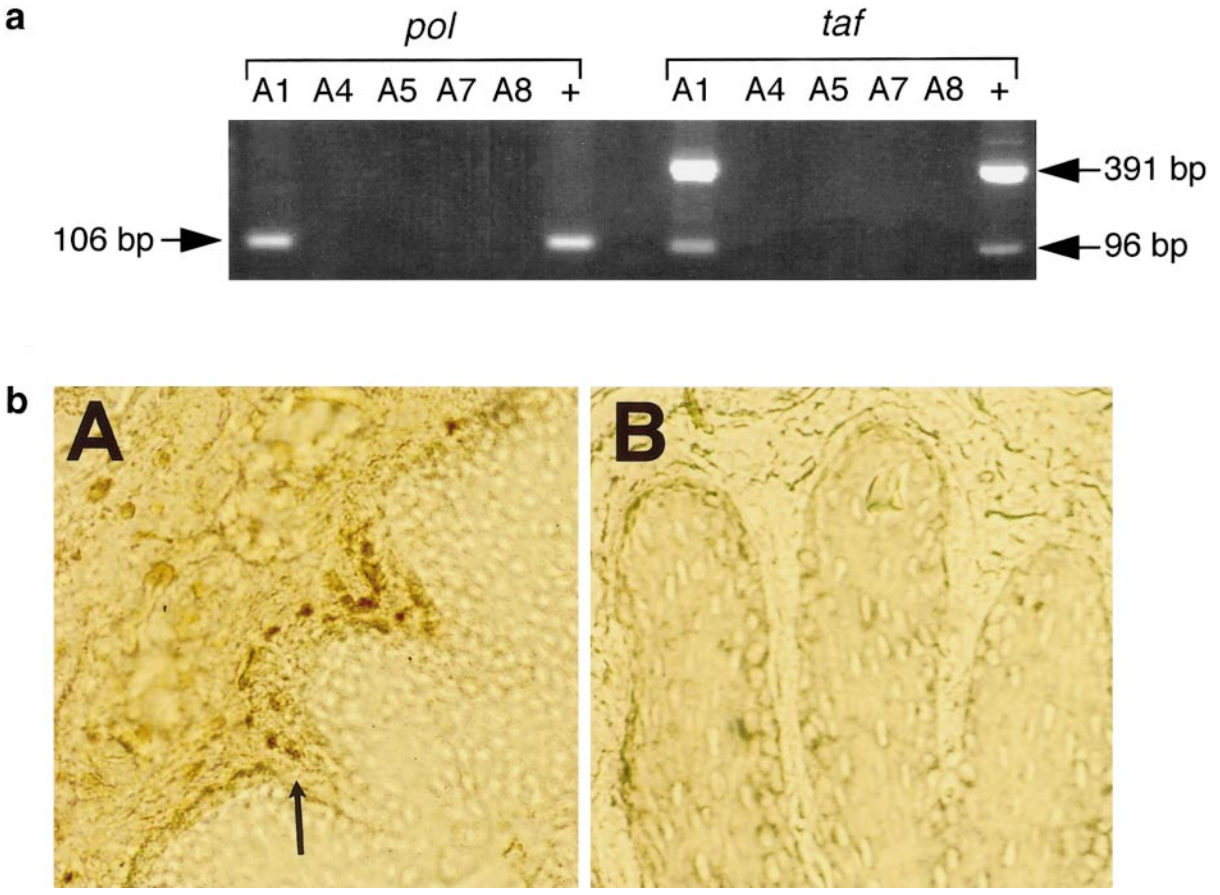

FIG. 3. (a) RT-nested PCR amplification products separated on an ethidium bromide-stained agarose gel. cDNA from oral mucosa of AGM A1, A4, A5, A7, and A8 was amplified using *pol* and *taf* primers. (+) cDNA from SFV-3-infected human fibroblasts. (b) *In situ* hybridization of oral mucosa of AGMs A1 (A) and A8 (B; negative control) using an SFV-3 *env*-specific antisense probe. Arrow indicates one of multiple SFV-positive cells along the stroma–epithelium borderline.

amplification was not sufficient to obtain the positive signal. None of the other samples analyzed (Table 1) contained detectable levels of viral RNA. Assessment of the efficacy of the DNase treatment and of the sensitivity of the *pol* RT-PCR was performed as described under Materials and Methods. The latter control revealed a detection minimum of 200 copies of target RNA in the presence of 5 μg of tRNA.

In situ detection of FV infected cells in the oral mucosa

Oral mucosa samples from AGMs A1, A4, A5, A7, and A8 were analyzed by *in situ* hybridization. Fresh frozen tissue sections were hybridized with antisense RNA probes specific for the *env, taf,* or *pol* region of SFV-3. Only the oral mucosa of AGM A1, previously found to be positive by RT-PCR, gave a positive signal by *in situ* analysis. Distinct positive cells were detected with all SFV-3 probes in the stroma underlying the basal epithelium (Fig. 3b, panel A). Positive cells often colocalized with small inflammatory infiltrates present in this area. Similar infiltrates were also seen in some sections of uninfected tissue. Thus, the causative role of FV in the induction of these infiltrates is questionable. Hybridization with sense probes or with an irrelevant probe was always negative as was hybridization of samples of oral mucosa from SFV-3-negative AGM (Fig. 3b, panel B). RNase treatment of the sections before hybridization always resulted in the loss of the hybridization signal (not shown). Histopathological examination did not reveal any lesion, neither in this mucosal area nor in the other organs listed in Table 1.

DISCUSSION

FV tropism *in vitro* has been well characterized (Mergia *et al.,* 1996), but important questions about cell tropism and viral gene expression *in vivo* have not yet been answered. Experimental infections of mice and rabbits with SFV and HFV (Swack and Hsiung, 1975; Brown *et al.,* 1982; Schmidt *et al.,* 1997; Saib *et al.,* 1997) have produced contradictory results regarding the distribution of viral genomes in different organs, the frequency of virus isolation, and the induction of pathological changes. We therefore analyzed the *in vivo* behavior of SFV in AGMs

that represent one of the natural hosts of these viruses. The present study clearly shows that SFV infection progresses toward latency in a wide range of cells. Since $CD8^+$ lymphocytes represent a main reservoir of SFV proviral DNA among peripheral blood cells (von Laer *et al.,* 1996), the ubiquity of these cells may cause false-positive results by PCR analysis. Therefore, the animals investigated in the present study were euthanized by perfusion with PBS alone or with PBS and PFA before sample collection. We found that most tissue samples harbor one viral copy per nanogram of cellular DNA (Fig. 1) which corresponds to one copy in about 150 cells (given that a typical mammalian cell contains about 7 pg of genomic DNA). It has been shown previously that circulating blood lymphocytes of the animals investigated in this study (Von Laer *et al.,* 1996) harbor one copy of proviral DNA in 10^5 to 10^3 cells; this observation rules out the possibility that contaminating lymphocytes had produced positive results. Moreover, it is well known that FV can be isolated in monolayer cultures obtained from organs of infected monkeys, thus proving the presence of FV in solid tissues (Hooks and Gibbs, 1975). The wide distribution of proviral genomes observed *in vivo* might be the result of transient virus delivery by lymphocytes actively replicating the virus. It would be of interest to identify the productively infected cells in the very early stages after infection to clarify how FV is spread to the different organs.

In a transgenic mouse model FV transgene expression and cytopathicity have been observed, with specific neuronal loss in the hippocampus and cerebral cortex (Bothe *et al.,* 1991); ensuing reports on a direct correlation between FV and encephalopathy in an orangutan (McClure *et al.,* 1994) and neurological disorders in humans (Westarp *et al.,* 1992) have been challenging, but a causal link between FV infection and such diseases has never been proved (Rösener *et al.,* 1996; Schweizer *et al.,* 1995). In the present study no histopathological lesions could be observed in any of the organs analyzed, especially the regions involved in the transgenic mouse model. Moreover, a semiquantitative analysis revealed no significant difference in the amount of proviral DNA harbored in the CNS and other organs. This finding indicates that in contrast to what has been described in FV-transgenic mice, infection with FV is apathogenic and no tissue-specific tropism is observed in the natural host.

In addition to proviruses containing the complete transactivator gene *taf,* proviral genomes carrying a specific 295-bp deletion in the *taf* gene were detected in all four monkeys investigated. Similar deletions, described in the transactivator gene *bel-1* of cultured HFV (Saib *et al.,* 1995), have also been found in the chimpanzee FV (Herchenröder *et al.,* 1994) and in bovine foamy virus (Renshaw and Casey, 1994). It has been proposed that the predominance in experimentally infected rabbits of either the defective or the wild-type *bel-1* gene might be associated with the establishment of latent or productive infections in different compartments (Saib *et al.,* 1997); however, these experiments have given no evidence of viral replication in the organs predominantly harboring the wild-type form. The same authors suggest that, relative to acute infections, in chronic infections a structural gene shut-off might be accompanied by conserved *bet* expression (Saib *et al.,* 1997). Here we found that in persistently infected AGMs both wild-type and defective *taf* genes are randomly distributed in most of the organs, although the methods used do not allow one to further establish whether both *taf* forms are harbored in the same cell. However, very low levels of viral transcripts (*pol* and *taf*/*bet*) could exclusively be detected in the oral mucosa of one of the monkeys investigated. Our finding shows that a deletion in the transactivator gene analogous to that described for HFV occurs also *in vivo* in naturally infected monkeys. Interestingly, all four AGMs naturally infected in the wild more than 20 years ago carry the same type of *taf* deletion. This observation suggests an essential role of *taf*-deleted proviral genomes *in vivo* and argues against the possibility that *taf* deletions represent a cell culture epiphenomenon unrelated to any *in vivo* function. However, the role of the defective *taf* gene in the establishment of FV persistence still remains unclear. The random distribution of both proviral forms observed here suggests that the occurrence of *taf* deletions is not directly related to the development of different stages of viral persistence, as hypothesized by Saib *et al.* (1995).

Although virus isolation from throat swabs was repeatedly successful in all four AGMs (including swabs taken immediately before sampling of tissues), viral transcripts could exclusively be detected in the oral mucosa of AGM A1. The apparent contradiction in this result could be resolved by the following possibilities: (i) the number of cells harboring FV RNA is unknown; thus we cannot exclude the possibility that the concentration of viral RNA in some negative samples was below the detection level of RT-PCR (200 copies for the *pol* RT-PCR); (ii) exogenous factors such as viral or bacterial coinfection and cytokine production might play a major role in regulating viral replication (Fauci, 1996; Falcone *et al.,* 1998); (iii) the productively infected cells might not be homogeneously distributed in the oral cavity; i.e., not all tissue samples taken from the oral mucosa and analyzed by RT-PCR and ISH contained the rare infected cells. Accordingly, the ISH analysis revealed that the productively infected cells are localized in small scattered foci. It has now been well established that accidental transmission of SFV from nonhuman primates to humans is rare and occurs only under conditions of severe injury (Schweizer *et al.,* 1997; Heneine *et al.,* 1998). It appears that the amount of virus that is orally shed by persistently infected monkeys is enough to promptly infect susceptible cells *in vitro* but that a larger amount of virus in saliva is needed for

efficient transmission through bites. Taken together, our results show that natural infection by SFV-3 is associated with the persistence of proviral genomes in all organs and with minimal levels of viral replication in the oral mucosa. The ubiquity of viral genomes indicates a wide range of FV infectable cells *in vivo*. Thus, missing restriction of target cells further encourages the development of FV-derived vectors for somatic gene transfer. Although systemic use of such vectors may appear rather unsuitable, they might be superior tools for topical application and *ex vivo* transduction of cells inaccessable to common vector systems.

MATERIALS AND METHODS

Animals

Four African green monkeys [*Chlorocebus* (*Cercopithecus*) *aethiops*], A1, A4, A5, and A7, had been kept as blood donors at the Department of Virology in Freiburg after capture in Kenya in 1978–1980. All animals were naturally infected with SFV-3 as proven by serology in 1980. It is likely that the infection occurred in the wild. As negative controls, tissue samples were obtained from a FV-negative AGM (A8) euthanized for other purposes at the Chiron Behring primate facilities in Marburg, Germany.

Animal perfusion and sample collection

The four animals were anesthesized by intramuscular injection with Ketanest/xylazin and euthanized by perfusion with cold PBS, pH 7.0, through the aorta. In two cases (A1 and A4) an additional perfusion with buffered 4% paraformaldehyde (PFA) was performed. Tissue samples were directly frozen in liquid nitrogen for DNA and RNA extraction or further fixed in 4% PFA for histological studies. For *in situ* hybridization analysis, samples were snap-frozen in prechilled *n*-hexane (Merck, Darmstadt, Germany) and subsequently in liquid nitrogen.

Nucleic acid preparation

DNA was extracted using the QIAmp Tissue Kit (Quiagen, Hilden, Germany) according to the manufacturer's recommendations. For RNA isolation, tissue samples of 50 mg were homogenized by use of an Ultraturrax-T25 homogenizer in a 1-ml Ultraspec RNA Isolation System (Biotecx, Houston, TX) and RNA extraction was performed according to the manufacturer's instructions. The RNA pellet was resuspended in Tris/EDTA buffer and further purified by phenol–chloroform–isoamyl alcohol extraction and ethanol precipitation according to standard procedures (Sambrook *et al.,* 1989). Quality of RNA was checked by electrophoresis in 1% denaturing agarose gel as described (Sambrook *et al.,* 1989). Prior to retrotranscription, samples were treated with 10 U/μg RNA of RNase-free DNase (Boehringer Mannheim, Mannheim, Germany) for 1 h at 37°C in the presence of 20 U of RNase inhibitor (Boehringer Mannheim) and 2 mM $MgCl_2$.

For cDNA synthesis, 1–5 μg DNase-treated RNA was retrotranscribed using random primers and a first-strand cDNA synthesis kit (Pharmacia, Freiburg, Germany) according to the manufacturer's recommendations.

Polymerase chain reaction and Southern blotting

For the detection of FV DNA, amplification of the SFV-3 *pol* region was performed by nested PCR as previously described (Schweizer and Neumann-Haefelin, 1995). The suitability of each DNA sample for PCR was tested by amplification of the cellular single-copy gene c-*myc* (Schweizer *et al.,* 1995). For the detection of the *taf* deletion, PCR was performed under the same conditions using the primer pairs Taf 1 5′ GTA TCA TGG CTT CCT GGG A 3′, Taf 4 5′ CCA GAA GGG TCC TCC AGA CAT 3′ for the first round and Taf 2 5′ ATT TAA AGA TGA GAA ATT GGG T 3′, Taf 3 5′ CCA TGG CGT CAC CAC TGG AAG 3′ for the second round; *taf* amplification gives rise to a 486-bp fragment in the wild-type virus and a 191-bp fragment in the defective form. Specificity of visualized bands by ethidium bromide was confirmed by hybridization with a radiolabeled *taf*-specific RNA probe on Southern blots prepared by standard procedures (Sambrook *et al.,* 1989).

For the detection of FV transcripts, cDNA was amplified by nested PCR under standard conditions with the following primer pairs. For the detection of unspliced *pol* and *taf* transcripts, primers giving rise to small amplification products were used. For the *pol* region of SFV-3, Pol 3 5′ GCT ACT CTA GCT GCG CCT CC 3′, and Pol_{in} 2, 5′ GAA GGA GCC TTA GTG GGG TA 3′ were used for the first round and Pol 4, 5′ ATT GAG GCC TGA ACG ACC TG 3′, and Pol 5, 5′ ATC GAC TAC TAC AAG GAC AT 3′, were used for the second round (amplification product of 106 bp). For the *taf* region of SFV-3, Taf 7, 5′ GGA TAT GAA GAT AAA GAA GC 3′, and Taf 11, 5′ CCA CAC CCC ATC TGG AGC G 3′, were used for the first round, and Taf 2, 5′ ATT TAA AGA TGA GAA ATT GGG T 3′, and Taf 10, 5′ CTG TAT CTT CTA ACA GGA TT 3′, were used for the second round (amplification products of 391 bp for the undeleted form and of 96 bp for the deleted form). For the cellular gene c-*myc,* MLA, 5′ GCC CCG AGC CCC TGG TGC TCC A 3′, and Myc 6, 5′ GCA GAA GGT GAT CCA GAC TC 3′, were used for the first round, and MLI, 5′ GAC ACC GCC CAC CAC CAG CAG C 3′, and Myc 5, 5′ CCT TTT GCC AGG AGC CTG CCT 3′, were used for the second round (amplification product of 99 bp).

Assessment of the sensitivity of RT-PCR

The sensitivity of RT-PCR to detect FV *pol* transcripts was established by using an *in vitro* transcribed FV RNA and a procedure similar to that described for the detec-

tion of Borna disease virus (Sauder and de la Torre, 1998). Briefly, the plasmid PMSO2 (nucleotides 1 to 6825 of SFV-3/LK-3 cloned into pSPT19; Schweizer *et al.,* 1989) was linearized with *Bst*EII and *in vitro* transcribed by T7 RNA polymerase using an RNA transcription kit (Boehringer Mannheim), in the presence of [^{32}P]CTP. After *in vitro* transcription, template DNA was eliminated by DNase treatment for 15 min at 37°C. *In vitro* transcribed RNA (nucleotides 5870–6825) was analyzed by gel electrophoresis and the concentration was determined by spectrophotometry. By measuring the percentage of incorporated radioactivity, and by knowing the number of cytosine residues present in the template DNA fragment, the amount of moles and molecules of RNA that have been transcribed can be calculated. Dilutions up to 1 copy/μl were prepared in tRNA (1 mg/ml). RT-nested PCR with primers Pol 3, Pol$_{in}$ 2, Pol 4, and Pol 5 was performed under standard conditions (von Laer *et al.,* 1996). RT-nested PCR was negative when the reverse transcriptase was omitted, indicating that the RNA preparation was free of template plasmid DNA.

Molecular cloning and nucleotide sequencing

Amplification products (*taf* region) were molecularly cloned using the TA cloning kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions and sequenced by the dideoxy chain termination method employing a T7 sequencing kit (United States Biochemicals) and SP6 and T7 primers homologous to flanking regions of the TA vector.

DIG labeling of RNA probes

Sense and antisense complementary RNA probes were synthesized by use of the DIG RNA Labeling Kit (Boehringer Mannheim) in the presence of DIG-labeled nucleotides.

To generate an *env*-specific probe, bases 6935 to 7727 of SFV/LK-3 were cloned into the *Bam*HI sites of pSPT19. For the synthesis of the sense *env* probe, the plasmid was linearized with *Bam*HI and T7 polymerase was used; for the antisense probe, *Hin*dIII and SP6 polymerase were used. For the synthesis of the *taf* probe, bases 9848 to 10,781 of LK-3 were cloned into the *Kpn*I and *Pst*I sites of pSPT19; for the sense probe the plasmid was linearized with *Eco*RI and T7 polymerase was used; for the antisense probe, *Hin*dIII and SP6 polymerase were used. For the synthesis of the *pol* probe, the 465-bp *pol* amplification product was cloned into the *Eco*RI sites of pCRII by use of the TA cloning kit; for the sense probe the plasmid was linearized with *Hin*dIII and T7 polymerase was used; for the antisense probe, *Xba*I and SP6 polymerase were used. *In vitro* transcription was performed for 2 h at 37°C and subsequently stopped by degrading the plasmid with 1 μl of DNase for 15 min at 37°C. To reduce the size of the transcripts, alkaline hydrolysis was performed according to a published protocol (Schaeren-Wiemers and Gerfin-Moser, 1993).

Preparation of tissue sections for *in situ* hybridization and histological examination

Tissue sections (10 μm) were cut from frozen tissue samples embedded in optimal conditioned medium (Tissue Tek, Bayer, Leverkusen, Germany) by use of a cryostat; sections were mounted onto Superfrost Plus slides (Menzel Glaser, Braunschweig, Germany), air-dried, and fixed in Streck tissue fixative (STF; Streck Laboratories, Omaha, NE) for 4–6 h. Sections were immediately used for *in situ* hybridization or kept at −80°C until use. For histological examination tissue samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin wax. Sections were cut at 5 μm and stained with hematoxylin and eosin for examination.

In situ hybridization

In situ hybridization was performed according to a published protocol (Schaeren-Wiemers and Gerfin-Moser, 1993), with modifications. Briefly, tissue sections were treated with 0.1 M HCl for 10 min, rinsed in PBS, and digested for 10 min with Proteinase K (1 μg/ml in 20 mM Tris–HCl, 2 mM $CaCl_2$). Slides were washed with PBS, postfixed for 10 min in STF, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 20 min. Sections were then prehybridized for 2–4 h at room temperature with a hybridization buffer consisting of 50% formamide, 5× SSC (1× SSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 1× Denhardt's solution, 10% dextran sulfate, 250 μg/ml tRNA, and 500 μg/ml denatured salmon sperm DNA. Hybridization with DIG-labeled probes (20–200 ng/ml probe in the hybridization buffer) was performed overnight at 45–50°C. After hybridization slides underwent a 5-min wash in 5× SSC at the hybridization temperature to remove coverslips, followed by a 30-min treatment with RNase (20 μg/ml in 10 mM Tris–HCl, 1 mM EDTA) at 37°C and a 1-h wash in 0.2× SSC at the hybridization temperature. For the detection of the hybridization signal the Tyramide signal amplification system was used (DuPont, Boston, MA) according to the manufacturer's recommendations.

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