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Construction of Infectious Feline Foamy Virus Genomes: Cat Antisera Do Not Cross-Neutralize Feline Foamy Virus Chimera with Serotype-Specific Env Sequences

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Full-length genomes of the feline foamy virus (FFV or FeFV) isolate FUV were constructed. DNA clone pFeFV-7 stably directed the expression of infectious FFV progeny virus indistinguishable from wild-type, uncloned FFV isolate FUV. The *env* and *bel 1* genes of pFeFV-7 were substituted for by corresponding sequences of the FFV serotype 951 since previous studies implicated a defined part of FFV Env protein as responsible for serotype-specific differences in serum neutralization (I. G. Winkler, R. M. Flügel, M. Löchelt, and R. L. P. Flower, 1998. *Virology* 247: 144–151). Recombinant virus derived from chimeric plasmid pFeFV-7/951 containing the hybrid *env* gene and the parental clone pFeFV-7 were used for neutralization studies. By means of a rapid titration assay for FFV infectivity, we show that progeny virus derived from plasmid pFeFV-7 was neutralized by FUV- but not by 951-specific antisera, whereas pFeFV-7/951-derived chimeric virus was neutralized by 951-specific antisera only. Both recombinant proviruses will be useful for repeated delivery of foreign genes for therapeutic gene applications into cats.

INTRODUCTION

Spumaretroviruses or foamy viruses (FV) are considered as potential vectors for targeted gene delivery due to their apparent apathogenicity and peculiar aspects in their mode of replication and gene expression (Mergia and Luciw, 1991; Löchelt and Flügel, 1995; Rethwilm, 1995; Coffin, 1996; Yu *et al.,* 1996; Linial, 1999). We started to work on feline foamy virus (FFV) to establish an animal model for investigating the potential and applicability of FV-derived vectors for targeted gene delivery and to study FV replication in its natural host. Administering FFV-based vectors into cats represents an experimental system to investigate the versatility of spumaretroviral vectors, e.g., by interfering with replication and pathogenicity of feline immunodeficiency virus (FIV). Those studies are of general interest as infection of cats with FIV results in virtually identical clinical symptoms characteristic for HIV-induced AIDS in man (Elder *et al.,* 1998). FFV was repeatedly isolated from and detected in naturally infected cats that were apparently healthy or suffered from diverse diseases, and its role as a copathogen for FIV is still under debate (Riggs *et al.,* 1969; Hackett *et al.,* 1970; Flower *et al.,* 1985; Bendecchi *et al.,* 1992; Loh, 1993; Winkler *et al.,* 1999).

Our previous studies showed that FFV is clearly distinct from the closely related primate FVs, but major aspects of gene expression and replication are conserved among known FVs (Bodem *et al.,* 1996, 1998a,b; Winkler *et al.,* 1997). Although different FV isolates from the same host species usually differ only slightly in their genetic coding capacity (Löchelt and Flügel, 1995), two distinct serotypes of FFV have been characterized (Flower *et al.,* 1985; Helps and Harbor, 1997; Winkler *et al.,* 1997). Sequencing members of each of these FFV serotypes revealed that the major difference in the coding capacity is confined to the central and carboxyl-terminal part of the Env SU protein (Helps and Harbor, 1997; Winkler *et al.,* 1997). This part of FFV Env was also found to be the major target for neutralizing antibodies (Winkler *et al.,* 1998).

To extend these studies, we constructed FFV genomes with serotype-specific differences in Env and Bel 1 (also designated Tas for transactivator of spumaviruses) and show that they are not cross-neutralized in a rapid neutralization assay.

RESULTS

Construction of FFV-FAB indicator cells for FFV titration

To establish FFV-FAB cells for rapid and sensitive titration of FFV infectivity analogous to those constructed

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for the titration of human foamy virus (HFV) on BHK cells (Yu and Linial, 1993), a plasmid containing part of the FFV U3 promoter upstream of a nuclear targeted β-gal gene was established. Transient transfection of pFeFV-U3-β-gal together with an FFV Bel 1 expression plasmid resulted in the strong expression of β-gal, whereas without Bel 1 coexpression, no β-gal activity was detectable at the single-cell level (data not shown). Plasmid pFeFV-U3-β-gal was transfected together with a plasmid coding for G418 resistance (Sambrook *et al.,* 1989). G418-resistant clones were selected by single-cell cloning. One single-cell clone turned out to stably contain plasmid pFeFV-U3-β-gal and showed β-gal expression only upon FFV infection or FFV Bel 1 transfection (data not shown). FFV-FAB cells allow the sensitive detection and titration of cell-culture-adapted FFV strains FUV and 951 and FFV isolated directly from FFV-infected cats (data not shown). The titer determined is expressed as (blue) focus forming unit per milliliter (FFU/ml; Yu and Linial, 1993).

Construction of infectious FFV-FUV genome pFeFV-7

To construct infectious, full-length FFV DNA clones, FFV DNA was amplified by long-template PCR using specific primers and DNA from FFV-FUV-infected CRFK cells as template as described previously (Winkler *et al.,* 1997). Amplicons were either subcloned or directly inserted into already existing FFV DNA clones as described under Material and Methods (Winkler *et al.,* 1997). Colinear FFV DNA from nt position 17 to 11,700 in the pBluescript backbone was subcloned into vector pAT153. Several independent and genetically stable recombinant clones were analyzed after transfection into FFV-permissive CRFK cells; most of them had only a low level of infectivity. Because FFV clone 13 showed moderate viral infectivity, it was used to fully restore FFV infectivity by substituting FFV DNA sequences from nt 5980 to 10,137 by FFV DNA obtained by direct PCR amplification from FFV-infected CRFK cells. The resulting FFV clone pFeFV-7 was genetically stable in bacteria. To determine the infectivity of clone pFeFV-7, DNA was transfected into permissive CRFK cells and viral infectivity was determined by infecting CRFK cell-derived FFV-FAB cells. Cell-free supernatants from CRFK cells harvested 3 days after transfection with plasmid pFeFV-7 reproducibly yielded titers of $\sim 10^5$ FFU/ml when assayed on FFV-FAB cells. After longer incubation, the titer increased gradually to $\sim 10^7$ FFU/ml. Virus derived from pFeFV-7-transfected cells was infectious for CRFK cells, induced the formation of syncytia, and maintained a titer of $\geq 10^7$ FFU/ml upon serial passage on CRFK cells indistinguishable from uncloned, wild-type FFV. Supernatants from untreated or pUC18-transfected CRFK cells did not induce the characteristic nuclear blue staining of FFV-infected FFV-FAB cells.

Sequencing of pFeFV-7 DNA revealed minor alterations of the primary nt sequence and the deduced amino acid sequence in comparison to the sequence described previously (Winkler *et al.,* 1997): A5379G, Pol N815D; T5555C, silent; T6787C, Env F157L; T7086C, silent; A7279G, Env N321D; G8258A, Env R647K; A8750G, Env D811G; G9067A, Env D917N; C9135T, silent; A9557G, silent. It is unknown whether these differences reflect the genetic variability of FFV in cultured cells or are due to PCR amplification or a combination of both.

Construction of Env-chimeric FFV genome pFeFV-7/951

As virions derived from pFeFV-7 were neutralized by FUV-serotype-specific cat sera (see below), we decided to construct recombinant FFV genomes that will not be neutralized by a preexisting immunity against FFV strain FUV. To this end, the majority of the *env* gene together with *bel 1* were substituted by corresponding sequences from the distinct serotype 951 (Flower *et al.,* 1985). By this procedure, we wanted to confirm that the known divergent sequences in FFV Env proteins were responsible for the strain-specific serum neutralization (Winkler *et al.,* 1998). Primers corresponding to the 951-like FFV isolate F17 (Helps and Harbor, 1997) located at nt positions 6418 and 9901 were used to amplify FFV DNA sequences from CRFK cells infected with FFV isolate 951. The resulting PCR fragment was digested with *Kpn*I and inserted into the corresponding sites of plasmid pFeFV-7. DNA clone pFeFV-7/951 was genetically stable and directed the synthesis of infectious FFV particles, however, the titer was only $\sim 5 \times 10^3$ FFU/ml 3 days after transfection when compared to the parental clone pFeFV-7. Sequencing of the chimeric provirus across the exchanged sequence confirmed its hybrid structure. Significant sequence differences between the published FFV Env and Bel 1 protein sequences and clone pFeFV-7/951 were apparent and are shown in Figs. 1 and 2, respectively. As expected, the Env sequence of pFeFV-7/951 strongly corresponded to the FFV isolate F17 (Helps and Harbor, 1997), which was recently shown to represent an 951-like FFV strain (Winkler *et al.,* 1998). The homology of the Bel 1 transactivator sequence is quite remarkable (Fig. 2). It is possible that the lower infectivity of clone pFeFV-7/951 can be partially attributed to sequence differences in the activation domain of Bel 1 (He *et al.,* 1993) although the levels of β-gal expression in infected FFV-FAB cells was indistinguishable when compared to the parental clone pFeFV-7 (data not shown).

Analysis of FFV protein expression directed from recombinant genomes

The pattern of FFV-protein expression directed from recombinant genomes pFeFV-7 and pFeFV-7/951 was compared to that of FFV-FUV-infected CRFK cells. With antisera directed against bacterially expressed do-

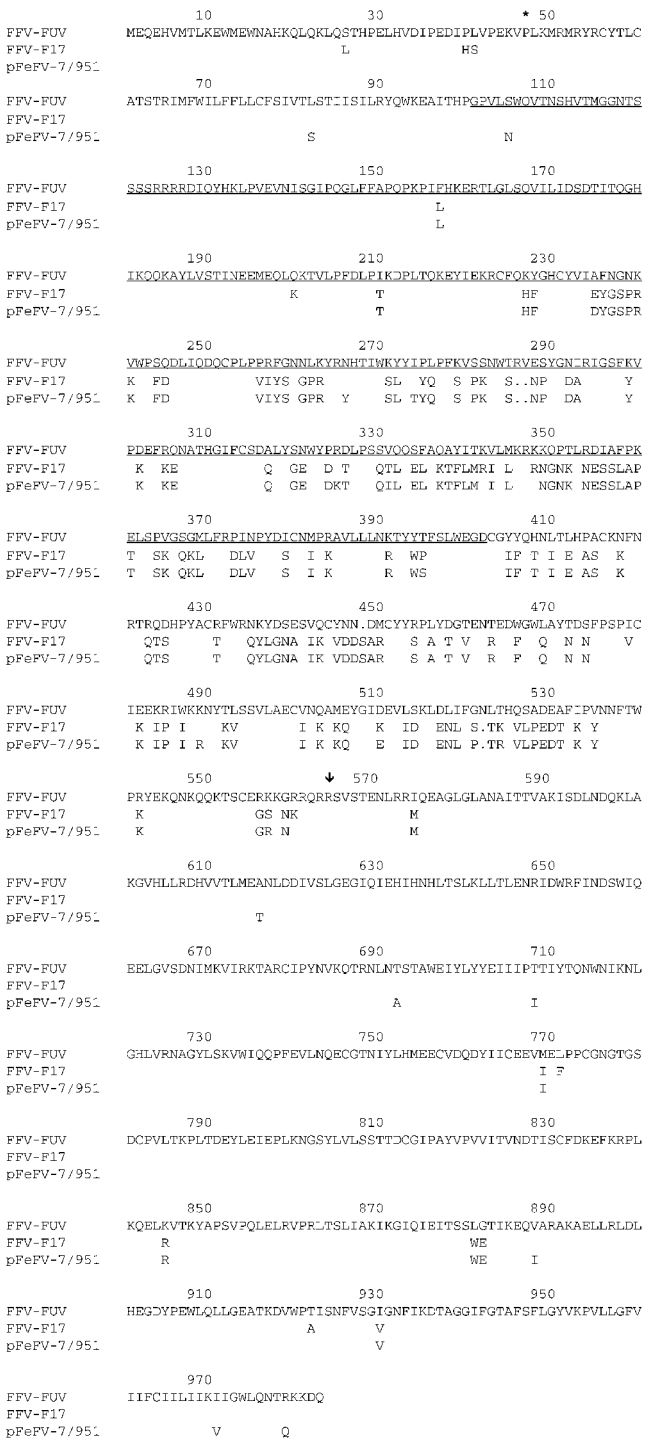

FIG. 1. Sequence analysis of pFeFV-7/951-encoded Env proteins. Comparison of deduced Env sequences of the prototype FFV FUV sequence (top; Winkler *et al.,* 1997), the FFV 951-like isolate F17 (middle; Helps and Harbor, 1997), and chimeric clone pFeFV-7/951 (bottom line). The FFV-FUV prototype Env sequence is shown in the single-letter amino acid code; only divergent residues are given for the other sequences; dots represent gaps in the sequences. The position of the predicted cleavage site between the SU and TM domain is given by a vertical arrow, the site corresponding to the 5′ end of the exchanged sequences in clone pFeFV-7/951 is marked by an asterisk, and FFV-FUV Env sequences expressed to generate the Env SU-specific antiserum are underlined.

mains of the amino-terminal part of FFV Gag (Fig. 3A Gag-N), the Pol PR domain (Fig. 3B, PR), the SU domain of FFV Env (Fig. 3C), and the FFV Bel 2/Bet proteins (Fig. 3D), significant differences were not detectable in gene expression when wild-type FFV-infected cells (Fig. 3, lanes 2) were compared with cells transfected with recombinant plasmids pFeFV-7 (lanes 3) and pFeFV-7/951 (lanes 4). In FFV-infected and pFeFV-7- and pFeFV-7/951-transfected cells, the sera specifically detected the full-length and terminally processed Gag proteins (arrowheads in A), the unprocessed Pro-Pol precursor and the PR-RT-RNase H Pol proteins (long and short arrows in B), the Env precursor (diamond in C), and the 43-kDa Bet protein (solid arrow in D). Thus FFV gene expression from recombinant plasmid pFeFV-7 and pFeFV-7/951 is indistinguishable from uncloned, cell-culture-derived FFV virus stocks. The FFV SU antiserum directed against FFV-FUV SU clearly recognized the 951-like Env protein of clone pFeFV-7/951 as expected because the amino-terminal part of the recombinant antigen used for immunization is highly conserved between both serotypes (see Fig. 1). In general, gene expression of cloned and uncloned FFV strongly resembles the gene expression profile and degree of proteolytic processing of Gag, Pol, and Env proteins when compared with the prototypic HFV.

Neutralization characteristics of recombinant FFV virions

To investigate the neutralization behavior of the FFV recombinants, ~200 FFU as determined on FFV-FAB cells of virus stocks obtained after transfection with plasmids pFeFV-7 and pFeFV-7/951 were incubated with sera from naturally FFV-infected Cats 10 or 12 (FUV-like) or Cats 11 or 14 (951-like),and serum from an FFV-negative cat as control (Winkler *et al.,* 1998). Neutralization assays were performed as previously described (Winkler *et al.,* 1998) and quantitated on FFV-FAB cells. Sera and dilutions thereof were considered as neutralizing when the given initial virus titer was reduced by ≥75%. Data from a representative serum neutralization experiment performed in duplicate are summarized in Table 1. It is evident that serum from FFV-FUV-infected Cat 12 neutralized progeny virus from clone pFeFV-7 but not that derived from plasmid pFeFV-7/951, whereas serum from Cat 14, which had been infected with an 951-like FFV isolate (Winkler *et al.,* 1998), neutralized virus derived form plasmid pFeFV-7/951 but not that from pFeFV-7 (Table 1). The FFV-negative control serum did not show any neutralization activity toward any of the recombinant viruses tested. We conclude that epitopes relevant for serum neutralization of FFV are primarily if not exclusively located in Env.

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                        10         20         30         40         50         60
                         .          .          .          .          .          .
FFV-FUV          MASKYPEEGPITEGVEEDFNSHSTSGLDLTSGNKEEPLISLALLSMHTSKIVVWIRDHFF
FFV-F17          MAAKYPEEGPITEGVEEDFNSHSTSGLDLTSGNKEEPLISLALLSMHTSKTVIWIRDHFF
pFeFV-7/951      MASKYPEEGPVTEGVEEDFNSHSTSGLDLTSGNKEEPLISLALLSMHTSKIVIWIRDHFF
                 **.*******.**************************************** *.*******

                        70         80         90        100        110        120
                         .          .          .          .          .          .
FFV-FUV          VKILSFGGKQKLYYICNQCHKGIPESGYITLNTKYYLYEKGPTETGTKGLTLMRRHVQNS
FFV-F17          VKILSFGGKQKLYYICNQCHKGIPESGYIVLNTKYYLYEKGPIETGTKGLTLMRRHVQNS
pFeFV-7/951      VKILTFGGKQKLYYICNQCHKGIPESGYIILNTKYYLYEKGPTETGTKGLTLMRRHVQNS
                 ****.************************ ************ ******************

                       130        140        150        160        170        180
                         .          .          .          .          .          .
FFV-FUV          PCFLNSRKESGTPKTDPTRPATSYSLCRSDYQEAGCSRPTPSNSESVCNGLGQPSERGHT
FFV-F17          PCFLNSRKESGPPKTDPTRPATSYSLCRSDYQEAGCSRPTPSNSESVCNGLGQPSERGHT
pFeFV-7/951      PCFLSSRKESGRPKTNPTRPTTSCSPCRSGHQEAGCSWPTPSNSESVCDGLGQPSECGHT
                 **** ****** *** ****.** * ***  ****** ********** ******* ***

                       190        200       209
                         .          .         .
FFV-FUV          SGESGGIMEEVSFISWLEGLWGEGFDYAN
FFV-F17          SGESGGTMEEVSFVSWLEGLWGEGFDYAN
pFeFV-7/951      SGESGGTMEEVSFISWLEGLWEEGYDHTN
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FIG. 2. Sequence analysis of pFeFV-7/951-encoded Bel 1 (Tas) proteins. Comparison of deduced Bel 1 transactivator sequences of the prototype FFV-FUV sequence (top; Winkler *et al.,* 1997), the FFV-951-like isolate F17 (middle; Helps and Harbor, 1997), and the chimeric clone pFeFV-7/951 (bottom line). Asterisks below the aligned sequences mark identical residues, dots mark conservative sequence exchanges.

DISCUSSION

Here we describe the construction of FFV genomes that direct the expression of infectious FFV particles. The recombinant FFV genomes are genetically stable and direct the expression of FFV particles that can be continuously propagated in cultured cells. The parental wt provirus is based on the FFV serotype FUV and its sequence almost completely corresponds to that published previously (Flower *et al.,* 1985; Winkler *et al.,* 1997). A derivative of FFV-FUV-based plasmid pFeFV-7, desig-

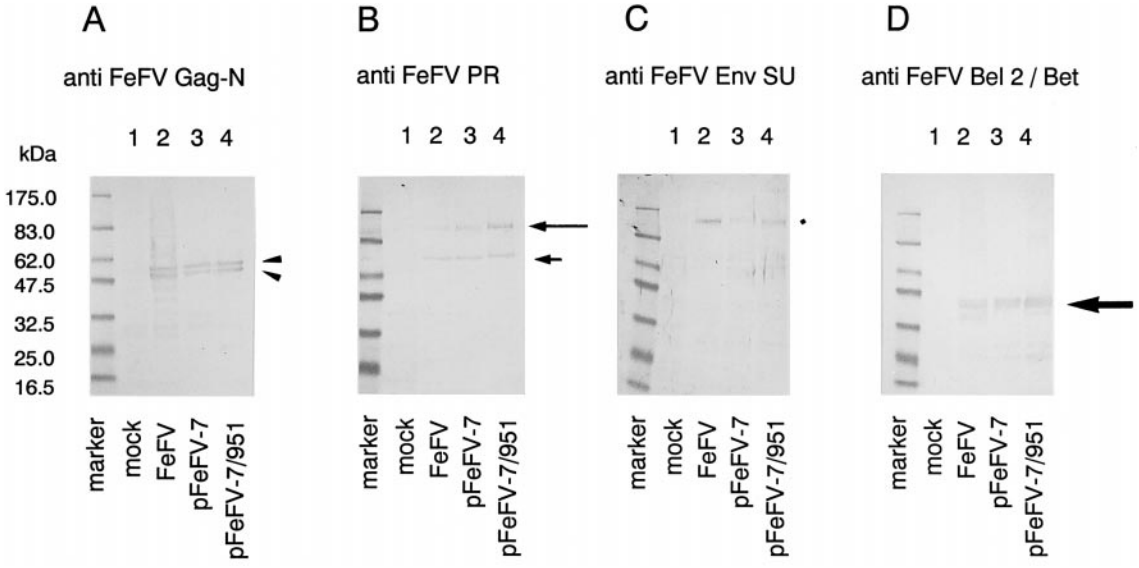

FIG. 3. Comparison of gene expression of uncloned and cloned FFV. Protein expression of recombinant clones pFeFV-7 (lanes 3) and pFeFV-7/951 (lanes 4) upon transfection into CRFK cells compared to antigen derived from FFV-infected CRFK cells (lanes 2). Cell-associated antigens were harvested 4 days after transfection or FFV infection and mock infection (lanes 1). The preparation of cell-associated antigen, immunoblotting, and the sera used were done described previously (Bodem *et al.,* 1998a). FFV-specific antigens were detected using antisera directed against the aminoterminal part of FFV Gag (A; Gag-N), the PR domain of Pol (B; PR), part of FFV Env SU (C), and Bel 2/Bet proteins (D.). The sera as specified above the blots detected the full-length and terminally processed Gag proteins (arrowheads in A), the unprocessed Pol precursor and the PR-RT-RNase H proteins (long and short arrows in B), the Env precursor (diamond in C), and the 43-kDa Bet protein (solid arrow in D). The apparent molecular masses of prestained protein markers separated in parallel are shown at the left margin.

TABLE 1

Serum Neutralization of Cloned FFV Env Variants[a]

	Antiserum from		
	FFV-negative cat	FFV-FUV-infected Cat 12	FFV-951-infected Cat 14
pFeFV-7	0[b]	1:80–1:160	0[b]
pFeFV-7/951	0[b]	0[b]	1:160–1:320

[a] Neutralization titers were recorded at the cat antiserum dilution that inhibited the number of blue cells in FFV-FAB cells by at least 75%.

[b] Reduction of the FFV titer was not detectable.

nated pFeFV-7/951, contains *env* and *bel 1* sequences from the distinct FFV serotype 951 (Winkler *et al.,* 1998) and codes for FFV particles with different neutralization characteristics as anticipated. The genetic differences between plasmids pFeFV-7 and pFeFV-7/951 are primarily confined to the central and carboxyl-terminal part of Env SU as expected from previous studies (Helps and Harbor, 1997; Winkler *et al.,* 1997, 1998; Wang and Mulligan, 1999) although the activation domain of FFV Bel 1 also shows a significant degree of sequence heterogeneity. By means of monospecific antisera directed against defined structural and nonstructural FFV-FUV proteins including FUV Env SU, these clones are indistinguishable from each other and from uncloned FFV. Infectivity derived from both recombinant genomes can be efficiently quantitated using CRFK cell-derived indicator cells. Importantly, FFV-FAB cells are suited for titration of FFV derived directly from naturally and experimentally FFV-infected cats (data not shown), confirming that a sensitive detection system for cell-culture-derived and field virus of both FFV serotypes has been established. However, as expected from our previous study, progeny virus derived from either FFV proviral genome can be clearly discriminated by means of strain-specific neutralization and PCR assays.

Our results show that the epitopes that elicited neutralizing antibodies against FFV are confined to the Env protein, most likely to the distinct central and carboxyl-terminal part of FFV SU that shows a low degree of sequence conservation between both serotypes. Other parts of Env that had not been exchanged in our mutant as well as other structural and nonstructural FFV proteins are not likely to contribute to serum neutralization because almost no cross-neutralization between the wild-type and Env chimeric viruses was detectable in this and a previous study (Winkler *et al.,* 1998).

According to our data, the parental FFV FUV clone pFeFV-7 and its Env chimeric derivative pFeFV-7/951 for which cross-neutralization is not observed, represent a promising pair of vectors for the repeated administration of recombinant FFV-based vectors for targeted gene delivery applications in cats. Engineered vectors derived from both FFV clones carrying therapeutic proteins may be administered consecutively into cats without clearance of the therapeutic second vector due to pre-established neutralizing antibodies induced by previous vector applications.

To our knowledge this is the first report of a pair of gene therapy vectors based on the same retroviral backbone that do not exhibit serum cross-neutralization, possibly allowing repeated and successful administration of the therapeutic vectors. Experiments to investigate whether successful administration of the two FeFV-based vectors is possible will be performed.

MATERIALS AND METHODS

Virus, cells, and transfection

FFV strains FUV and 951 have been described previously and were propagated in CRFK cells (Flower *et al.,* 1985; Winkler *et al.,* 1998). Electroporation of 10 μg plasmid DNA into CRFK cells was done at 975 μFd and 160 V in 2-mm cuvettes as described (Winkler *et al.,* 1997). FFV-FAB cells were selected and maintained in CRFK cell medium supplemented with 0.5 μg/ml G418 (Boehringer Mannheim, Germany).

Construction of recombinant DNA

To construct infectious, full-length FFV DNA clones, FFV DNA sequences from nt position 5118 to 7431 (Winkler *et al.,* 1997) were amplified by long-template PCR with primers 5′-CCTCATGCTTACGGGAATAATCTGGCTG-3′ (at FFV genome position 5118–5145, Winkler *et al.,* 1998) and 5′-GAATAGCATACCAGAGCCTACAGGGCTC-3′ (FFV position 7404–7432) and DNA from FFV-infected CRFK cells as template as described (Winkler *et al.,* 1997). Amplicons were cloned into plasmid pCRII (Invitrogen, Groningen, The Netherlands) by standard techniques (Sambrook *et al.,* 1989; Bodem *et al.,* 1996). Correct clones were digested with *Nde*I (in the FFV sequence) and *Ecl*136II (in pCRII). FFV DNA of ~2.3 kb was inserted into FFV DNA clone 7 (Winkler *et al.,* 1997) digested with *Cla*I, blunt-ended by Klenow DNA polymerase, and digested with *Nde*I. The resulting FFV clones 24 and 28 extended from nt position 17 to 7431. In parallel, the FFV insert from plasmids 4, 6, and 8 harboring FFV-DNA sequences from nt position 8636 to the 3′ end of the 3′-LTR (Winkler *et al.,* 1997) were subcloned into pBluescript KS using common flanking restriction enzyme sites from the polylinkers. In the resulting clones 5, 7, and 15, the 3′ end of the genome is followed by *Cla*I and *Apa*I sites from the PCR primers and the polylinker sites of the vector. FFV DNA sequences from nt position 7163 to 9057 (primers 5′-CCAATTGGACAAGAGTAGAATCCTATGG-3′ and 5′-TTCTCCAAGGAGCTGCAGCCACTCTGG-3′) were

amplified by long-template PCR and cloned into plasmid pCRII as described above, resulting in clone V. To obtain full-length proviral DNA clones, the cloned *Apa*I (in the vector) to *Pml*I fragment of clones 24 and 28, the *Pml*I to *Pst*I fragment from clone V, and the *Pst*I to *Apa*I fragment from clones 5, 7, and 15 described above were combined in three-component ligations. Resulting DNA clones contained FFV DNA from nt position 17 to 11,700 in the pBluescript backbone. The FFV insert between the flanking *Eag*I and *Cla*I sites was subcloned into the correspondingly digested vector pAT153 (Sambrook *et al.,* 1989). Finally, FFV DNA sequences from nt 5775 to 10,522 were amplified by long-template PCR using DNA from FFV-infected CRFK cells and primers 5′-TTTGCTCAGTGGGCAAAGGAAAGGAATATACAATTGG-3′ and 5′-GTTGACACTGATTTATATGGCACAATAATTCCTCTC-3′. The DNA was directly digested with *Bst*Z17I (nt position 5980) and *Bsa*I (nt position 10,137). The FFV DNA fragment of ~4.2 kb was used to substitute for the corresponding sequences of FFV clone 13. FFV clone pFeFV-7 turned out to be genetically stable in bacteria.

An FFV reporter plasmid containing part of the FFV U3 region upstream of a β-galactosidase (β-gal) gene containing a nuclear localization signal was constructed by excising the HFV promoter from plasmid pHSRV5LG (Yu and Linial, 1993) by *Sma*I and *Pst*I digestion. FFV-LTR sequences from FFV nt position 33 to 1095 in the U3R region were amplified by PCR using primers 5′-TCCCCCGGGAATACTCTCTGCTGCC-3′ (nucleotide position 5118–5145 of FFV genome, Winkler *et al.,* 1997) and 5′-AAACTGCAGCCAAGTCTGTGAGAA-3′ (FFV position 7404–7432) and cloned FFV DNA as template. The amplicon was digested with *Sma*I and *Pst*I and inserted into the correspondingly cleaved vector backbone of plasmid pHSRV5LG (Yu and Linial, 1993).

Virus neutralization assays

Sera used for neutralization assays have been described (Winkler *et al.,* 1998). The titer of FFV derived from cleared supernatants of transfected CRFK cells was determined and infectivity was diluted to a final titer of ~200 FFU/ml in complete cell culture medium. Serially diluted cat antisera were added, incubated for 1 h at 37°C under gentle shaking, and added onto FFV-FAB cells grown in 24-well plates (Winkler *et al.,* 1998). After 2 h of absorption, the virus was removed and new medium was added. The remaining infectivity was determined 2 days later as described above for FFV-FAB cells. Neutralization was scored positive when the given titer was reduced by ≥75% and expressed as the highest serum dilution that still resulted in FFV neutralization.

Preparation of monospecific antisera and immunoblotting

Defined domains of FFV *gag* and *env* genes were amplified by PCR using pFeFV-7 DNA as template, subcloned into pCRII plasmids, excised with restriction enzymes whose recognition sites had been introduced into the primers, and cloned between the *Nde*I to *Bam*HI sites of prokaryotic expression plasmids pET16b. To express FFV Gag residues 1–210, primers 5′-TCATATGGCTCGAGAATTAAATCCTC-3′ and 5′-AGGATCCTAAAATACTCCTTCAATAGCGGC-3′ were used; the amplicon was excised using *Nde*I and *Bam*HI. To express residues 101–402 of Env SU, primers 5′-CATATGGGCCCAGTCTTAAGCTGG-3′ and 5′-AGATCTCCTTCCCATAGTGAG-3′ were used; FFV sequences were excised with *Nde*I and *Bgl*II. The FFV protease (PR; Pol residues 1–140) was PCR-amplified using primers 5′-GGGGGTACCGACGACGACGACAAGATGGATCTGCTGAAGCCG-3′ and 5′-CGCGGATCCTTAATTCTCCCAACTTTGCCATAA-3′; excised with *Kpn*I and *Bam*HI and inserted into the correspondingly cleaved pET32a vector. Expression and purification of recombinant proteins were done as described previously (Bodem *et al.,* 1998a). The immunization of rabbits was performed by Eurogentec, Seraing, Belgium. The FFV Bel 2/Bet antiserum and immunoblotting have been described recently (Bodem *et al.,* 1998a).

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REFERENCES

Bendecchi, P., Matteucci, D., Baldinotti, F., Guidi, G., Abramo, F., Tozzini, F., and Bendinelli. M. (1992). Prevalence of feline immunodeficiency virus and other retroviral infections in sick cats in Italy. *Vet. Immunol. Immunopathol.* **31,** 337–345.

Bodem, J., Löchelt, M., Delius, H., and Flügel, R. M. (1998a). Detection of subgenomic cDNAs and mapping of feline foamy virus mRNAs reveals complex patterns of transcription. *Virology* **224,** 417–426.

Bodem, J., Löchelt, M., Winkler, I., Flower, R. P., Delius, H., and Flügel, R. M. (1996). Characterization of the spliced pol transcript of feline foamy virus: The splice acceptor site of the pol transcript is located in gag of foamy viruses. *J. Virol.* **70,** 9024–9027.

Bodem, J., Zemba, M., and Flügel, R. M. (1998b). Nuclear localization of the functional Bel 1 transactivator but not of the Gag Proteins of the feline foamy virus. *Virology* **251,** 22–27.

Coffin, J. M. (1996). Retroviridae: The viruses and their replication. In "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), pp. 1767–1847. Raven Press, New York.

Elder, J. H., Dean, G. A., Hoover, E. A., Hoxie, J. A., Malim, M. H., Mathes, L., Neil, J. C., North, W., Sparger, E., Tompkins, M. B., Tompkins, W. A., Yamamoto, J., Yuhki, N., Pedersen, N. C., and Miller, R. H. (1998). Lessons from the cat: Feline immunodeficiency virus as a tool to develop intervention strategies against human immunodeficiency virus type 1. *AIDS Res. Hum. Retroviruses* **10,** 797–801.

Flower, R. L., Wilcox, G. E., Cook, R. D., and Ellis, T. M. (1985). Detection

and prevalence of serotypes of feline syncytial spumaviruses. *Arch. Virol.* **83,** 53–63.

Hackett, A. J., Pfiester, A., and Arnstein, P. (1970). Biological properties of a syncytia-forming agent isolated from domestic cats (feline syncytia-forming virus). *Proc. Soc. Exp. Biol. Med.* **135,** 899–904.

He, F, Sun, J. D., Garrett, E. D., and Cullen, B. R. (1993). Functional organization of the Bel-1 trans activator of human foamy virus. *J. Virol.* **67,** 1896–1904.

Helps, C. R., and Harbor, D. A. (1997). Comparison of the complete sequence of feline spumavirus with those of the primate spumaviruses reveals a shorter gag gene. *J. gen. Virol.* **78,** 2549–2564.

Linial, M. L. (1999). Foamy viruses are unconventional retroviruses. *J. Virol.* **73,** 1747–1755.

Löchelt, M., and Flügel, R. M. (1995). The molecular biology of primate spumaviruses. In "The Retroviridae" (J. A. Levy, Ed.), Vol. 4, pp. 239–292. Plenum Press, New York.

Loh, P. (1993). Spumaviruses. In "The Retroviridae" (J. A. Levy, Ed.), Vol. 2, pp. 361–397. Plenum Press, New York.

Mergia, A., and Luciw, P. (1991). Replication and regulation of primate foamy viruses. *Virology* **184,** 475–482.

Rethwilm, A. (1995). Regulation of foamy virus gene expression. *Curr. Top. Microbiol. Immunol.* **193,** 1–24.

Riggs, J. L., Oshiro, L. S., Taylor, D. O. N., and Lennette, E. H. (1969). Syncytium forming agent isolated from domestic cats. *Nature* **222,** 1190–1191.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Wang, G., and Mulligan, M. J. (1999). Comparative sequence analysis and predictions for the envelope glycoproteins of foamy viruses. *J. Gen. Virol.* **80,** 245–254.

Winkler, I., Bodem, J., Haas, L., Zemba, M., Delius, H., Flower, R., Flügel, R. M., and Löchelt, M. (1997). Characterization of the genome of feline foamy virus and its proteins show distinct features different from those of primate spumaviruses. *J. Virol.* **71,** 6727–6741.

Winkler, I. G., Flügel, R. M., Löchelt, M., and Flower, R. L. P. (1998). Detection and molecular characterisation of feline foamy virus serotypes in naturally infected cats. *Virology* **247,** 144–151.

Winkler, I. G., Löchelt, M., and Flower, R. L. P. (1999). Epidemiology of Feline Foamy Virus and Feline Immunodeficiency Virus infection in domestic and feral cats: A seroepidemiological study. *J. Clin. Microbiol.* **37,** 2848–2851.

Yu, S. F., Baldwin, D. N., Gwynn, S. R., Yendapalli, S., and Linial, M. L. (1996). Human foamy virus replication—a pathway distinct from that of retroviruses and hepadnaviruses. *Science* **271,** 1579–1582.

Yu, S. F., and Linial, M. L. (1993). Analysis of the role of the bel and bet open reading frames of human foamy virus by using a new quantitative assay. *J. Virol.* **67,** 6618–6624.